

THE EFFECT OF VARYING HEAVY METAL BALANCES IN THE
NUTRIENT MEDIUM, ON THE GROWTH AND DEVELOPMENT OF
ASPERGILLUS SP. AND PENICILLIUM SP.

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by

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SUMMARY.

The study was conducted to investigate the effects of:

- (i) varying the level of supply of the heavy metals iron, manganese, copper and zinc.
- (ii) varying the ratio between different pairs of metals in the medium. In particular the iron:manganese and zinc:copper ratios were studied.

Initially the two fungi Aspergillus niger van Tieghem, (variety and strain) and Penicillium notatum Westling, were used. Penicillium notatum Westling was subsequently discarded, in favour of Penicillium glaucum Link, because it did not sporulate freely in liquid culture.

The fungi were grown in controlled nutrient solutions, and during the course of the growth and development, the form and sporulation of the felts was noted. After a period of growth, the felts were removed, dried and weighed. The pH of the liquor was measured. The results were studied to determine the effect of varying levels of supply of the heavy metals, and of the varying heavy metal ratios in the culture solution.

In the investigation of the effect of varying the level of supply of individual heavy metals, optimum concentrations were demonstrated for copper and manganese.

Increasing the concentration of pairs of heavy metals simultaneously was found to influence the appearance and degree of symptoms of toxicity. Cultures of Aspergillus and Penicillium were found to be able to tolerate concentrations of copper, in particular, considerably greater than the observed optima, when zinc was present in equal concentration.

Citric acid, and subsequently ethylene-diaminetetra-acetic acid,

were used as chelating agents, in order to prevent the precipitation of the metals in the culture solution during autoclaving. It was found that the use of chelating agents markedly reduced symptoms of toxicity.

There was no conclusive evidence that the iron:manganese ratio in the culture medium was an important factor in the growth and development of cultures of Aspergillus and Penicillium. However, there is considerable evidence that in cultures of Penicillium, the zinc:copper ratio in the medium is of some importance in the determination of the dry weight yield trends. This effect was not demonstrated in cultures of Aspergillus.

CHAPTER I.Introduction.

It is well established that while trace elements are necessary for normal growth and development of plants, when in excess they may be injurious. Thus numerous workers have carried out investigations directed to determine the factors controlling the uptake, and the amount of any element which has to be absorbed in order to produce injury. There is abundant evidence that the amount of any element which has to be absorbed before injury becomes apparent, is, at least in part determined by the amount of other elements present in the medium and in the plant. Prominent among this evidence, is that of Somers and Shive (1942) who demonstrated that in soya bean the absorption of manganese is influenced by the presence of iron salts in the medium. Thus it was found possible to reduce or eliminate the symptoms of manganese toxicity by the presence of iron.

Investigations of this nature have, to my knowledge, been confined to studies on flowering plants. The present investigation is an attempt to determine whether similar interactions between heavy metals could be observed in the nutrition of Aspergillus niger van Tieghem and Penicillium species. A review of the relevant literature is therefore not out of place here.

Review of the literature.

Work on the nutritional requirements of the fungi was initiated by the discovery of Pasteur that yeast could grow on an artificial medium containing only sugar, ammonium salt and yeast ash. Raulin (1869) was the first worker to demonstrate that, in addition to the usual nutrients such as potassium, magnesium and phosphorous, certain other elements, notably zinc and iron, had to be furnished in the medium of Ascophora nigrans (Aspergillus niger.) in order to obtain the maximum dry weight yield.

It was not until the introduction of an efficient method for the purification of the culture medium that the work became quantitative. Prior to this it was not appreciated how minute the requirements of the heavy metals were, and how easily sufficient amounts of the metals could be obtained in the form of contaminants. The contaminants found in the nutrient solutions are derived from three sources: reagents, water and containers.

Complete removal of traces of heavy metals is practically impossible, even when chemically pure reagents are used. A purification technique may however reduce the contaminants to almost negligible values. Several authors have reported methods which are satisfactory for the purification of the culture solution. The earliest technique, that of Steinberg 1919, relied on the heating of the nutrient solution in the presence of excess CaCO_3 (15 gms./litre.), in an autoclave at a pressure of 20 lbs. per square inch for twenty minutes, and then filtering.

Where large scale experiments are undertaken, in particular when large volumes of the culture solutions are required, it is unsatisfactory to have to purify the complete medium simultaneously and for this reason Stout and Arnon (1939) developed a modified method from Steinberg. The modified method enabled the different constituent solutions to be purified separately. Using this method they were able to reduce the metal contaminants from 500 - 5000 parts per billion to consistently less than ten parts per billion. They also report that redistillation of water could reduce the metal content from 10 - 100 parts per billion to less than one part per billion.

Glassware from different sources varies widely with respect to the influence it exerts, by way of dissolution of metals from the glass. Of the several makes of glass tested, quartz has proven to be the best (Javillier 1914 and Richter 1901.), whilst that of Pyrex quality is quite satisfactory (Steinberg 1918, Sommer and Lipham 1926). Steinberg

1919 has shown that with repeated usage of the culture vessels, reduced yields of Aspergillus niger could be obtained due to the gradual leaching out of metals from the glass.

The results on the influence of specific elements have often proved somewhat contradictory. This is, however, not surprising, since very varied results may be obtained from different strains of the same fungus, under the same cultural conditions (Perlman 1941). There emerge, however, certain effects which have been confirmed by several workers.

The essentiality of zinc for growth in fungi has been confirmed many times, (Chesters and Rolinson 1951a.). The most notable effect is the increase in the dry weight yield, which may be as much as 2,309 times, depending on the amount of zinc pre-existent in the medium, (Steinberg 1919). Porges 1932, reported that when a zinc salt is added to the medium, the surface pellicle of Aspergillus niger becomes much thickened and corrugated in appearance. In zinc deficient cultures the pellicle is smooth, and in cultures with a high degree of purity, no pellicle is formed, the growth being entirely submerged.

Zinc deficient cultures of Aspergillus niger are profusely covered with black spores, whilst cultures with a zinc level of 0.01% produce spores of an olive-green to grey colour (Porges 1932). Sporulation in Aspergillus niger is reported to be decreased in the absence of iron and manganese, but not in the absence of zinc or copper, (Steinberg 1945). As the critical concentration of zinc for spore inhibition in Aspergillus niger is approached, the spore colour changes from black to yellow-brown to yellow, (Roberg 1928). Subsequent increases in the zinc concentration result in the production of a white, practically sterile, felt. Zinc has also been shown to stimulate growth and to repress sporulation in Neurospora crassa and Neurospora sitophila (Bonner 1943 and Stokes 1943). Zinc does not always suppress sporulation. In Rhizopus nigricans, no sporulation takes place until zinc is added to the medium

(Foster and Waksman 1939). Spore colour change in Aspergillus niger has been utilised for the determination of the concentration of iron and zinc in the blood (Nagasako 1939).

Sohman 1934 recorded two optimum concentrations of zinc sulphate in the growth of Aspergillus niger. However, different strains of the same species show different optimum concentrations of zinc for maximum yield (Mosseray 1932, Steinberg 1919). Penicillium waksmanii is able to grow in a saturated solution of copper sulphate and in concentrated solutions of zinc sulphate or manganese chloride (Hubert, Biol. Abst. 14.7573).

In general, zinc acts adversely upon the accumulation of organic acids in fungal cultures, but citric acid accumulation is favoured at levels of zinc too low for normal growth (Chasters and Rolinson 1951a). Lockwood (1940) showed that, in submerged cultures of Rhizopus oryzae, zinc did not influence acid production, whilst, in surface cultures, it reduced acid production. This zinc effect is not specific: citric acid accumulation is favoured by other mineral deficiencies (Chesters and Rolinson, 1951b).

Steinberg (1919) and Roberg (1928) have shown that in highly purified solutions the addition of iron gave growth which was 43.7 and 75 times that when iron was omitted. Sporulation and spore pigment are closely bound up with iron metabolism in fungi. Raulin (1869) observed diminished sporulation in iron poor cultures, but this was not subsequently confirmed by either Coupin (1903) or Sauton (1910). Preparations of aspergillin, the spore pigment of black Aspergillus species, have been shown to contain 0.26% iron (Quilco and Di Capua 1933). Aspergillus is reported by Nicholas (1952) to be able to utilise both ferrous and ferric iron, the latter of which may be bound by the release into the medium of a substance or substances, which are not citrate (Garribaldi and Neilands, 1956). Iron is lost from solutions at neutral or alkaline pH's, but the inclusion of a chelating agent reduces the loss (Fries 1945, Hopkins and Wann 1926).

Iron has been shown to be essential for maximum yields of citric acid in Aspergillus niger (Perlman 1947, Shu and Johnson 1947), but manganese, zinc, and other ions are also reported to affect citric acid production, (Perlman 1947).

Growth reductions in manganese deficient cultures are always less than corresponding iron and zinc cultures (Steinberg 1936). This indicates that the manganese requirements are very small. Bertrand (1912) reports that, at a concentration of one part in ten billion, the element gives a decided stimulation in growth. Apart from the effect manganese has on yield, when deficient it results in a pronounced decrease in sporulation (Perlman 1949b, Foster 1939). The necessity of manganese for sporulation in Aspergillus niger has been shown by Steinberg (1935).

In submerged cultures of Aspergillus niger, the addition of manganese reduced citric acid production. In surface cultures of the same strain, it had no effect on acid production (Shu and Johnson 1947a and b). Perlman (1947), however, reports that it has little effect on submerged cultures of other strains.

Very significant growth increases have been obtained by the addition of copper salts to the nutrient medium (Bortels 1927). Wolff and Emmerie (1930) have shown that copper is an essential trace element in Aspergillus niger, and the same is true of A. flavus and A. glaucus (Mulder 1939). The copper requirements of fungi, for normal growth, are about 0.01 - 0.1 p.p.m. (Chesters and Rolinson 1951b and c). Higher concentrations are normally toxic, although Starkey and Waksman (1943) have shown that some fungi make visible growth even in saturated solutions of copper sulphate.

The most striking effect is the reduction in pigmentation of the coloured spore of Aspergillus sp. when copper is deficient (Bortels 1927, Javillier 1939, Mulder 1939). Pigment production is reported to be lower in Penicillium notatum, when additional copper is present in the medium, (Clayton et al 1944).

In solutions with a very low copper content, Aspergillus forms a well developed, white, completely sterile felt (Mulder 1938). Addition of copper leads to the formation of spores. The intensity of pigmentation of the spores increases with increased copper, from yellow to yellow-brown to black, (Roberg 1928). Yoshimura (1936) reports that, while this element stimulated conidia formation in Aspergillus niger, it had little effect on A. oryzae.

Copper is presumed to favour organic acid production, as evident by pH. changes (Mulder 1938).

Observations on the effect of the heavy metals on cytology are few. Iwanoff (1904) claimed that high zinc concentrations increased the vacuolisation of the hyphal cells, and furthered greater branching of the mycelium. Sakamura and Yoshimura (1933) and Yoshimura (1934), showed that zinc, copper and other heavy metals could cause the formation of peculiar spherical shaped cells in the mycelium of Aspergillus oryzae and A. niger. A distinct parallelism was noted with the degree of impurity especially with copper. Copper was even capable of transforming the sterigmata and conidia into abnormal cells (Yoshimura 1936).

The main aim of the investigation was to examine the effect of varying heavy metal balances in the culture medium on the growth and development of Aspergillus niger, (the strain was not known with any certainty, but it appears to be Aspergillus niger van Tieghem, series and strain), and Penicillium species. Thus a brief review of the literature pertinent to this problem will not be out of place here.

Considerable research has been carried out on the interactions of different elements in plant nutrition. Prominent among these is the iron : manganese interaction. Thus, as early as 1902, Aso, demonstrated that, in peas, radish, wheat and barley, the symptoms of manganese toxicity could be reduced, or eliminated altogether, by the presence of iron. Subsequent investigators have indicated that maximum growth only occurs when the iron and manganese supplies are present in a

TABLE 1. Effects of soya beans grown in water culture of different proportions of iron and manganese. (From Somers and Shive, 1942).

Concentration in substrate (p.p.m.)		Ratio Fe/Mn in substrate	Ratio soluble Fe/soluble Mn.		Dry weight per plant grams.	Condition of Plants
Iron	Manganese		In leaves	In roots		
0.005	0.000	-	2.65	5.04	3.47	Normal
0.005	0.002	2.50	2.37	3.36	3.78	Normal
0.005	0.010	0.50	2.05	2.73	3.62	Slight Fe deficiency (Mn excess)
0.005	0.250	0.02	1.00	1.44	2.86	Medium Fe deficiency (Mn excess)
0.005	2.00	0.0025	0.80	1.35	1.50	Severe Fe deficiency (Mn excess)
0.005	5.00	0.0010	0.83	1.29	1.57	Very severe Fe deficiency (Mn excess)
0.500	0.000	-	5.66	15.8	1.51	Very severe Mn deficiency (Fe excess)
0.50	0.002	250.0	5.44	14.5	2.22	Severe Mn deficiency (Fe excess)
0.50	0.010	50.0	4.47	12.4	3.01	Medium Mn deficiency (Fe excess)
0.50	0.250	2.0	1.95	2.24	3.78	Normal
0.50	2.00	0.25	0.72	1.26	3.16	Slight Fe deficiency (Mn excess)
0.50	5.00	0.10	0.58	1.17	3.02	Medium Fe deficiency (Mn excess)
3.00	0.000	-	9.82	17.4	1.37	Very severe Mn deficiency (Fe excess)
3.00	0.002	1500	9.85	15.1	1.39	Very severe Mn deficiency (Fe excess)
3.00	0.250	12.0	2.48	4.15	3.78	Normal
3.00	2.00	1.5	1.50	2.51	4.05	Normal
3.00	5.00	0.6	0.76	1.77	3.70	Slight Fe deficiency (Mn excess)

certain ratio. Among these were Pugliese (1913), who considered the optimum ratio of iron to manganese in the culture solution to be 1 to 2.5 for the growth of wheat, Scharrer and Schropp (1934), who found that with maize in water culture the growth of roots attained a maximum when the iron manganese ratio was 7 to 1, and Somers and Shive (1942), who showed clearly that in soya beans, the dry weight and condition of the plant was related to the iron : manganese ratio and not to the absolute concentrations.

The results of Somers and Shive (Table 1) showed that, if the ratios were above 2.5, the symptoms were of one kind, and, if the ratios were below 1.5, then the symptoms were of another kind. Both kinds of symptoms were quite distinct. The former could be described as iron excess or manganese deficiency, and the latter as iron deficiency or manganese excess. It was found possible to correct the pathological symptoms by altering either the iron or manganese concentrations in such a way as to bring the iron : manganese ratio within the range 1.5 to 2.5. This work received support from the report (Somers, Gilbert and Shive, 1942) that in the respiration of soya beans, the respiration rates were always lower when the iron : manganese ratio was outside 1.5 to 2.5, than when it was within it.

Twyman (1951) working with tomatoes, lettuce and oats, found that, in general, with a constant level of supply, increase in the supply of iron as ferrous sulphate with tartaric acid, ferric citrate or ferric ammonium citrate led to a reduction in the manganese content in the leaves. These results are summarised in Table 2.

TABLE 2. Effect of varying iron supply on the absorption of manganese (Data from Twyman, 1951).

Species	Manganese concentration in medium	Manganese concentration in tissues with different concentrations of iron in medium.				
		0.005	0.010	0.050	0.500	3.0
Tomato	0.000	-	-	0.0	0.2	1.0
	0.005	-	-	2.0	3.0	2.0
	0.050	-	-	31.0	20.0	12.0
Oats	0.000	5	6	-	4	6
	0.002	12	10	-	3	2
	0.005	34	30	-	7	8
	0.010	53	54	-	9	6
	0.050	220	183	-	34	21
	0.250	529	454	-	92	66

The conclusions of Somers and Shive have, however, only received partial support from subsequent workers. Nicholas (1949) and Twyman (1951) concluded that the ratio of total iron to total manganese was not very significant in determining manganese deficiency or toxicity, although Twyman was of the opinion that the ratio might be important in the metabolism of healthy plants.

Stiles (1961) has suggested that the differentiation of the iron and manganese in the plant into active and inactive fractions, might reveal a more precise relationship. This would explain why characteristic deficiency has been observed in plants containing high levels of manganese, and why deficiency symptoms were not evident in plants with a low total content of manganese. In the former, a high proportion of the manganese would be in an inactive state, whereas in the latter, all the manganese would be in a highly active state.

Evidence of an iron : manganese interaction in the fungi is, as far as I am aware, limited to a few reports, none of which deal with the iron : manganese balance effect. Thus Bertrand (1912) noted that iron, zinc and manganese were all nutritive elements functioning synergistically in growth and conidia formation in Aspergillus niger. Brack (1947) reported that, in cultures of Penicillium patulum, the addition of manganese to a medium containing iron was beneficial to sporulation and patulin yield, but reduced gentinyl alcohol production. It has also been reported that, in cultures of Penicillium citrinum, iron may be replaced by manganese but not by copper or zinc (Lochhead et al 1946).

A relationship between iron and copper has also been reported. The presence of copper in concentrations greater than two p.p.m. prevented the accumulation of penicillin in cultures of Penicillium citrinum, but this effect was nullified by the addition of iron (Knight and Frazier, 1945 and Koffler et al, 1945). In cultures of Penicillium patulum, copper in the presence of iron reduced the production of gentinyl alcohol and patulin, but increased sporulation. Brown and Holmes (1955) reported a relationship between iron and copper, but different plants were found to exhibit different behaviour in this respect. In maize, deficiency of copper resulted in an accumulation of iron throughout the plant. Working on subterranean clover, Greenwood and Hallsworth (1960) described a study of complex interactions between copper, molybdenum, calcium and phosphorous.

As far as I am aware there is no evidence of zinc interacting with other heavy metals. However, the uptake of zinc has been shown to be determined to a large extent by the pH of the soil. Thus Camp (1945) found that zinc deficiency was likely to occur on alkaline or even neutral soils.

CHAPTER II.

EXPERIMENTAL METHODS.Introduction.

The culture technique was essentially that of Steinberg (1936), using the optimum dibasic solution to which molybdenum was added. Good quality distilled water was used and the stock solutions were purified by the method of Stout and Arnon (1939).

The value of the water culture technique for the investigation of plant nutrition is widely recognised, because it permits strict control of the supply of nutrients. Every effort was made to ensure that the solutions were free from contaminants and that the containers were chemically inactive.

Water Purification.

All water used in this investigation was tap water which had been distilled through an electric tin lined still, and subsequently purified by either of the following methods:

Glass redistillation. The distilled water obtained from the electric still was used to fill a constant level "Pyrex" glass still as described by Wilson and Strickler (1939), and the redistilled water was collected in a "Pyrex" glass aspirator. This method was only used during the initial stages of the investigation.

Demineralisation. The apparatus used is shown in figure 1. Water from the electric still was used to fill the "Pyrex" aspirator (A), from which it was siphoned by way of a "Pyrex" tube (p), into a monobed resin column (R). The demineralised water was collected in the "Pyrex" aspirator (D). The rate of flow was controlled by the screw-clip (S).

The resin column contained "Amberlite" MB3, in three layers ($1\frac{1}{2}$ inches deep) separated by "Pyrex" glass wool (V). Care was taken to ensure that the capillary (C), never drained the water from the column

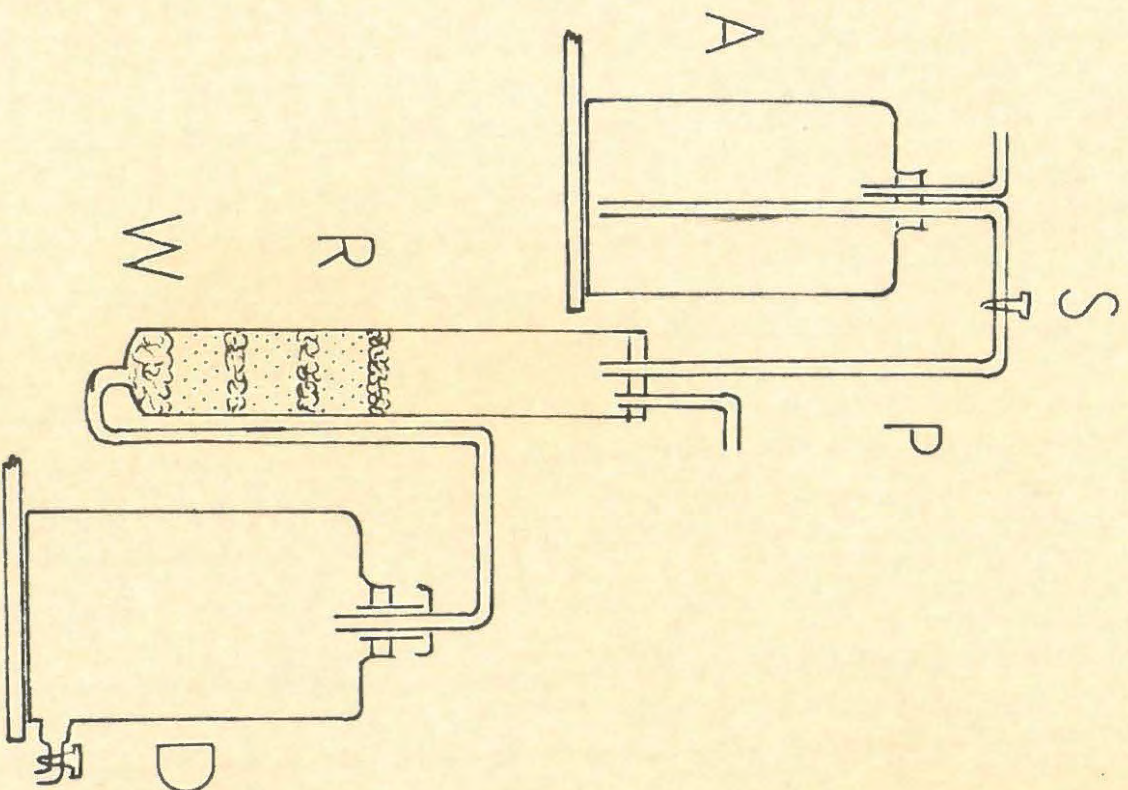


FIGURE 1. Diagram of the apparatus used for production of purified water.

thus allowing the resin to be exposed. Degeneration of the resin was shown by a colour change from green to orange. The resin was changed whenever the resin in the second layer showed colour change.

Containers and experimental vessels.

All the glassware used during the course of this investigation was of "Pyrex" quality. The culture vessels were wide-mouth 250 ml. Erlenmeyer flasks. Stock solutions were stored in 1 litre polythene bottles.

The containers and glassware were cleaned in the following manner. They were first washed in tapwater with teepol followed by thorough rinsing in tapwater. This was followed by rinsing in a 1 : 1, HCl : water solution, then thorough rinsing in purified water.

Preparation and purification of sucrose solution.

Sucrose of "Analar" grade was used throughout the investigation. A concentration of 50 grams of sucrose per litre of culture solution was used. The sucrose solution was purified by passage through a resin column. The apparatus used is shown in figure 2.

A concentrated solution of sucrose was prepared in a 5 litre "Pyrex" flat-bottomed flask (A). The solution was siphoned into two resin columns (R), the flow being controlled by screwclips (S). The purified solution was collected in a second 5 litre flat-bottomed "Pyrex" flask (B). The resin columns contained "Amberlite" MB3 in a single layer $1\frac{1}{2}$ - 2 inches deep. "Pyrex" glass wool (w) was placed above and below the resin in order to keep it in place. When the sucrose solution had been purified the resin was cleaned by allowing purified water to flow through the columns. The resin columns were stored with water covering the resin.

Preparation of stock solutions.

Reagents of "Analar" quality were used throughout the course of the investigation.

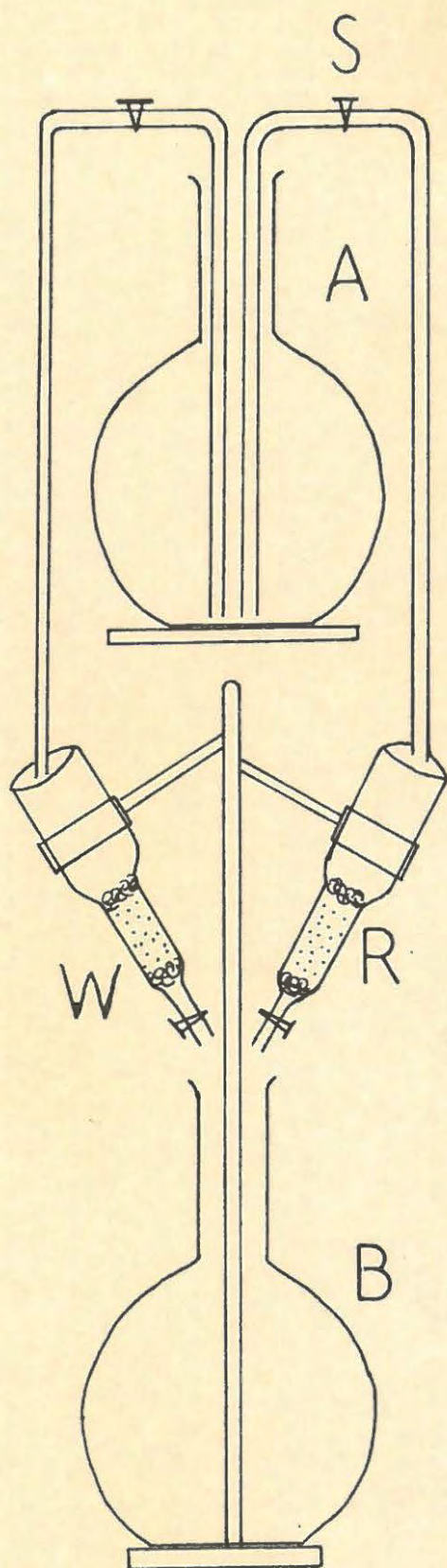


FIGURE 2. Diagram of the apparatus used for the purification of sucrose.

(i) Ammonium nitrate.

400 grams of NH_4NO_3 were dissolved in 1 litre of purified water. To this was added:

13 grams of CaCO_3

5 ml. 1.0 Molar $\text{Ca}(\text{NO}_3)_2$

5 ml. 1.0 Molar K_2HPO_4 .

This solution was shaken thoroughly and steamed for one hour. The cool solution was filtered and the filtrate was stored in a 1 litre polythene bottle.

The required concentration of NH_4NO_3 in the culture solution was 2.0 grams per litre, thus 5 ml. of the stock solution was added for each litre of culture solution.

(ii) Magnesium sulphate.

120 grams of MgSO_4 was dissolved in 1 litre of purified water. To this was added:

13 grams of CaCO_3

5 ml. 1.0 Molar $\text{Ca}(\text{NO}_3)_2$

5 ml. 1.0 Molar K_2HPO_4

This solution was shaken thoroughly and then autoclaved at 15 lbs. pressure for one hour. The solution was then filtered through Whatman number one filter paper, and the filtrate stored in a 1 litre polythene bottle.

The required concentration of MgSO_4 in the culture solution was 0.6 grams per litre, thus, 5 ml. of the stock solution was added for each litre of culture solution.

(iii) Dipotassium phosphate.

96 grams of K_2HPO_4 was dissolved in 1 litre of purified water. To this was added:

13 grams of CaCO_3

5 ml. of 1.0 Molar $\text{Ca}(\text{NO}_3)_2$.

The solution was shaken thoroughly and autoclaved at 15 lbs. pressure for one hour and then filtered through Whatman number one paper. The filtrate was stored in a 1 litre polythene bottle.

The required concentration of K_2HPO_4 in the culture solution was 0.48 grams per litre, thus, 5 ml. of the stock solution was added for each litre of culture solution.

(v) Micronutrients.

The stock solutions of the micronutrients, iron, copper, zinc, manganese and molybdenum, were made up in three ways. In all cases the concentration of the stock solution was calculated so that a 1 ml. sample added to 50 mls. resulted in the required concentration. All stock solutions were stored in 1 litre polythene bottles.

(a) Solutions of the five metals were made up separately to enable the study of their separate effects.

(b) A stock solution containing copper, zinc and molybdenum was made up in order to allow known amounts of iron and manganese to be added to the culture medium.

(c) A stock solution of iron, manganese and molybdenum was prepared in order to allow known amounts of copper and zinc to be added to the culture medium.

The solutions referred to in (a) above were prepared in the following manner:

Iron.

0.99555 grams of $FeSO_4 \cdot 7H_2O$ was weighed and dissolved in purified water in a volumetric flask. The volume was made up to 1 litre. A 1 ml. sample of this in 50 ml. gave an iron concentration in the medium of four parts per million.

Manganese.

0.81195 grams of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was weighed and dissolved in purified water in a volumetric flask. The volume was made up to 1 litre. A 1 ml. sample of this in 50 ml. gave a manganese concentration of four parts per million.

Copper.

0.00390 grams of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was weighed and dissolved in purified water in a volumetric flask. The volume was made up to 1 litre. A 1 ml. sample of this solution in 50 ml. gave a copper concentration of 0.02 parts per million.

Zinc.

0.01920 grams of $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ was weighed and dissolved in purified water in a volumetric flask. The volume was made up to 1 litre. A 1 ml. sample of this solution in 50 ml. gave zinc concentration of 0.14 parts per million.

Molybdenum.

0.00915 grams of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ was weighed and dissolved in purified water in a volumetric flask. The volume was made up to 1 litre. A 1 ml. sample of this solution in 50 ml. gave a concentration of molybdenum of 0.01 parts per million.

In solutions (b) and (c) referred to above, where a single stock solution containing different concentrations of the micronutrients was prepared, the concentration of the micronutrients was calculated so that a 1 ml. sample in 1 litre of stock culture solution, supplied the micronutrients in the required concentration.

Solution (b) was prepared in the following manner:

0.07854 grams $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

0.38415 grams $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$

0.18404 grams $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$.

The above were dissolved together in purified water in volumetric

flask, the volume being made up to 1 litre. A 1 ml. sample of this stock solution in 1 litre of culture solution gave the following concentrations of the micronutrients:

Copper 0.02 parts per million.

Zinc 0.14 parts per million.

Molybdenum 0.01 parts per million.

Solution (c) was prepared in the same manner:

19.91047 grams $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

16.23885 grams $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$.

0.18404 grams $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$.

The above were dissolved in purified water in a volumetric flask and the volume was made up to 1 litre. A 1 ml. sample of this stock solution in 1 litre of stock culture solution gave the following concentrations of the micronutrients.

Iron 4 parts per million.

Manganese 4 parts per million.

Molybdenum 0.01 parts per million.

Dispensing of stock culture solution.

The solution containing sucrose, magnesium sulphate, ammonium nitrate, di-potassium hydrogen phosphate and the micronutrients as required was placed in a "Pyrex" aspirator (A), see figure 3. The aspirator was connected to two automatic 50 ml. pipettes (P). In this manner 50 ml. of the culture solution could be dispensed into each culture vessel.

Varying the heavy metal balance.

A standard sample of 1 ml., as a source of supply of each metal under consideration was used throughout the investigation. The balance was varied by adding samples of different concentrations of the heavy metals under consideration.

The different concentrations of the heavy metals were prepared in the following manner: In each experiment, a solution of each heavy

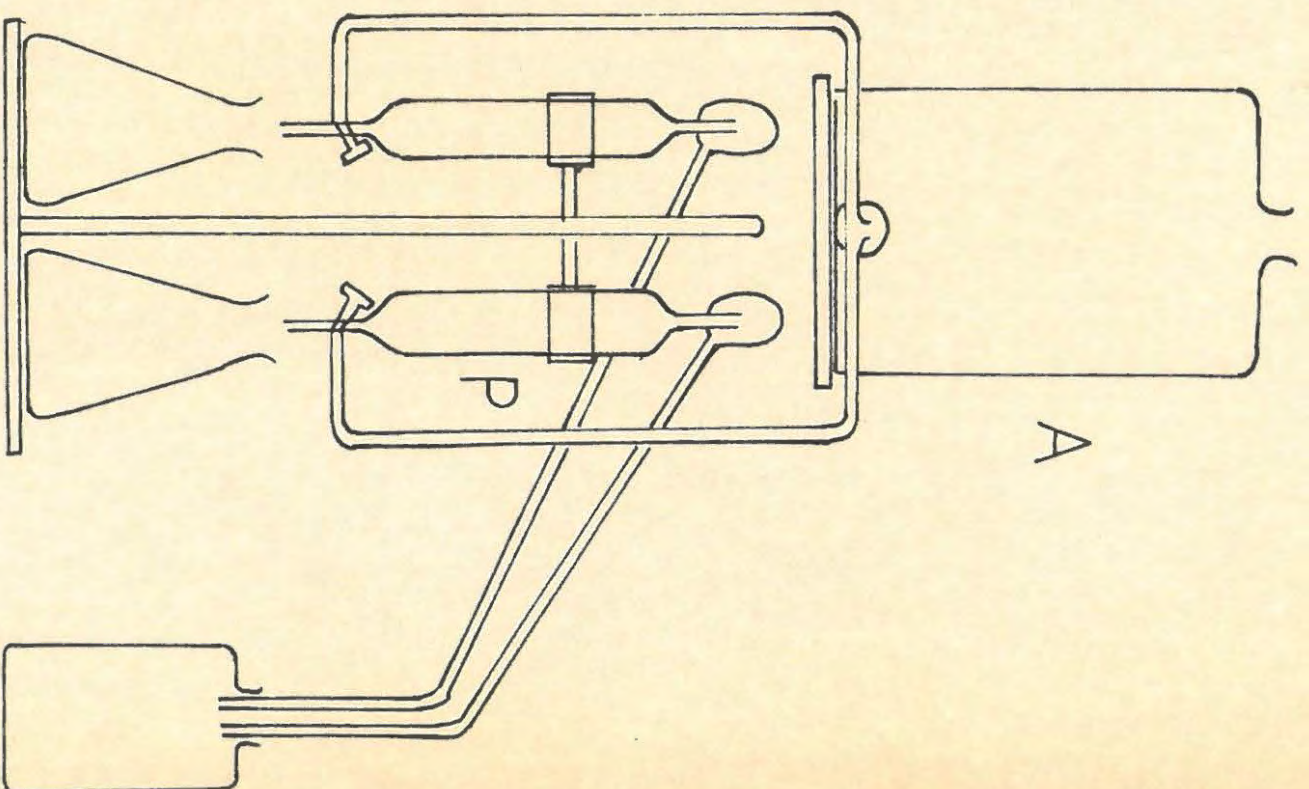


FIGURE 3. Diagram of the apparatus used for dispensing the culture solution.

metal under consideration was prepared. The concentration in each solution was calculated so that a 1 ml. sample in 50 ml. supplied the maximum concentration required. Less concentrated solutions were prepared by subsequent dilution of the first prepared solution. In this way a series of different concentrations of each heavy metal was prepared.

During the course of the investigation, experiments were carried out in which the heavy metal supplies were prepared with a chelating agent. Two agents were used, citric acid and ethylene diamine-tetra-acetic acid, and in each case they were supplied in equivalent amounts. The amounts used are given in the text of each experiment.

Sterilising of culture medium.

The flasks containing the culture medium were plugged with cotton wool and autoclaved at 15 lbs. pressure for 20 minutes. Care was taken to ensure that the flasks were arranged in a random manner in the autoclave, and that the cotton wool plugs were covered by paper to prevent water condensing on them. Where all the flasks could not be autoclaved at the same time, they were autoclaved in random samples.

Maintenance of cultures.

Three fungi were used in the investigation; Aspergillus niger, Penicillium notatum and Penicillium glaucum. They were cultured on 2.5 malt, 2% agar slopes. Agar slopes were sterilised at 15 lbs. pressure for 20 minutes and then stored in a cold room.

The slopes were inoculated and incubated in the dark at 25°C. The incubation period was 9 - 14 days in the case of Aspergillus and 17 - 21 days in both Penicillium notatum and Penicillium glaucum. These slopes were used in the preparation of the spore suspension.

Preparation of spore suspension.

Two slopes of the required fungus were used, in each case, as a source of spores.

200 mls. of the basic culture solution was placed in a 300 ml. conical flask. This was autoclaved at 15 lbs. pressure for 20 minutes. Four drops of teepol were added to the culture solution and after shaking, about 5 ml. of the solution was poured into the tube containing the fungal culture. The tube was then rapidly rotated between the palms of the hands to dislodge the spores. This spore suspension was returned to the flask. This was repeated for the second tube. The flask was then shaken thoroughly to ensure that the aggregates of spores were broken down. The function of the teepol was to reduce the surface tension, thereby aiding the separation of the spores.

Sterilising of pipettes for inoculation.

Clean 1 ml. pipettes were loosely plugged at the mouth, with cotton wool, leaving no threads protruding. These were then placed in a copper canister and sterilised by heating in an hot air oven at 135^o-145^oC. for two hours.

Inoculation.

A standard inoculum of 1 ml. was used throughout the investigation. The spore suspension was shaken between samples in order to eliminate variations between samples. The culture vessels were inoculated in a random manner so that if there was any difference due to the spore suspension it would be distributed throughout the experiment.

Incubation.

Incubation was in the dark at 25^oC. The culture vessels were randomly arranged in the incubator in order to avoid any differences, as a result of convection, being confined to any section of the experiment.

Investigation of results.

(a) Visual effects.

Prior to harvesting the mycelium was studied to ascertain any visual effects as a result of the varying heavy metal balances.

The mycelium produced was described under the following headings:

1. Amount of mycelium covering the surface of the culture medium.

An attempt was made to distinguish between cultures in which the felt completely covered the surface of the culture medium, and those in which the felt only partially covered the surface, either as a single piece or as a number of small pieces or "pellets".

2. Structure of the felt.

This was considered from two aspects:

(a) Compactness: described by assigning the following symbols:

Ct. - cottony.

SCp. - slightly compact.

MCp. - moderately compact.

VCp. - very compact.

(b) Wrinkling of the surface: described by assigning the following symbols:

Sm - smooth.

SW - slightly wrinkled.

MW - moderately wrinkled.

HW - heavily wrinkled.

VHW - very heavily wrinkled.

3. Sporulation.

The degree of sporulation was described by a number according to the scale where:

0 complete sterility.

5 felt moderately covered with spores.

10 felt densely covered with spores.

This system had the disadvantage that it was difficult to assign an index to cultures in which a few pellets of mycelium were produced, but which were densely covered with spores. Thus the method was used purely as a means of assessing general trends in the sporulation.

Morphological investigations.

Small amounts of the mycelium were removed from the cultures and examined to determine any effects on the cellular morphology of the mycelium and spores. Particular attention was paid to the effect on branching of the hyphae.

Harvesting of felts.

The mycelium was removed from the culture medium by filtration through 15 cm. "Whatman" No. 1. filter paper. The felts were washed twice with distilled water.

In the case of Aspergillus, where a considerable amount of submerged mycelium was produced, the culture medium was thoroughly stirred to ensure that all the mycelium was filtered off.

Trial runs in which culture solution was passed through the filter paper, followed by two washings with distilled water were carried out. In this way when the correction factor for the weight of the filter paper was obtained (Table 1 of Preliminary Investigations).

Drying of filter paper and mycelium.

The filter papers containing the mycelium were folded so that the mycelium was completely enclosed within the paper. The filter papers were then placed on drying trays. The drying trays were made up of a frame, which was subdivided by slats into a number of compartments. The bottom of the tray was made of wire gauze to ensure that convection in the oven was not prevented.

The trays containing the pieces of filter was then placed in an hot air oven at 70 C. for three days. The trays were then removed to a dessicator, containing calcium chloride, until they were cool after which they were weighed.

Weighing of filter paper and mycelium.

The pieces of filter paper containing the mycelium were removed from the dessicator and weighed immediately, in order to avoid, as far as possible, uptake of moisture from the air by the filter paper. The

balance used throughout the investigation was a "Mettler" model B.

Measurement of pH. of the filtrate.

The pH. of the culture solution after growth was measured, using a portable "Beckman" pH meter. Samples of culture medium were autoclaved at 15 lbs. pressure for 20 minutes and the pH. of these samples was measured (Table 2 of Preliminary Investigations). In this way it was possible to determine the effect of growth of the fungi on the pH. of the culture medium.

Statistical methods.

Two types of analysis were used, analysis of variance, and regression analysis. Examples of these are given in part 2 of the Appendix.

CHAPTER III.

PRELIMINARY INVESTIGATION.

EXPERIMENT 1. To determine the correction factor for the calculation of the dry weight yield of mycelium harvested.

The fungal growth in each culture vessel was harvested by filtering through 15 cm. Whatman No. 1 circles. To facilitate drying and weighing, the felt was treated together with the filter paper. In order to obtain the absolute weight of the mycelium, a correction factor for the weight of the filter paper had to be obtained. This was done in the following manner.

Basic culture solution (for composition see Table 1) was filtered through 80 separate 15 cm. circles of Whatman No. 1 filter paper. Each piece was subsequently washed twice with distilled water. The pieces of filter paper were then dried at 70°C for 48 hours in a hot air oven and then removed to a dessicator and allowed to cool before they were weighed. The mean weight for the pieces of filter paper was calculated and used as the correction factor throughout the course of the investigation.

Table 1. Composition of basic culture solution used in Experiments 1 and 2.

Constituent	Level of supply in gms/litre
Sucrose	50.0
Magnesium sulphate	0.6
Ammonium nitrate	2.0
Dipotassium hydrogen phosphate	0.48

Table 2. Dry weight, in grams, of 80 separate pieces of 15 cm. Whatman No. 1 filter paper, after filtering basic culture solution, followed by two rinses with distilled water.

1.44028	1.45102	1.44143	1.47385
1.47341	1.441401	1.43264	1.45350
1.49315	1.43963	1.47278	1.46398
1.46966	1.46582	1.43561	1.51022
1.45241	1.43996	1.41605	1.41724
1.44793	1.52570	1.41694	1.54705
1.43058	1.42944	1.51389	1.44522
1.44700	1.44674	1.44061	1.49993
1.46014	1.42148	1.44441	1.52103
1.49315	1.47741	1.44163	1.47015
1.47142	1.47231	1.43408	1.47341
1.44032	1.46015	1.43963	1.43963
1.50123	1.47364	1.45014	1.46015
1.44437	1.54017	1.44012	1.44421
1.47341	1.43014	1.43044	1.45014
1.42443	1.45025	1.49360	1.43331
1.50214	1.49957	1.44012	1.46773
1.52214	1.48642	1.40309	1.41632
1.45132	1.51112	1.46814	1.41637
1.51023	1.44412	1.48321	1.44213

Correction factor (mean weight of one piece of filter paper

= 1.46015 gms.

Weight of filter paper and mycelium = M

Weight of filter paper only (correction factor) = 1.46015 gms.

Therefore weight of mycelium only = M - 1.46015 gms.

EXPERIMENT 2. To determine the mean pH of culture solution after autoclaving at 15 lbs pressure for 20 minutes.

The report that zinc acts adversely on the accumulation of organic acids in fungal cultures (Chesters and Rolinson 1951b), and that iron is essential for maximum yields of citric acid in Aspergillus niger (Perlman 1947), stimulated an attempt to gauge acid production under different treatments.

The method employed was to standardise the pH of the culture solution before autoclaving, to ensure that the pH was suitable before inoculation, and then to sample the filtrate after harvesting the felts. It was found, however, that autoclaving the culture solution decreased the pH slightly. To overcome this, an experiment was carried out to determine the average pH of the culture solution after autoclaving.

Basic culture solution (for composition see Table 1) was prepared and the pH standardised at 7.13 using 0.1N HCl. This was dispensed in 50 cc. aliquots into 50 flasks, which were then plugged with cotton wool and autoclaved at 15 lbs. pressure for 20 minutes. When they had cooled to room temperature, the pH was measured. The pH values, obtained after autoclaving, are given in Table 3.

Using the mean of the 50 pH values as the pH level of the culture solution prior to inoculation, the change in pH due to acid production during growth could be measured. This change was taken as an index of acid production.

Table 3. The pH values of 50 samples of basic culture solution after autoclaving at 15 lbs. pressure for 20 minutes.

6.75	6.79	6.75	6.76	6.78	6.72	6.78	6.73	6.76	6.74
6.72	6.78	6.73	6.73	6.74	6.76	6.73	6.78	6.72	6.72
6.73	6.78	6.72	6.77	6.77	6.78	6.78	6.73	6.75	6.73
6.78	6.78	6.69	6.77	6.76	6.79	6.73	6.72	6.75	6.76
6.73	6.71	6.78	6.74	6.74	6.74	6.76	6.74	6.79	6.68

Standardised pH before autoclaving = 7.13

Mean pH after autoclaving = 6.75

EXPERIMENT 3. To investigate the effect of varying the level of supply of iron, manganese, copper and zinc in the culture medium on the growth and development of Aspergillus niger and Penicillium notatum.

Perlman (1949) has reported that very varied results can be obtained from different strains of the same fungus, under the same conditions. Thus, in order to investigate the effect of varying the level of supply of the heavy metals, iron, manganese, copper and zinc, it was decided to carry out a series of preliminary experiments.

This experiment was designed to indicate:-

- (i) at what level of supply of the different heavy metals a toxic effect became apparent, and
- (ii) whether, within the range of tolerance of each heavy metal, a range of concentrations could be found, within which Aspergillus niger and Penicillium notatum were most sensitive to small changes in the level of supply.

The design of the experiment is shown in Table 4.

The method used in the experiment was to dispense, into a series of nine flasks, culture solution, (for composition see Table 5) which was deficient in one of the heavy metals to be studied. The metal omitted was subsequently added to supply the different concentrations required. The series of stock solutions used for each metal was prepared by dilution of a single concentrated stock solution, which was prepared so that a 1 ml. sample in 50 ml. gave a concentration of 26.2144 p.p.m. From Table 4 it will be apparent that the dilution factor was 4. Thus using the stock solution first, the second concentration was prepared by a 1 in 3 dilution (1 volume solution in 3 volumes purified water). Similarly the third solution was produced by a 1 in 3 dilution of the second solution. This procedure

repeated until nine solutions had been prepared. This was carried out for iron, manganese, copper and zinc, so that four series of nine treatments in each were prepared. In each series only one factor, the concentration of one of the heavy metals, was varied.

Since this experiment was preliminary in nature, no replications of the treatments were prepared.

Table 4. The plan of Experiment 3, showing four series of flasks, one for each of iron, manganese, copper and zinc, and the levels of supply of the variable in parts per million.

	Series 1	Series 2	Series 3	Series 4
	Iron	Manganese	Copper	Zinc
	0.0004	0.0004	0.0004	0.0004
	0.0016	0.0016	0.0016	0.0016
	0.0064	0.0064	0.0064	0.0064
	0.0256	0.0256	0.0256	0.0256
	0.1024	0.1024	0.1024	0.1024
	0.4096	0.4096	0.4096	0.4096
	1.6384	1.6384	1.6384	1.6384
	6.5536	6.5536	6.5536	6.5536
	26.2144	26.2144	26.2144	26.2144

Owing to the presence of a precipitate in the flasks containing high levels of iron it was decided to use an equivalent amount of Ethylene diaminetetra-acetic acid (E.D.T.A.) in the preparation of the iron solution. The stock solution from which dilutions were made, was prepared by dissolving 0.87358 gms. E.D.T.A. and 0.65240 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ together in purified water in a 100 ml. volumetric flask.

The stock solutions of manganese, copper and zinc were prepared as follows:

Manganese: 0.5321 gms. of $MnSO_4 \cdot 4H_2O$ was dissolved in purified water in a 100 ml. volumetric flask.

Copper : 0.51484 gms. of $CuSO_4 \cdot 5H_2O$ was dissolved in purified water in a 100 ml. volumetric flask.

Zinc : 0.35965 gms. $ZnSO_4 \cdot H_2O$ was dissolved in purified water in a 100 ml. volumetric flask.

Table 5. The composition of the culture solution used in Experiment 3.

Element omitted	Constituents of Nutrient Solution							
	Ammonium nitrate gms./litre	Magnesium sulphate gms./litre	Dipotassium hydrogen phosphate gms./litre	Iron p.p.m.	Manganese p.p.m.	Copper p.p.m.	Zinc p.p.m.	Molybdenum p.p.m.
Iron	2.00	0.60	0.48	-	4.0	0.02	0.14	0.01
Manganese	2.00	0.60	0.48	4.00	-	0.02	0.14	0.01
Copper	2.00	0.60	0.48	4.00	4.0	-	0.14	0.01
Zinc	2.00	0.60	0.48	4.00	4.0	0.02	-	0.01

Results.

The dry weight yields of mycelium are given in Table 6, for Aspergillus niger, and in Table 7, for Penicillium notatum. The pH values of the filtrate are given in Tables 1 and 2 of the appendix for Aspergillus and Penicillium respectively.

- (1) The effect of varying the level of iron supply on the growth, sporulation and yield of Aspergillus niger and Penicillium notatum.

There is evidence in Tables 6 and 7 that increases in the iron supply up to 26.2144 p.p.m. resulted in increased dry weight yields,

TABLE 6. The dry weight yield, in grams, obtained with Aspergillus niger, for nine different treatments of iron, manganese, copper and zinc.

Concentration of Fe.Mn.Cu. and Zn in p.p.m.	Dry Weight Yield in Grams.			
	Iron level varied	Manganese level varied.	Copper level varied	Zinc level varied
0.0004	0.43743	0.52339	0.57331	0.33888
0.0016	0.41345	0.60850	0.61263	0.36091
0.0064	0.64766	0.55682	0.73919	0.33424
0.0256	0.56857	0.60152	0.59880	0.36624
0.1024	0.41350	0.74019	0.63262	0.43162
0.4096	0.58237	0.62360	0.67945	0.69902
1.6384	0.67220	0.41023	0.58836	0.30544
6.5536	0.68755	0.65314	0.60330	0.77030
26.2144	0.63983	0.52091	0.21880	0.87862

TABLE 7. The dry weight yield, in grams, obtained with Penicillium notatum, for nine different treatments of iron, manganese, copper and zinc.

Concentration of Fe.Mn.Cu. and Zn. in p.p.m.	Dry Weight Yield in Grams.			
	Iron level varied	Manganese level varied	Copper level varied	Zinc level varied
0.0004	0.23064	0.32494	0.25633	0.35136
0.0016	0.17234	0.39930	0.33861	0.39165
0.0064	0.30983	0.40117	0.29517	0.23438
0.0256	0.21596	0.35184	0.35072	0.35332
0.1024	0.33675	0.43138	0.28019	0.32290
0.4096	0.40791	0.27366	0.31194	0.40967
1.6384	0.35896	0.36299	0.42706	0.27716
6.5536	0.47838	0.30302	0.21964	0.36510
26.2144	0.69132	0.25944	0.27789	0.31694

in both Aspergillus and Penicillium. These increases of approximately 57% in Aspergillus and 59% in Penicillium, can only be considered as yield trends, since within the trends considerable variations in the yields were evident. With the evidence available, the slightly diminished yield obtained with Aspergillus at high levels of iron supply cannot be interpreted as being due to iron toxicity.

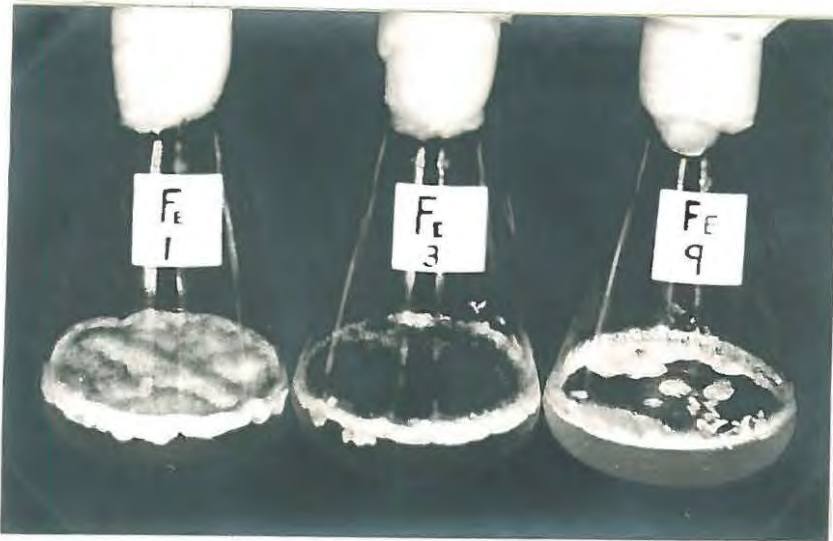
In both Aspergillus and Penicillium (Plates 1 and 2) the wrinkling of the surface of the felt could be correlated with the level of iron supply in the culture medium. There was a marked progressive increase in the wrinkling of the felt with increased iron supply (Plates 1 and 2).

Sporulation in Penicillium (Plate 2) was very slight in all treatments and no trends were evident. In Aspergillus (Plate 1) however there was some indication that sporulation was increased when the iron supply was raised to approximately 1.6384 p.p.m. Subsequent increases in the level of iron supply resulted in depressed sporulation.

- (2) The effect of varying the level of manganese supply on the growth, sporulation and yield of Aspergillus niger and Penicillium notatum.

Cultures of Aspergillus (Table 6) showed an approximately 42% increase in the dry weight yield as the level of manganese supply was increased from 0.0004 p.p.m. to 0.1024 p.p.m. There was a tendency for the yield to be depressed when the level of manganese supply was increased above 0.1024 p.p.m. The dry weight yield tended to increase with increased manganese supply up to 0.1024 p.p.m., after which level subsequent increases in the supply resulted in diminished yields.

The dry weight yield trends observed in Penicillium (Table 7) were very similar to those of Aspergillus. In general progressive increases in the yield were obtained with increases in the manganese



26.2144 p.p.m. 0.1024 p.p.m. 0.0004 p.p.m.

PLATE 1. The effect of increasing the iron supply
on the growth of Aspergillus niger.



26.2144 p.p.m. 0.1024 p.p.m. 0.0004 p.p.m.

PLATE 2. The effect of increasing the iron supply
on the growth of Penicillium notatum.

supply up to 0.1024 p.p.m. There was some evidence that the yield was progressively diminished by increases in the supply of manganese between 0.1024 p.p.m. and 26.2144 p.p.m.

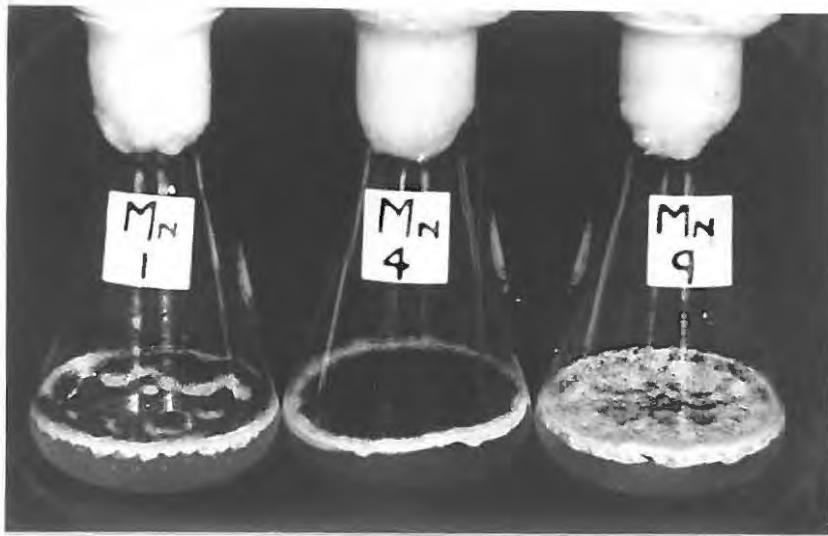
Thus there appeared to be a tendency, in both Aspergillus and Penicillium for the dry weight yield to increase progressively with increased manganese supply between 0.0004 p.p.m. and 0.1024 p.p.m., and to decrease progressively with increases in the supply of manganese between 0.1024 and 26.2144 p.p.m. The diminished yields observed in both Aspergillus and Penicillium at high manganese levels can be interpreted as being due to manganese toxicity.

The felts produced in cultures of Aspergillus and Penicillium showed a progressive wrinkling of the surface as the manganese supply was increased (Plates 3 and 4).

Increases in the manganese supply up to 0.1024 p.p.m. were beneficial to sporulation in cultures of Aspergillus (Plate 3). Above this level sporulation was slightly diminished, although dense sporulation was evident when the level of manganese supply was 26.2144 p.p.m. Cultures of Penicillium (Plate 4) showed very little sporulation and were almost sterile at all levels of manganese supply.

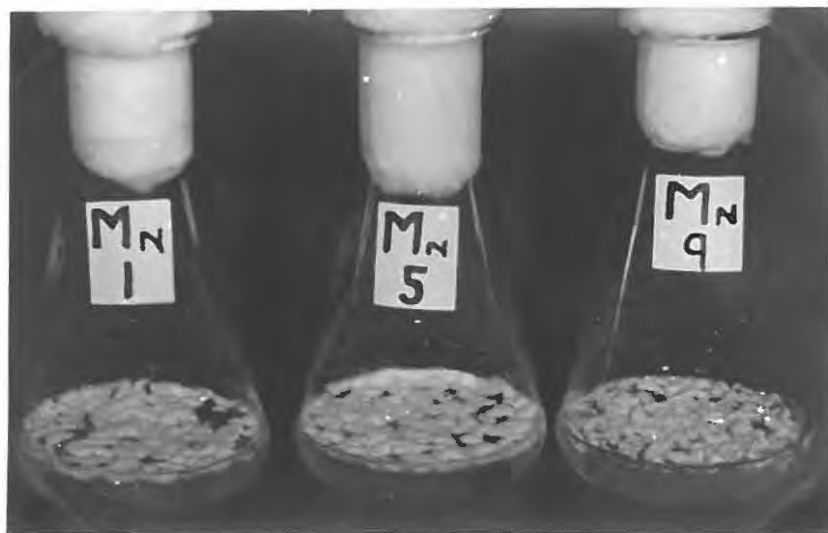
- (3) The effect of varying the level copper supply on the growth, sporulation and yield of Aspergillus niger and Penicillium notatum.

In cultures of Aspergillus (Table 6), increasing the supply from 0.0004 to 0.0064 p.p.m. had a favourable effect on the dry weight yield, but increases above this level markedly depressed the yields. It is noteworthy that the increase in the dry weight yield, obtained when the copper supply was increased to 0.0064 p.p.m. was approximately 29% whereas the yield was diminished by about 60% when the level of copper was increased to 26.2144 p.p.m. This very marked trend in the yield is probably due to copper-toxicity.



26.2144 p.p.m. 0.4096 p.p.m. 0.0004 p.p.m.

PLATE 3. The effect of increasing the manganese supply on the growth of Aspergillus niger.



0.0004 p.p.m. 0.4096 p.p.m. 26.2144 p.p.m.

PLATE 4. The effect of increasing the manganese supply on the growth of Penicillium notatum.

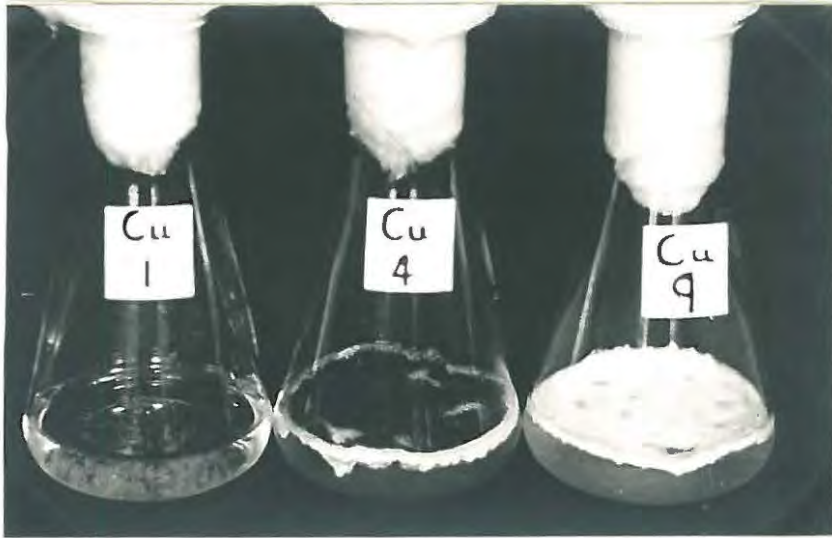
Penicillium (Table 7) cultures receiving copper supplies of between 0.0004 and 1.6384 p.p.m., gave progressively increased yields with increased copper supply but cultures receiving over 1.6384 p.p.m. copper showed a marked tendency to have their yields depressed. Thus whilst increases in the supply of copper up to 1.6384 p.p.m. were advantageous, increases above these levels resulted in copper toxicity.

There is evidence (Plates 5 and 6) that the level of copper supply determined the degree of wrinkling of the felt, in both Aspergillus and Penicillium. Increases in the copper supply resulted in increased wrinkling of the felt. Wrinkling was determined primarily by the level of copper supply since with Aspergillus (Plate 5) the felt produced at high copper levels (26.2144 p.p.m.) was more wrinkled than that produced at low levels (0.0004 p.p.m.), although, the yield was approximately 60% lower at the high levels, than at the low levels.

There is some indication (Plate 5) that the supply of copper could be raised to 0.1024 p.p.m. resulting in increased sporulation in cultures of Aspergillus. Above this level however there was a tendency for the sporulation to be suppressed. Thus at high levels of copper supply (26.2144 p.p.m.) the felt was sterile. The cultures of Penicillium (Plate 6) at levels of copper supply between 0.0004 and 26.2144 p.p.m. were all almost sterile and thus no trends were evident.

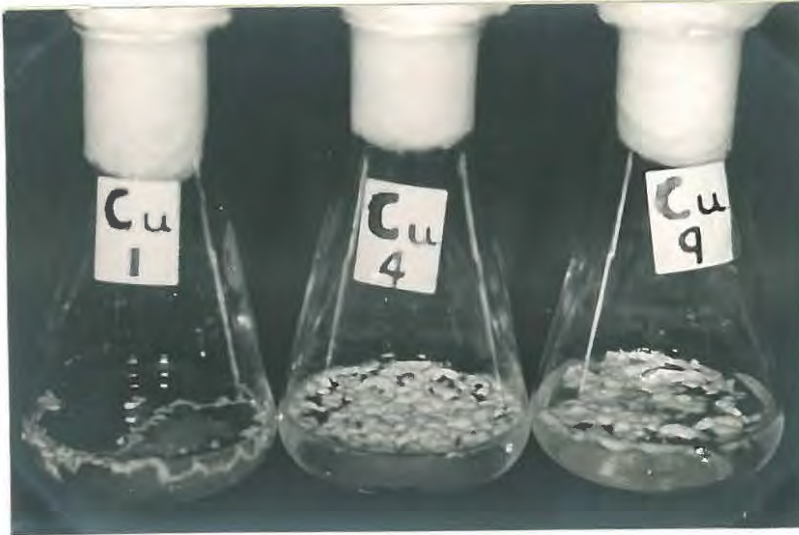
- (4) The effect of varying the level of a supply of zinc on the growth, sporulation and yield in Aspergillus niger and Penicillium notatum.

There was a marked progressive increase in the dry weight yield of Aspergillus cultures as the zinc supply was increased to 26.2144 p.p.m. (Table 6). At low levels of zinc supply, however,



26.2144 p.p.m. 0.4096 p.p.m. 0.0004 p.p.m.

PLATE 5. The effect of increasing the copper supply
on the growth of Aspergillus niger.



26.2144 p.p.m. 0.4096 p.p.m. 0.0004 p.p.m.

PLATE 6. The effect of increasing the copper supply
on the growth of Penicillium notatum.

(0.0004 - 0.1024 p.p.m.) the overall increase in the yield (27%) was not as marked as it was at high level (26.2144) where the yield had increased by 159%. Thus zinc appeared to exert a greater effect on the yield when the level was between 0.1024 - 26.2144 p.p.m. Cultures of Penicillium (Table 7) gave increased dry weight yields of about 16% when the zinc level was increased to 0.4096 p.p.m. Above this level there was some indication that the yields were depressed, but with the evidence available this was not conclusive.

There was a progressive increase in the wrinkling of the felt in cultures of Aspergillus and Penicillium (Plates 7 and 8) with increases in the zinc supply from 0.0004 to 26.2144 p.p.m.

At low levels of zinc supply the felts produced in cultures of Aspergillus (Plate 7) were profusely covered with black spores. There was some indication that increases in the supply of zinc up to 0.1024 p.p.m. resulted in slight increases in the sporulation, but increases above this level suppressed sporulation markedly. At high zinc levels (26.2144 p.p.m.) cultures of Aspergillus were almost sterile the spores produced being of an olive-brown colour. Cultures of Penicillium (Plate 8) were almost sterile for all levels of zinc between 0.0004 and 26.2144 p.p.m., and thus no trends in the sporulation were evident.

Discussion. In order to facilitate the discussion, the results obtained with Aspergillus niger and Penicillium notatum in Experiment 3 are summarised in Tables 8 and 9 respectively. The results obtained for each heavy metal studied are discussed separately.

Iron. Steinberg (1919) and Roberg (1928) have shown that large increases in the yield of Aspergillus niger could be obtained by the addition of iron to the culture solution. These results are confirmed, since an increase in the iron supply gave 57% increase in the yield in Aspergillus and 59% increase in Penicillium. The

TABLE 8. Summary of results obtained with *Aspergillus niger* in Experiment 3.

Iron, Manganese, Copper, Zinc Level Varied	ASPECT OF RESULTS CONSIDERED				
	Dry Weight Yield	Form of Felt	Degree of Sporulation	Changes in pH	Toxicity
Iron	Progressive increase in the yield with increased iron supply.	Felt becomes progressively wrinkled with increases in Fe supply.	Progressive increase in sporulation with increase in Fe supply to 0.1024 p.p.m. Subsequent increases result in diminished sporulation.	pH values consistently low, (1.98-2.36). No trends evident.	Iron levels above 0.1024 p.p.m. toxic to sporulation. No toxic symptoms in yield data.
Manganese	Progressive increase in the yield with increase in Mn supply to 0.1024 p.p.m. Subsequent increases result in diminished yield.	Felt becomes progressively wrinkled with increase in Mn supply.	Progressive increase in sporulation with increase in Mn supply to 0.4096 p.p.m. Subsequent increases result in slightly diminished sporulation.	pH values consistently low, (1.96 - 2.09). No trends evident.	Mn levels above 0.1024 and 0.4096 p.p.m. toxic to yield and sporulation respectively.
Copper	Progressive increase in the yield with increase in Mn supply to 0.0064 p.p.m. Subsequent increases result in diminished yield.	Felt becomes progressively wrinkled with increase in Fe supply. Growth entirely submerged at high levels (26.2144 p.p.m.)	Felt sterile at low levels. Progressive increase in sporulation with increase in Cu supply to 0.4096. Subsequent increases result in diminished sporulation.	pH values consistently low (1.95 - 3.39). No trends evident.	Cu levels above 0.0064 and 0.4096 p.p.m. toxic to yield and sporulation respectively.
Zinc	Progressive increase in the yield with increased Zn supply.	Felt becomes progressively wrinkled with increase in Fe supply.	Progressive increase with increase in Zn supply to 0.1024 p.p.m. Subsequent increases change spore colour, diminishes sporulation.	pH values consistently low (1.96 - 2.62). No trends evident.	Zn levels above 0.1024 p.p.m. toxic to sporulation. No toxic symptoms in yield.

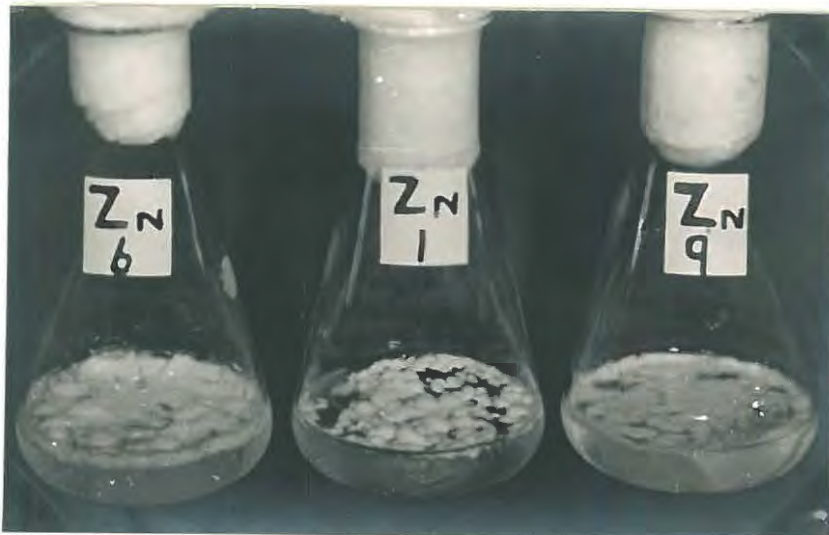
TABLE 9. Summary of Results obtained with *Penicillium notatum* in Experiment 3.

Iron, Manganese, Copper, Zinc Level Varied.	ASPECT OF RESULTS CONSIDERED				
	Dry Weight Yield	Form of Felt	Degree of Sporulation	Changes in pH	Toxicity
Iron	Progressive increase in the yield with increased supply.	Felt becomes progressively wrinkled with increased Fe supply.	Felts almost sterile. No trends evident.	pH values consistently low (2.36 - 3.31). No trends evident.	No symptoms of iron toxicity.
Manganese	Progressive increase in the yield with increase in Mn supply to 0.1024 p.p.m. Subsequent increases result in diminished yield.	Felt becomes progressively wrinkled with increased Mn supply.	Felts almost sterile. No trends evident.	pH values consistently low (2.42 - 2.66). No trends evident.	Mn levels above 0.1024 toxic to yield. No toxic symptoms in sporulation.
Copper	Progressive increase in the yield with increased Cu to 1.6384 p.p.m. Subsequent increases result in diminished yield.	Felt becomes progressively wrinkled with increased Cu supply.	Felts almost sterile. No trends evident.	pH values consistently low (2.35 - 2.59). No trends evident.	Cu levels above 1.6384 toxic to yield. No toxic symptoms in sporulation.
Zinc	Progressive increase in the yield with increased Zn to 0.4096 p.p.m. Subsequent increases result in diminished yield.	Felt becomes progressively wrinkled with increased Zn supply.	Felts almost sterile. No trends evident.	pH values consistently low (2.47 - 2.88). No trends evident.	Zn levels above 0.4096 toxic to yield. No toxic symptoms in sporulation.



26.2144 p.p.m. 0.1024 p.p.m. 0.0004p.p.m.

PLATE 7. The effect of increasing the supply of zinc
on the growth of Aspergillus niger.



0.0256 p.p.m. 26.2144 p.p.m. 0.0004 p.p.m.

PLATE 8. The effect of increasing the zinc supply
on the growth of Penicillium notatum.

report of diminished sporulation in iron poor cultures of Aspergillus, which was not subsequently confirmed by either Coupin (1903) or Sauton (1910), is confirmed. Sporulation in Aspergillus niger increased slightly with increases in the iron supply up to 0.1024 p.p.m. Further increases in the iron supply resulted in diminished sporulation (Plate 2).

Iron has been shown to be essential for citric acid production in Aspergillus niger (Perlman 1947, Shu and Johnson 1947). In the present investigation, the pH of the filtrate, after the harvesting of the felt, was used to gauge acid production. From Tables 1 and 2 of the Appendix, it is apparent that even in iron deficient (0.0004 p.p.m.) cultures, the pH of the filtrate was very low, 2.29 in Aspergillus and 2.61 in Penicillium. At high iron levels (26.2144 p.p.m.), the pH values were 2.01 in Aspergillus and 3.31 in Penicillium. No conclusions could be drawn with respect to the effect of iron on acid production.

Manganese. Bertrand (1912) and Steinberg (1936) report that increasing the supply of manganese results in increased yield. This was confirmed for increases in the manganese supply up to 0.1024 p.p.m. above which slightly decreased yields were obtained. Apart from the effect of manganese on yield, increasing the supply of manganese up to 0.1024 p.p.m. increases sporulation in Aspergillus niger. This is in agreement with Perlman (1947b) and Foster (1939), who reported that manganese deficient cultures showed diminished sporulation.

Although the addition of manganese to submerged cultures of Aspergillus is reported to reduce citric acid production (Shu and Johnson 1947 a and b), it appears to have little effect on the pH of surface cultures of Aspergillus niger and Penicillium notatum. The pH at low (0.0004 p.p.m.) manganese levels for Aspergillus and Penicillium were 2.04 and 2.55 respectively, and at high levels, (26.2144 p.p.m.), 2.01 and 2.52 respectively. This supports the

report by Shu and Johnson (1947 a and b), that additions of manganese have little effect on citric acid production in surface cultures of Aspergillus.

Copper. Chesters and Rolinson (1951 b and c) report that the copper requirements for normal growth in Aspergillus niger and Streptomyces griseus are between 0.01 p.p.m. and 0.10 p.p.m. Maximum yield in the present investigation was obtained when the level of supply was 1.6384 p.p.m. in Penicillium and 0.4096 p.p.m. in Aspergillus.

Bortels (1927), Javillier (1939) and Mulder (1939) have demonstrated reduction in pigmentation in Aspergillus niger when copper is deficient. This was not evident in the present work. The strain of Aspergillus niger used was not a copper sensitive strain. Clayton et al. (1944) have reported reduced pigmentation in copper deficient cultures of Penicillium notatum. This observation is not confirmed.

Mulder (1938) reported that copper was presumed to favour organic acid production, as evident by pH changes. In this investigation the pH values recorded for low levels of copper (0.0004 p.p.m.) were 2.06 and 2.50 for Aspergillus and Penicillium respectively. At high copper levels (26.2144 p.p.m.) the values were 3.39 and 2.52. The values of 3.39 for Aspergillus would suggest less acid production at high copper levels. This cannot be considered conclusive since there are no replications of treatments and since the pH value recorded when the level of copper supply was 6.5536 p.p.m. was 2.12.

The concentration of copper in the culture medium does not affect the pH of the culture medium to any marked extent.

Zinc. The most notable effect of zinc on the growth of Aspergillus is the increase in the dry weight yield (Steinberg 1919). This is confirmed in the present work, and also demonstrated for Penicillium notatum. The increased thickening and wrinkling of the surface of the pellicle, due to the addition of zinc, reported by Porges (1932) is also confirmed.

Porges (1932), reported that zinc deficient cultures of

Aspergillus niger were profusely covered with black spores whilst cultures in which the zinc level was 0.01% produce olive-green spores. These results are confirmed, but, contrary to the report by Steinberg (1948), the sporulation appears to be slightly decreased in zinc deficient cultures. At zinc levels of 1.6384 p.p.m. the spores of Aspergillus niger change from black to olive-brown. This has also been reported by Roberg (1928), where the colour change was reported to indicate the critical level for spore inhibition. When the zinc level was increased to 26.2144 p.p.m., the felt was entirely sterile.

Zinc is reported to act adversely of the accumulation of citric acid in cultures of Aspergillus niger (Chesters and Rolinson 1951a and Lockwood 1947). In the present investigation only slight decreases in the pH were recorded at the high levels of zinc supply (26.2144 p.p.m.). The pH values being 2.62 and 1.96 for Aspergillus and 2.88 and 2.47 for Penicillium, at high and low levels of zinc supply respectively.

Since the pH values are low at all levels of zinc supply no conclusions can be drawn with respect to the effect of zinc on acid production.

Conclusion.

Optimum levels of supply were apparent for manganese (0.1024 p.p.m.) in both Aspergillus and Penicillium, for copper 1.0064 p.p.m. in Aspergillus and 1.6384 p.p.m. in Penicillium and for zinc (0.4096 in Penicillium only). There was no evidence that cultures of Penicillium and Aspergillus were more sensitive to certain ranges of supply than to others.

There was little doubt that the method used was satisfactory for the study of the effect of varying the level of supply of different heavy metals on the growth and development of Aspergillus niger and Penicillium notatum. However marked discrepancies in the yield trends were evident, presumably due to contaminants present in the

culture medium or as a result of being introduced with the spore suspension. These could, to a certain extent, be overcome by replicating each treatment.

EXPERIMENT 4. To investigate the effect, on the growth and development of Aspergillus niger and Penicillium notatum, of varying the levels of supply of different pairs of heavy metals, when the ratio between the levels of supply of each metal was maintained at 1.0.

Previous workers (Bertrand 1912 Lockhead et al. 1946 and Brack 1947) have reported interactions between heavy metals, but the results are contradictory, and no work has been done on the fungi to ascertain whether different combinations of heavy metals function synergistically in growth when supplied at particular ratios. Many workers (Aso 1902, Pugliese 1913, Beck 1916, Scharrer and Schropp 1934 and Somers and Shive 1942) have shown that in certain flowering plants, maximum growth occurs when iron and manganese are present in a certain ratio.

In order to select different combinations of heavy metals for further investigation, it was decided to carry out a preliminary experiment to determine which combinations would prove most suitable for further investigations. This was done by preparing six series of nine culture flasks, and dispensing into each flask of a series, a 50 cc aliquot of culture solution, from which a known pair of heavy metals had been omitted (Table 10). Nine different levels of supply were selected for investigation (Table 10) and these were prepared by making separate solutions of each heavy metal, from which a 1 ml. sample in 50 ml. gave a concentration of 26.2144 p.p.m. These solutions were subsequently diluted by a factor of 4 (1 volume solution and 3 volumes purified water) to supply the nine different

concentrations from which 1 ml. samples were taken. In this way according to requirements, the iron: manganese, iron: copper, iron: zinc, manganese: copper, manganese: zinc or the copper:zinc ratio in the medium could be kept constant at 1.0 whilst the absolute supplies of the metals was varied.

Since this experiment was preliminary in nature, no replications of the different treatments were prepared. The result obtained can, however, be compared with those obtained in Experiment 3 because the two experiments were carried out simultaneously, using the same metal solutions, culture solution, and spore suspension. This was desirable so that the effect of varying the level of supply of two metals together could be compared with the effect of varying the level of supply of each separately, in the presence of adequate supplies of the other.

Results. The dry weight yields of mycelium are given in Tables 12 and 13, and the pH values in Tables 3 and 4 of the appendix, for Aspergillus and Penicillium respectively.

Table 11. The maximum % increase in the dry weight yield calculated from the yield obtained at the 0.0004 p.p.m. level of supply.

Treatment.	% increase in yield	
	Aspergillus	Penicillium
Iron: manganese	40	415
Iron:copper	71	124
Iron:zinc	129	173
Manganese:copper	19	10
Manganese:zinc	116	48
Copper:zinc	81	102

Table 11 gives the maximum percent increase in the yield calculated from the yield obtained at the 0.0004 p.p.m. level of supply. From this Table it is evident that considerable increases in the dry weight yield were obtained, with Aspergillus and Penicillium, in all treatments except with the Manganese:copper effect. The iron:manganese and copper:zinc balances were selected for further investigation because, in the former, iron and manganese have been shown to interact in the growth of flowering plants (Somers and Shive 1942) and in the latter, because whilst copper was highly toxic to yield in Aspergillus when supplied at high levels (26.2144 p.p.m.) in Experiment 3 (Table 6), no toxicity was evident in this Experiment (Table 11) when copper and zinc were supplied at high levels (26.2144 p.p.m.) together, with a copper:zinc ratio of 1.0.

The results obtained with the iron:manganese or the copper:zinc balances are considered in detail.

- (i) The effect of varying the level of supply of iron and manganese in the culture solution with a constant iron:manganese ratio of 1.0.

In cultures of Aspergillus and Penicillium, when the iron:manganese balance was maintained at unity, the absolute supply of iron and manganese could be increased with advantage (Tables 12 and 13), resulting in a 40% and 415% increase in the yield respectively. From Table 12 it can be seen that in Aspergillus there were considerable variations in the yields at low levels of supply (0.0004 - 0.1024 p.p.m.), but when the level of supply was above 0.1024 p.p.m. there was a marked tendency for the yield to increase progressively with increased iron and manganese supply. Although variations in the yield were also evident in cultures of Penicillium there is evidence (Table 13) that raising the iron and manganese

Table 12. The dry weight yield in grams obtained with Aspergillus niger, for six different combinations of iron, manganese, copper and zinc, and nine different treatments.

Concentration of each metal in pair in p.p.m.	Dry Weight Yield in Grams					
	Iron Manganese Ratio 1.0	Iron Copper Ratio 1.0	Iron Zinc Ratio 1.0	Manganese Copper Ratio 1.0	Manganese Zinc Ratio 1.0	Copper Zinc Ratio 1.0
0.0004	0.58606	0.43878	0.40010	0.59281	0.49913	0.55089
0.0016	0.49864	0.31423	0.24825	0.43666	0.33129	0.27512
0.0064	0.45992	0.33991	0.33348	0.67487	0.24578	0.31620
0.0256	0.49726	0.32616	0.39639	0.65994	0.34116	0.66392
0.1024	0.51363	0.29987	0.27128	0.57001	0.44085	0.68413
0.4096	0.62761	0.53070	0.69309	0.70604	0.57547	0.67813
1.6384	0.69865	0.69560	0.73810	0.48610	0.47673	0.94929
6.5536	0.65273	0.54355	0.91812	0.68737	1.07730	0.99633
26.2144	0.81867	0.75232	0.83145	0.21974	0.91589	0.91766

Table 13. The dry weight yield in grams, obtained with Penicillium notatum, for six different combinations of iron, manganese, copper and zinc, and nine different treatments.

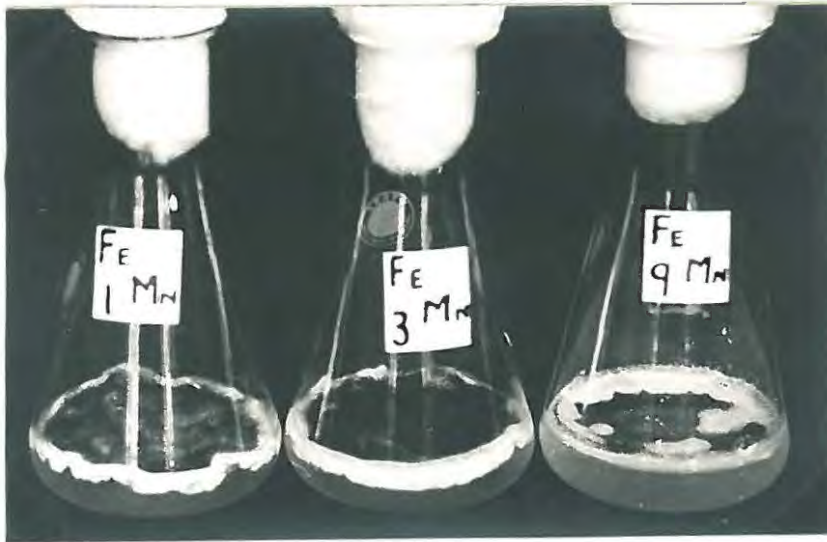
Concentration of each metal in pair in p.p.m.	Dry Weight Yield in Grams					
	Iron Manganese Ratio 1.0	Iron Copper Ratio 1.0	Iron Zinc Ratio 1.0	Manganese Copper Ratio 1.0	Manganese Zinc Ratio 1.0	Copper Zinc Ratio 1.0
0.0004	0.12795	0.29096	0.26007	0.39470	0.28224	0.20361
0.0016	0.19701	0.15023	0.16296	0.43484	0.22310	0.33538
0.0064	0.23691	0.24076	0.18325	0.42301	0.25047	0.32292
0.0256	0.18560	0.33790	0.29720	0.25382	0.24629	0.33938
0.1024	0.23024	0.21696	0.24368	0.38691	0.41921	0.41227
0.4096	0.29289	0.33446	0.19532	0.32765	0.32991	0.28581
1.6384	0.36092	0.33365	0.38149	0.28168	0.25763	0.30814
6.5536	0.24806	0.32489	0.43811	0.32555	0.39936	0.37995
26.2144	0.65919	0.65159	0.71076	0.27496	0.26622	0.16722

supply from low levels (0.0004 p.p.m.) resulted in progressively increased yields. Although with the evidence available no concrete conclusions can be drawn, it is noteworthy that the yield obtained with Aspergillus and Penicillium at high levels (26.2144 p.p.m.), were 25% and 166% respectively, higher than the yield obtained when the level of supply was 6.5536 p.p.m. These marked increases in the yield are considerably larger than increases between any other two consecutive yields in the series. This is also evident in the iron:copper and manganese:zinc investigations.

Cultures of Aspergillus and Penicillium receiving 26.2144 parts per million of iron and manganese showed no toxic symptoms, although with increased iron and manganese supply the surface of the felt became progressively more wrinkled in both Aspergillus and Penicillium (Plates 9 and 10). In cultures of Aspergillus (Plate 9) raising the supply of iron and manganese to 26.2144 p.p.m. resulted in increased sporulation, but in Penicillium (Plate 10) sporulation in all cultures was very low and varying the level of supply of iron and manganese had little effect.

- (ii) The effect of varying the level of supply of copper and zinc in the culture solution, with a constant copper:zinc ratio of 1.0.

There is evidence in Tables 12 and 13 that increases in the supply of copper and zinc in the culture medium of Aspergillus and Penicillium may be beneficial. In Aspergillus (Table 12) progressively increased yields were obtained when the level of copper and zinc was increased. With the evidence available, however, the high yield obtained at low levels of supply (0.0004 p.p.m.), and the slightly diminished yield at high levels (26.2144 p.p.m.) cannot be conclusively interpreted. The former is probably due to contamination whilst the latter may or may not represent a toxic



26.2144 p.p.m. 1.6384 p.p.m. 0.0004 p.p.m.

PLATE 9. The effect of *Aspergillus niger* of varying the levels of supply of iron and manganese with a constant iron:manganese ratio of 1.0.



26.2144 p.p.m. 0.1024 p.p.m. 0.0004 p.p.m.

PLATE 10. The effect on *Penicillium notatum* of varying the levels of supply of iron and manganese with a constant iron:manganese ratio of 1.0.

symptom. Cultures of Penicillium (Table 13) showed a tendency for the dry weight yield to increase with increased copper and zinc supply, up to 0.1024 p.p.m., above which there was some indication that the yields were depressed.

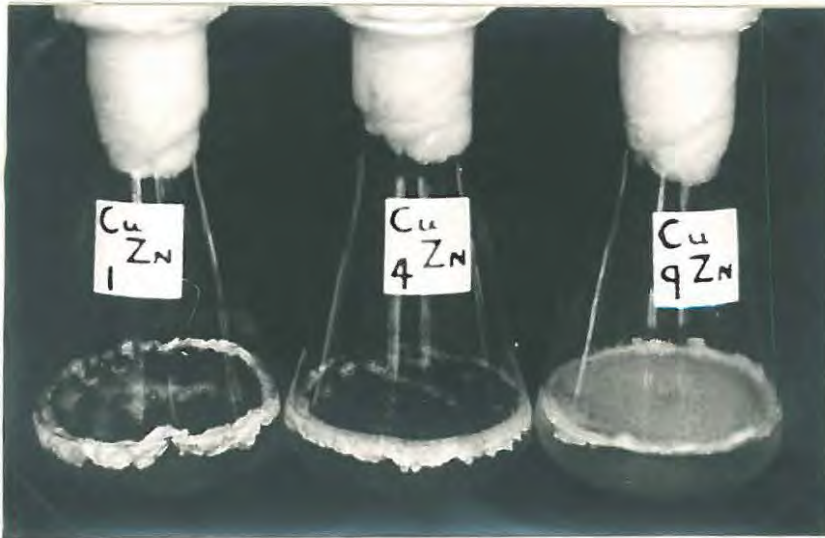
Cultures of Aspergillus and Penicillium (Plates 11 and 12) receiving low levels of copper and zinc supply (0.0004 p.p.m.) showed little wrinkling of the felt. With increased supply the felt became progressively more wrinkled. Increasing the supply of copper and zinc was beneficial to sporulation in Aspergillus (Plate 11) although there is some indication that sporulation may have been slightly suppressed at high levels (26.2144 p.p.m.) of copper and zinc supply. Sporulation was low in all cultures of Penicillium, and there was no evidence of a response to varying supplies of copper and zinc.

Discussion. The experiment was designed to investigate the effect of varying the level of supply of different pairs of heavy metals, when the ratio between the metals was maintained at unity. Using four heavy metals, iron, manganese, copper and zinc, there were six possible pairs, and of these two, the iron:manganese and the copper:zinc pairs were selected for further investigation. (See page 36). The discussion of the results is confined to these states of supply, and the results obtained were summarised in Table 14.

- (i) Varying the level of supply of iron and manganese
when the ratio of iron to manganese is 1.0.

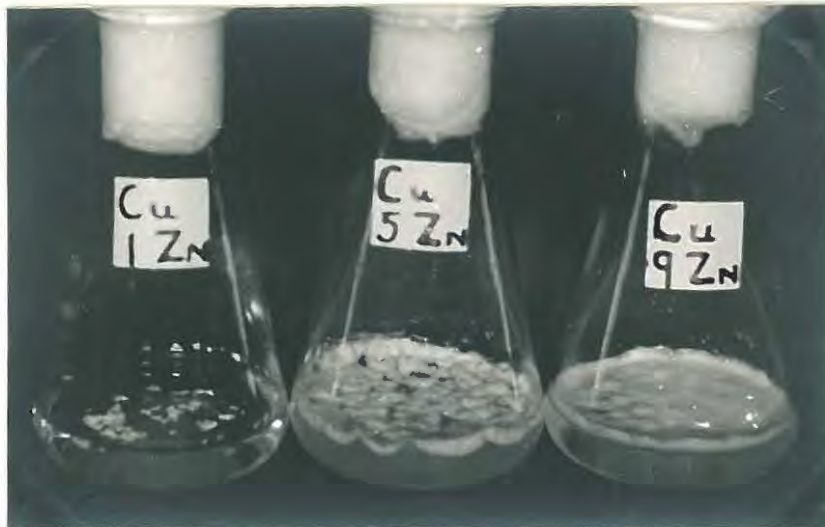
Aso (1902) demonstrated that the presence of iron brings about a reduction in the absorption of manganese in peas, radish, wheat and barley. Subsequent workers (Pugliese 1913, Beck 1916, Scharrer and Schropp 1934 and Somers and Shive 1942) have shown that maximum growth occurs when iron and manganese are present in a certain ratio.

Considering iron and manganese in Experiment 3, by virtue of



26.2144 p.p.m. 0.4096 p.p.m. 0.0004 p.p.m.

PLATE 11. The effect on Aspergillus niger of varying the levels of supply of copper and zinc with a constant copper:zinc ratio of 1.0.



26.2144 p.p.m. 0.1024 p.p.m. 0.0004 p.p.m.

PLATE 12. The effect on Penicillium notatum of varying the levels of supply of copper and zinc with a constant copper:zinc ratio of 1.0.

TABLE 14. Summary of the results obtained with Aspergillus niger and Penicillium notatum in Experiment 4.

Ratio Fe:Mn, Cu:Zn = 1.0 Level Varied	ASPECT OF RESULTS CONSIDERED					
	Dry Weight Yield	Form of Felt	Degree of Sporulation	Changes in pH	Toxicity	
Aspergillus	Iron : Manganese	Progressive increase in the yield with increased Fe and Mn supply.	Felt becomes progressively wrinkled with increased Fe and Mn supply.	Progressive increase in sporulation with increased Fe and Mn supply.	pH values consistently low (1.95 - 2.20). No trends evident.	No symptoms of toxicity.
	Copper : Zinc	Progressive increase in the yield with increased Cu Zn up to 6.5536. High levels possibly toxic.	Felt becomes progressively wrinkled with increased Cu and Zn supply.	Progressive increase in sporulation with increased Cu and Zn supply. High levels possibly toxic.	pH values consistently low (1.91 - 2.57). No trends evident.	High levels of Cu and Zn (26.2144 p.p.m.) may be slightly toxic to yield, and sporulation.
Penicillium	Iron : Manganese	Progressive increase in the yield with increased Fe and Mn supply.	Felt becomes progressively wrinkled with increased Fe and Mn supply.	Felts almost sterile. No trends evident.	pH values consistently low (2.36 - 3.24). No trends evident.	No symptoms of toxicity.
	Copper : Zinc	Progressive increase in the yield with increased Cu Zn to 0.1024 p.p.m. Subsequent increases diminished yield.	Felt becomes progressively wrinkled with increased Cu and Zn supply.	Felts almost sterile. No trends evident.	pH values consistently low (2.31 - 2.90). No trends evident.	Cu and Zn levels above 0.1024 p.p.m. toxic to yield.

the fact that when the iron level was varied the manganese level was kept constant at 4.0 p.p.m., and vice versa, the yields obtained represent yields obtained over a wide range of different iron:manganese balances. In fact when the iron level was varied the range in ratio of iron to manganese was 0.0001 to 6.5529, and when the manganese was varied and the iron kept constant at 4.0 p.p.m., the range in ratio was 10,000 to 0.1526. It may therefore be significant that iron toxicity was not evident, but the results obtained with manganese cannot be similarly explained. In the present experiment, the yields obtained with Aspergillus niger were markedly higher than the yields obtained for either the iron or manganese investigation in Experiment 3, (Tables 12 and 6 respectively), indicating that at all levels of supply an iron:manganese ratio of 1.0 is beneficial for growth. Also of importance is that the manganese toxicity evident in Aspergillus and Penicillium in Experiment 3 (Tables 6 and 7 respectively), is not evident when high levels of manganese are accompanied by high levels of iron.

- (ii) Varying the level of supply of copper and zinc when the ratio of copper to zinc is 1.0.

Considerably less work has been done on the interactions of copper and zinc than on iron and manganese interaction, and the work has dealt with interaction of copper with elements other than zinc. Greenwood and Hallsworth (1960) described a study of complex interactions shown by subterranean clover to variation in the supply of copper, molybdenum, calcium, phosphorous and nitrogen, and Brown and Holmes (1955) have reported a relationship between iron and copper but different plants were found to exhibit different behaviour in this respect.

In the same manner as with iron and manganese in Experiment 3 (Tables 6 and 7), the yields obtained were representative of a wide

range of ratios, whereas all the yields obtained in the present experiment (Tables 12 and 13) were obtained when the copper : zinc ratio was 1.0. In cultures of Aspergillus a copper:zinc ratio of 1.0 appeared to be beneficial to growth since the yields obtained were considerably higher than those obtained when a wide range of ratios was used. The marked copper toxicity in cultures of Aspergillus and Penicillium when copper alone was supplied at 26.2144 p.p.m. is not evident in Aspergillus and is considerably reduced in Penicillium, when high levels of copper are accompanied by high levels of zinc.

Conclusion.

There is evidence that increasing the levels of supply of different pairs of heavy metals, in particular iron and manganese and copper and zinc, results in increased dry weight yield, and in cultures of Aspergillus increased sporulation. Also evident is a marked reduction in symptoms of toxicity, when high levels of copper are accompanied by high levels of zinc. These results suggest that the iron manganese and copper:zinc ratios in the culture medium may be of some importance in determining the growth and development of cultures of Aspergillus niger and Penicillium notatum.

Before carrying out a detailed investigation it was decided to determine whether, using the technique applied in Experiments 3 and 4, the results obtained could be repeated.

EXPERIMENT 5.

The main object of this experiment was to determine whether, using the technique applied in Experiments 3 and 4, the results obtained with varying levels of supply of iron and manganese on cultures of Aspergillus niger and Penicillium notatum could be repeated; (Plan of experiment in Table 15). As a subsidiary object

the experiment was designed to observe the effect of supplying iron without a chelating agent. Thus in the experiment three sets of results were obtained:

- (1) Aspergillus - iron supplied with an equivalent of E.D.T.A.
- (2) Penicillium - iron supplied with an equivalent of E.D.T.A.
- (3) Aspergillus - no chelate supplied.

In the investigations where a chelate was used, three replications of each treatment were prepared.

Table 15. Plan of Experiment 5.

	Iron Level Varied.	Manganese Level Varied	Fe:Mn Ratio 1.0 Levels Varied
	0.0004	0.0004	0.0004
Levels of supply in p.p.m.	0.0016	0.0016	0.0016
	0.0064	0.0064	0.0064
	0.0256	0.0256	0.0256
	0.1024	0.1024	0.1024
	0.4096	0.4096	0.4096
	1.6384	1.6384	1.6384
	6.5536	6.5536	6.5536
	26.2144	26.2144	26.2144

Results. The results are given as follows:-

- (i) Iron supply prepared with a chelating agent:
 - Aspergillus - dry weight yield Table 16, pH values Table 5 of the appendix.
 - Penciillium - dry weight yield Table 17, pH values Table 6 of the appendix.
- (ii) Iron supply NOT prepared with a chelating agent:
 - Aspergillus - dry weight yield Table 18, pH values Table 7 of the appendix.

TABLE 16. The dry weight yield, in grams, obtained with *Aspergillus niger* for ten treatments of iron, manganese and iron:manganese ratio.

Metal or Metals Varied.	Level of Supply in p.p.m.									
	0	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144
		0.18489	0.33670	0.27412	0.34222	0.51381	0.80744	0.85880	0.55467	1.00578
Iron	0.14026	0.17865	0.15037	0.31118	0.39271	0.48242	0.37740	0.62651	0.75325	0.79710
		0.21958	0.23220	0.20303	0.44411	0.49466	0.54942	0.62101	0.65642	0.90322
		0.15155	0.18117	0.16948	0.17646	0.19965	0.21739	0.20484	0.16802	0.27114
Manganese	0.18038	0.10472	0.19555	0.16131	0.21879	0.23451	0.16773	0.29813	0.15522	0.17569
		0.33930	0.13721	0.14306	0.13612	0.14086	0.16340	0.19343	0.20326	0.28886
Iron:		0.10867	0.26904	0.20295	0.33563	0.64129	0.58130	0.75053	0.37521	0.87611
Manganese	0.14493	0.17058	0.20004	0.19631	0.38848	0.61910	0.38933	0.74314	0.74255	0.86535
Ratio = 1		0.18128	0.19580	0.26641	0.42951	0.59777	0.57612	0.51560	0.64437	0.95104

TABLE 17. The dry weight yield, in grams, obtained with *Penicillium notatum* for ten treatments of iron, manganese and iron:manganese ratio.

Metals or Metals Varied.	Level of Supply in p.p.m.									
	0	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144
		0.28316	0.27981	0.31257	0.33375	0.52520	0.65264	0.65038	0.70529	0.75874
Iron	0.24235	0.26582	0.28366	0.25571	0.36083	0.55633	0.63660	0.64949	0.82634	0.79885
		0.28608	0.28663	0.36413	0.33254	0.60673	0.60486	0.63892	0.68936	0.76774
		0.38783	0.28733	0.37157	0.29035	0.36032	0.40881	0.61238	0.60091	0.73446
Manganese	0.20956	0.34572	0.26511	0.28206	0.32044	0.36470	0.42820	0.53618	0.67035	0.59922
		0.24110	0.26463	0.36017	0.30027	0.31241	0.44410	0.62733	0.65196	0.65701
Iron:		0.32344	0.31111	0.35930	0.31625	0.32019	0.35772	0.31705	0.32894	0.28716
Manganese	0.24877	0.32336	0.33800	0.30986	0.27463	0.29032	0.35615	0.33738	0.32432	0.36962
Ratio = 1		0.25715	0.35018	0.37494	0.31391	0.63948	0.29663	0.23077	0.31183	0.38534

TABLE 18. The dry weight yield, in grams, obtained with Aspergillus niger for ten treatments of iron, manganese and iron:manganese ratio.

Level of Supply in p.p.m.	Metal or Metals Varied		
	Iron	Manganese	Iron:Manganese Ratio = 1.0
0	0.20128	0.32094	0.21017
0.0004	0.20000	0.24197	0.27843
0.0016	0.19259	0.29080	0.29000
0.0064	0.27682	0.22321	0.29672
0.0256	0.29634	0.22577	0.39360
0.1024	0.35965	0.29849	0.58306
0.4096	0.38838	0.28041	0.43512
1.6384	0.59973	0.21590	0.63236
6.5536	0.63300	0.28581	0.62664
26.2144	0.38430	0.18196	0.60542

1. The effect of varying the level of iron supply on the growth, sporulation and yield of Aspergillus niger and Penicillium notatum.

In cultures of Aspergillus and Penicillium the level of iron supply could be raised to 26.2144 p.p.m. with advantage (Tables 16 and 17 respectively). The diminished dry weight yield obtained at high levels of iron supply (26.2144 p.p.m.) with Aspergillus, when the iron was supplied without a chelating agent (Table 18) is not conclusive since there were no replications. There was a marked progressive increase in the wrinkling of the surface of the felt in Aspergillus and Penicillium when the level of iron supply was increased. Cultures of Aspergillus showed progressively increased sporulation with increased iron supply up to 26.2144 p.p.m. irrespective of the presence of a chelating agent, but all cultures of Penicillium showed a low level of sporulation and no trends were evident.

2. The effect of varying the level of manganese supply on the growth sporulation and yield of Aspergillus niger and Penicillium notatum.

The dry weight yields obtained from cultures of Aspergillus and Penicillium were not markedly affected by the level of manganese supply, (Tables 16, 17 and 18). However a progressive increase in the wrinkling of the felt was evident in both Aspergillus and Penicillium when the level of manganese supply was increased. Sporulation in Aspergillus was slightly increased by increased manganese supply, but in Penicillium, sporulation was very low in all treatments and no variation in the degree of sporulation could be attributed to the level of manganese supply.

3. The effect of varying the level of supply of iron and manganese with an iron:manganese ratio of 1.0.

Increases in the supply of iron and manganese supplies up to 26.2144 p.p.m., were beneficial to cultures of Aspergillus (Tables 16 and 18) and Penicillium (Table 17) as evidenced by the increase in the dry weight yield. In Aspergillus and Penicillium (Tables 16, 17 and 18) although the yields obtained when iron and manganese were varied together were higher than those obtained when manganese alone was varied, there is no evidence that they are markedly different from those obtained when iron alone was varied.

There was some indication that in Aspergillus and Penicillium increasing the supply of iron and manganese together resulted in progressive increases in the wrinkling of the felt. In cultures of Aspergillus there was a tendency for sporulation to increase with increased iron and manganese supply. This was not evident in Penicillium where sporulation was very low in all treatments.

Discussion. The results will be discussed, with reference to the results obtained in Experiments 3 and 4, under the following three main headings:

- (1) The relation between the iron and manganese supplies and the dry weight yield.
 - (2) The relation between visual symptoms and the iron and manganese supplies.
 - (3) The relation between the supply of iron and manganese and acid production as shown by pH studies.
- (1) The relation between the iron and manganese supplies and the dry weight yield.

Increases in the iron supply up to 26.2144 p.p.m. irrespective of whether a chelate was supplied, are considered to be beneficial

to cultures of Aspergillus and Penicillium since at high levels (26.2144 p.p.m.) there is no evidence of diminished yields. High levels of manganese, however, resulted in diminished yields in Aspergillus and Penicillium, in Experiment 3 but this is not confirmed in Experiment 4, although there was a general low response to changes in the level of manganese supply. There is little doubt that high levels of manganese supply are beneficial when accompanied by high levels of iron, since in Experiment 4 this resulted in the disappearance of the symptoms of manganese toxicity, and in Experiments 4 and 5 it resulted in a general increase in dry weight yield in response to changes in the absolute level of iron and manganese. This would suggest that in cultures of Aspergillus and Penicillium whilst high levels of iron are easily tolerated, and may be advantageous, high levels of manganese are only beneficial when accompanied by high levels of iron. Thus for absolute levels of supply up to 26.2144 p.p.m. the iron:manganese ratio must be 1.0 or more in order to obtain maximum dry weight yields.

- (2) The relation between visual symptoms and the iron and manganese supplies.

Increases in the supply of iron, manganese and iron and manganese together result in progressive increases in the wrinkling of the felt. There is little doubt that the degree of wrinkling is determined by the level of iron and manganese supplies, since at high levels of either iron and manganese, or both, the felt is wrinkled irrespective of the yield. Owing to a general low level of sporulation in Penicillium different degrees of sporulation could not be attributed to variations in the level of supply of iron and manganese. In Aspergillus however, there is evidence that sporulation is increased by increases in the supply of iron, manganese and iron and manganese together, although there was some indication that

high levels of manganese not accompanied by high levels of iron depressed sporulation. This latter fact appears to be the sole symptom showing that at high levels of supply a Fe:Mn ratio of 1.0 is beneficial, whilst a ratio below 1.0 is toxic to sporulation.

- (3) The relation between the supply of iron and manganese, and acid production as shown by pH changes.

In general the pH values recorded after harvesting the felt were very low, so that trends in acid production were difficult to discern, and this was made increasingly difficult by the variations obtained under the same treatment. There was evidence of one trend: In Experiment 5, with Penicillium (Table 6), where at high levels of iron supply the pH values were lower than at low levels of iron supply, indicating that high levels of iron stimulated acid production. However, with the evidence available no conclusions can be drawn with respect to the effect of varying the level of supply, or the ratio of iron to manganese, on acid production in cultures of Aspergillus and Penicillium.

General Conclusions from the Preliminary Investigation.

There was no doubt that the method used would be useful in water culture work on the effects of varying heavy metal balances on the growth and development of Aspergillus niger and Penicillium notatum.

Further experiments were designed to investigate a wide range of iron:manganese and copper:zinc ratios in the culture medium.

CHAPTER IV.

Included in this chapter, are four experiments dealing with the effect of varying the iron:manganese ratio in the culture medium on the growth and development of cultures of Aspergillus niger and Penicillium species.

Experiments 6 and 7 were essentially preliminary in nature, since they were designed primarily to investigate a wide range of iron:manganese ratios. These experiments were orthogonal with respect to iron and manganese treatment, and thus the effect of varying levels of supply of iron and manganese could be investigated by performing an analysis of variance. The effect of varying the iron:manganese ratio in the medium, was not analysed statistically.

In Experiments 8 and 9, the effect of varying the iron:manganese balance, on the dry weight yield of cultures of Aspergillus niger and Penicillium species was investigated statistically, by regression analysis. In Experiment 9, which was designed so that it was orthogonal with respect to level of iron supply and iron:manganese balance, the effect of varying the iron:manganese balance was also investigated by analysis of variance.

EXPERIMENT 6. A preliminary investigation of the effect of varying the iron:manganese ratio over a wide range on the growth and development of cultures of Aspergillus niger.

This experiment was designed primarily to investigate the effect of varying the iron:manganese balance in the culture solution on the growth and development of Aspergillus niger. The experiment was divided into two parts:

- (1) Where the maximum iron and manganese supplies were 1.9683 p.p.m., and
- (2) Where the maximum iron and manganese supplies were 51.2 p.p.m.

Part 1.

This was designed in the form of a grid (Table 18), so that a wide range of ratios of iron:manganese could be investigated. It was only possible to have one culture of each treatment, since more than one would have resulted in an unmanageable number of cultures. The different ratios were obtained by using 1 ml. samples of different concentrations of iron and manganese. The iron and manganese solutions were prepared by consecutive 1:2 dilution (1 volume solution in 2 volumes of purified water) of a concentrated solution, which was prepared so that a 1 ml. sample in 50 ml. gave a concentration of 1.9683 p.p.m. The concentrated solutions were prepared as follows:

Iron: 0.48937 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in purified water in a 1 litre volumetric flask. 1 ml. of this solution in 50 ml. gives an iron concentration of 1.9683 p.p.m.

Manganese: 0.39955 gms. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ dissolved in purified water in a 1 litre volumetric flask. 1 ml. of the solution in 50 ml. gives a manganese concentration of 1.9683 p.p.m.

Results. The dry weight yields are given in Table 19, and the result of the statistical analysis (analysis of variance) is given in Table 20. The results will be considered under the following three main headings:

- (a) The overall effect of increasing the iron supply, irrespective of the level of manganese in the medium.
- (b) The overall effect of increasing the manganese supply, irrespective of the level of iron in the medium.
- (c) The relation between the growth and development and the iron:manganese ratio in the medium.

TABLE 18. The plan of Experiment 6, showing the levels of supply of iron and manganese, and the resulting iron:manganese ratios.

Manganese Supply in p.p.m.	Iron Supply in p.p.m.									
	0.0001	0.0003	0.0009	0.0027	0.0081	0.0243	0.0729	0.2187	0.6561	1.9683
0.0001	1	3	9	27	81	243	729	2187	6561	19683
0.0003	0.333	1	3	9	27	81	243	729	2187	6561
0.0009	0.111	0.333	1	3	9	27	81	243	729	2187
0.0027	0.3741	0.111	0.333	1	3	9	27	81	243	729
0.0081	0.01234	0.3741	0.111	0.333	1	3	9	27	81	243
0.0243	0.004115	0.01234	0.3741	0.111	0.333	1	3	9	27	81
0.0729	0.001371	0.004115	0.01234	0.3741	0.111	0.333	1	3	9	27
0.2187	0.000457	0.001371	0.004115	0.01234	0.3741	0.111	0.333	1	3	9
0.6561	0.000152	0.000457	0.001371	0.004115	0.01234	0.3741	0.111	0.333	1	3
1.9683	0.000051	0.000152	0.000457	0.001371	0.004115	0.01234	0.3741	0.111	0.333	1

TABLE 20. Results of analysis of variance performed on the dry weight yields obtained in Experiment 6, Part 1.

Factor	^o F	S.S.	M.S.	Ratio	Significance
Iron	9	5.12628	.56959	34.15	Significant at 0.1%
Manganese	9	0.63770	.07085	4.24	Significant at 0.1%
Error	81	1.35091	.01667		

Least significant difference for the mean of each treatment of iron or manganese at the 0.05 probability level = \pm 0.10286 grams.

- (a) The overall effect of increasing the iron supply, irrespective of the level of manganese in the medium.

From the mean of the total for each iron treatment (Table 19) it is clearly evident that when the level of iron supply was varied within the ranges 0.0001 - 0.0084 p.p.m. and 0.0729 - 1.9683 p.p.m. there was no significant trend in the yield. However the yields obtained when the level of iron supply was between 0.0001 and 0.0084 p.p.m. were significantly different from those obtained when the level of iron supply lay within the range 0.0729 - 1.9683 p.p.m. Variations in the concentration of iron in the culture medium between these two ranges, i.e. variations in the range 0.0081 - 0.0729 p.p.m., resulted in very marked yield trends. Increases in the level of iron supply resulted in marked increases in the dry weight yield. This trend is therefore responsible for the highly significant iron effect, obtained when an analysis of variance was performed on the dry weight yield data (Table 20). Thus the effect on the yield of varying iron supply, irrespective of the level of manganese in the medium, may be summarised as follows: Increases in the level of iron supply up to 0.0081 p.p.m. had little effect, but

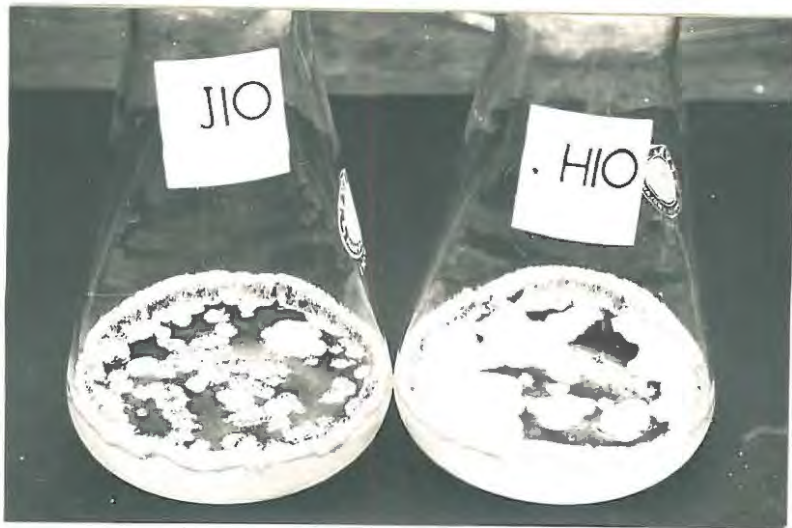
subsequent increases up to 0.0729 p.p.m. resulted in very marked increases in the yield. Further raising of the concentration of iron in the medium, up to 1.9683 p.p.m., although resulting in small increases in the yield, was not significant.

There is some evidence that increasing the level of iron supply resulted in a marked progressive increase in the wrinkling of the felt, (Plates 13, 14, 15 and 16). Thus at low levels of iron supply (Plates 13 and 15) there was little evidence of wrinkling, but at high levels of iron supply (Plates 14 and 16) the felts were very heavily wrinkled. The level of iron supply did not appear to play a significant role in the determination of sporulation.

- (b) The overall effect of increasing the manganese supply, irrespective of the level of iron in the medium.

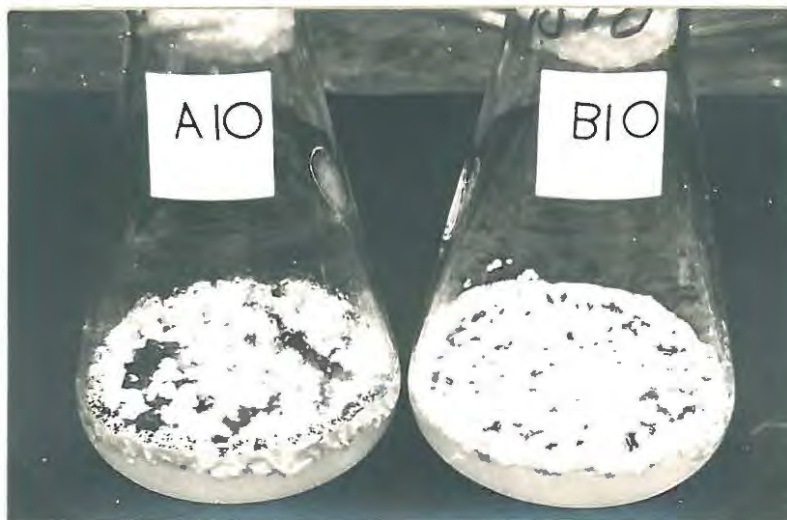
The results of analysis of variance (Table 20) showed the manganese effect to be less significant than the iron effect in determining the dry weight yield trends. From the mean for each manganese treatment (Table 19), it is clear that a significant increase in the yield was only obtained when the level of manganese supply was varied between 0.0001 and 0.0027 p.p.m. Even within this range, the differences between the yields obtained at two consecutive manganese levels, with the exception of the difference between the yield obtained at the 0.0009 and 0.0027 p.p.m. levels of supply, were not significantly different. Thus raising the level of manganese supply to 0.0027 p.p.m. resulted in progressively increased yield, but above this level, increasing the level of manganese had little effect.

There was no evidence that the level of manganese in the culture medium was important in determining the degree of wrinkling or sporulation of the felts, (Plates 13, 14, 15 and 16).



Fe.	0.0001 p.p.m.	0.0009 p.p.m.
Mn.	0.0001 p.p.m.	0.0001 p.p.m.
Fe/Mn	1.0	9.0

PLATE 13. The effect of low iron and manganese supplies on the growth of Aspergillus niger.



Fe.	1.9683 p.p.m.	0.6561 p.p.m.
Mn.	0.0001 p.p.m.	0.0001 p.p.m.
Fe/Mn	19683	6561

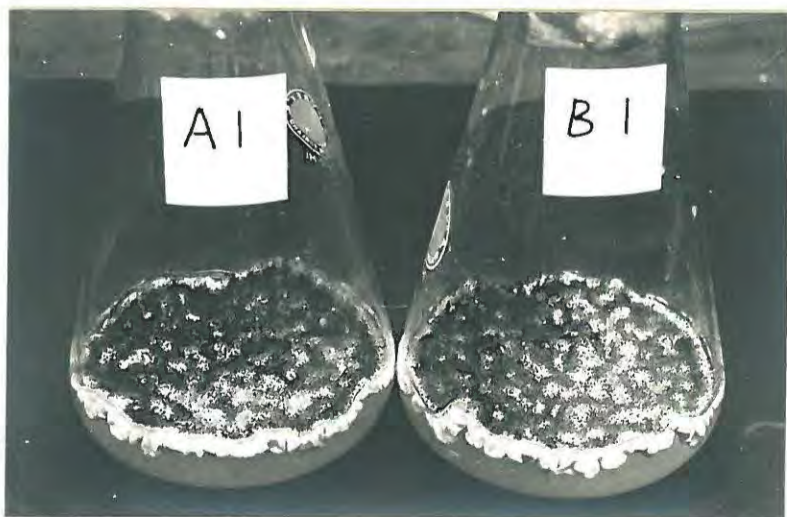
PLATE 14. The effect of high iron and low manganese supplies on the growth of Aspergillus niger.



Fe. 0.0009 p.p.m.
 Mn. 1.9683 p.p.m.
 Fe/Mn 0.000457

0.0003 p.p.m.
 1.9683 p.p.m.
 0.000152

PLATE 15. The effect of high manganese and low iron supplies on the growth of Aspergillus niger.



Fe. 1.9683 p.p.m.
 Mn. 1.9683 p.p.m.
 Fe/Mn 1.0

0.6561 p.p.m.
 1.9683 p.p.m.
 0.333

PLATE 16. The effect of high manganese and iron supplies on the growth of Aspergillus niger.

- (c) The relation between the growth and development and the iron:manganese ratio in the medium.

There is some evidence (Table 21), that increasing the iron:manganese ratio in the culture medium resulted in progressively increased dry weight yields. There was no evidence that any particular iron:manganese ratio or range of ratios was more satisfactory for maximum dry weight yield than others.

The iron:manganese ratio was, however, of some importance in determining the wrinkling of the felts. At low iron:manganese ratios, the felt had a cottony texture and showed no evidence of wrinkling (Plate 15). Increasing the ratio to 1.0 (Plate 14) resulted in the production of felts of a compact texture, but the wrinkling and sporulation was determined, in part, by the absolute level of iron and manganese supplies. Thus at low levels of supply there was only slight evidence of wrinkling and sporulation (Plate 13), but at high levels of supply the felts were heavily wrinkled and densely covered with spores. When the iron:manganese ratio in the culture medium was raised to 6561 and 19683, (Plate 14), the felts were very compact and heavily wrinkled. There was evidence that sporulation was suppressed at high iron:manganese ratios (Plate 14).

Thus while increasing the iron:manganese ratio in the culture medium resulted in the felts becoming progressively more compact and wrinkled, increasing the ratio above 1.0 resulted in suppressed sporulation.

Part 2.

The design of the second part of Experiment 6 is shown in Table 22. Basically this design was the same as that used in Part 1 except that six levels of iron and manganese supply were used

TABLE 21. The dry weight yields, in grams, obtained for each iron:manganese ratio in the culture medium, with *Aspergillus niger* in Experiment 6, part 1.

Fe/Mn ratio	0.00005	0.00015	0.00046	0.00137	0.00411	0.0123	0.0374	0.111	0.333	1.0	3.0	9.0	27.0	81.0	243	729	2187	6561	19683
Total	0.34700	0.64885	0.98180	1.11880	1.63837	2.05230	3.03285	4.49246	5.03959	5.84042	5.14255	4.72594	4.30115	3.96670	2.98405	1.91860	1.63558	1.57975	0.67020
Mean	0.34700	0.32442	0.32727	0.37260	0.32767	0.3405	0.43327	0.56156	0.55995	0.58404	0.57139	0.59074	0.61442	0.66112	0.59681	0.47975	0.54519	0.78982	0.67020

(0.05 - 51.2 p.p.m.), the dilution factor was 4 (i.e. 1 volume of solution to 3 volumes of purified water), and three replications of each treatment were prepared. The concentrated solution of iron and manganese from which a 1 ml. sample in 50 ml. gave a concentration of 51.2 p.p.m., were prepared as follows:

Iron: 1.2742 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in purified water in a 100 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives an iron concentration of 51.2 p.p.m.

Manganese: 1.03928 gms. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ dissolved in purified water in a 100 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives a manganese concentration of 51.2 p.p.m.

TABLE 21. The plan of Experiment 6, Part 2, giving the levels of supply of iron and manganese and the resulting iron:manganese ratios.

Manganese Supply in p.p.m.	Iron Supply in p.p.m.					
	0.05	0.20	0.80	3.20	12.80	51.20
0.05	1	4	16	64	256	1024
0.20	0.25	1	4	16	64	256
0.80	0.0625	0.25	1	4	16	64
3.20	0.0156	0.0625	0.25	1	4	16
12.80	0.0039	0.0156	0.0625	0.25	1	4
51.20	0.0009	0.0039	0.0156	0.0625	0.25	1

Results. The dry weight yields are given in Table 23, and the results of the statistical analysis (analysis of variance) in Table 24. The results will be considered under the following

TABLE 23. The dry weight yield, in grams, obtained from cultures of Aspergillus niger in experiment 6, part 2.

Manganese supply in p.p.m.	Iron Supply in p.p.m.						Total for each Mn treatment	Mean for each Mn treatment
	0.05	0.20	0.80	3.20	12.80	51.20		
	0.45505	0.60996	0.49590	0.47070	0.48998	0.32495		
0.05	0.37340	0.40505	0.62895	0.52140	0.61284	0.49946	8.83900	1.47317
	0.31495	0.47464	0.38256	0.54895	0.61721	0.61305		
	0.41846	0.54600	0.33755	0.60443	0.60000	0.40446		
0.20	0.33021	0.42330	0.59621	0.48506	0.73760	0.45020	9.00764	1.50127
	0.56141	0.49015	0.33785	0.54745	0.66590	0.47140		
	0.62195	0.60730	0.42448	0.53760	0.61430	0.48770		
0.80	0.53716	0.71435	0.43882	0.59761	0.60975	0.45005	9.58967	1.59328
	0.42860	0.31400	0.41511	0.70749	0.59485	0.48855		
	0.55250	0.38965	0.30806	0.53085	0.50511	0.52704		
3.20	0.38800	0.51620	0.97034	0.38725	0.51290	0.44012	8.98347	1.49724
	0.50320	0.50955	0.31055	0.54560	0.68750	0.45905		
	0.51245	0.69397	0.58816	0.65220	0.65425	0.40910		
12.80	0.32445	0.78031	0.71257	0.71140	0.65609	0.40600	10.79182	1.79864
	0.65735	0.55215	0.69586	0.77807	0.50935	0.46809		
	0.28920	0.66251	0.36945	0.68410	0.73212	0.53150		
51.20	0.42096	0.68149	0.66010	0.65125	0.59446	0.43770	9.93041	1.65507
	0.41600	0.38635	0.64775	0.72497	0.59170	0.44880		
Total	8.10530	9.75693	9.32027	10.65638	10.98591	8.31722		
Mean for each Fe treatment	1.35088	1.62615	1.55388	1.77615	1.83098	1.38620		

Least significant difference between means for each treatment at 0.05 probability level \pm 0.0092 grams.

three main headings:

- (a) The overall effect of increasing the iron supply irrespective of the level of manganese in the medium.
- (b) The overall effect of increasing the manganese supply irrespective of the level of iron in the medium.
- (c) The relation between growth and development and the iron:manganese balance in the medium.

TABLE 24. Results of analysis of variance performed on the dry weight yield data obtained in Experiment 6, Part 2.

Factor	⁰ F	S.S.	M.S.	Ratio	Significance
Iron	5	0.38802	0.07760	5.63	Significant at 0.1%
Manganese	5	0.15583	0.03117	2.26	Significant at 5%
Iron: Manganese	25	0.29514	0.01180	0.86	Not significant
Replication	2	0.01496	0.00748	0.005	Not significant
Error	70	0.96549	0.01379		

Least significant difference between means for each treatment at the 0.05 probability level
 = \pm 0.13035 grams.

- (a) The overall effect of increasing the iron supply, irrespective of the level of manganese in the culture medium.

Increasing the concentration of iron in the culture medium, from 0.05 to 3.20 p.p.m., resulted in a marked increase in the dry weight yield, (Table 23). This increase was shown, by analysis of variance to be highly significant, (Table 22). There is evidence that increasing the level of iron from 3.20 to 12.80 p.p.m. had little

effect on the yield, since the means of the total yields obtained for each of these levels were not significantly different. However, cultures receiving 51.20 p.p.m. of iron gave diminished yields (Table 23). This decrease in the yield was shown, by comparing the means of the total yield for each treatment, to be significant. Thus increasing the level of iron supply in the culture medium to 12.80 p.p.m. resulted in increased dry weight yields, but further increases resulted in diminished yields due to iron toxicity.

The level of iron in the culture medium did not exert a marked effect on the wrinkling of the felt, but there is some evidence that it was important in determining the degree of sporulation (Table 25). Thus increasing the supply of iron resulted in marked increases in the sporulation. There was no evidence, however, that high levels of iron were toxic to sporulation.

TABLE 25. ^{*} The degree of sporulation of cultures of Aspergillus niger in Experiment 6, Part 2. The values of each of three replications for each treatment are given.

* 1 = very sparse sporulation

10 = very dense sporulation.

Manganese Supply in p.p.m.	Iron Supply in p.p.m.					
	0.05	0.20	0.80	3.20	12.8	51.2
0.50	2,2,2.	2,2,4.	3,5,4.	4,2,3.	4,9,9.	10,8,6.
0.20	2,2,3.	2,2,2.	7,2,2.	5,7,7.	6,7,8.	8,7,5.
0.80	3,2,2.	3,3,8.	7,4,3.	6,5,5.	8,7,6.	8,8,9.
3.20	3,3,3.	3,3,3.	2,2,8.	3,3,3.	5,4,6.	9,7,7.
12.8	6,2,1.	7,9,5.	5,6,6.	9,8,7.	7,10,4.	8,5,9.
51.2	2,2,2.	6,6,5.	5,3,6.	5,5,5.	6,6,6.	9,7,8.

- (b) The overall effect of increasing the manganese supply, irrespective of the level of iron in the culture medium.

Increasing the level of manganese supply up to 128 p.p.m., irrespective of the level of iron in the medium, resulted in increased dry weight yields (Table 23). Although this tendency was shown by an analysis of variance to be significant, it was not as significant as the iron effect (Table 24). This was because, although the yields obtained at high levels of iron supply were significantly different from those obtained at low levels, the increase between two consecutive treatments tended to be less than the value for least significant difference, and were therefore not significant. However, increasing the yield from 3.20 to 12.80 p.p.m. and from 12.80 to 51.20 p.p.m. resulted in significant changes in the yield. Whilst raising the level of manganese supply from 3.20 to 12.80 p.p.m. resulted in a marked increase in the yield, subsequent increases to 51.20 p.p.m. resulted in diminished yields, due to manganese toxicity.

The concentration of manganese in the culture medium, although unimportant in determining the wrinkling of the felts, was important in sporulation. Thus increasing the manganese supply resulted in progressively increased sporulation (Table 25). There is some evidence however, that high levels of manganese (51.2 p.p.m.) were toxic to sporulation, since slightly diminished sporulation was evident in cultures receiving 51.2 p.p.m. of manganese.

- (c) The relation between growth and development, and the iron:manganese balance in the medium.

There is some evidence, (Table 26), that increasing the iron:manganese balance, in the medium, to 0.0625, resulted in increased dry weight yield, and that subsequent increases resulted in progressively

diminished yields. This would suggest that an optimal iron:manganese balance of approximately 0.0625 existed for dry weight yield. There was no evidence however that the iron:manganese ratio was important in the determination of trends in the wrinkling or sporulation of the felts.

Conclusions.

There is little doubt that, in cultures of Aspergillus niger, as the levels of iron and manganese were increased between 0.0001 and 12.80 p.p.m., there was a progressive increase in the dry weight yield, and sporulation. At the low levels of iron and manganese supply, between 0.002 and 0.02 p.p.m., the increase in the yield, as a result of increased iron or manganese supply, was very marked. There was some evidence that levels of iron and manganese above 12.8 p.p.m. were toxic, resulting in diminished dry weight yields.

There was no conclusive evidence that the iron:manganese balance in the culture medium was an important factor in determining the yield trends or general condition of the cultures. These results were further confused by the marked effect of increasing the absolute supply of iron and manganese in the culture medium. Thus, when low levels of iron and manganese were used, (Part 1) whilst an iron:manganese ratio of approximately 1.0 was optimal for sporulation, the dry weight yield increased progressively with increase in the ratio. When high levels of iron and manganese were investigated, the ratio was found to be of little importance in determining the degree of sporulation, but maximum yield was obtained when the iron:manganese ratio was 0.0625.

Since these results were contradictory, it was decided to conduct further experiments in which the effect of different iron:manganese ratios could be studied at low levels of supply, 0.064 - 4.096 p.p.m., and at high levels of supply, 2.0 - 128.0 p.p.m. In these

TABLE 26. The dry weight yields, in grams, obtained for each iron:manganese ratio in the culture medium with Aspergillus niger in Experiment 6, part 2.

				1.14340							
				1.31008		1.48965					
			1.58771	1.45945		1.50741					
	1.44370		1.63565	1.27161		1.54105					
	1.49425	1.41485	1.27841	1.63694		1.72003					
1.12616	2.02643	1.58895	1.84270	2.00350		1.43746					
	1.73035	1.99659	1.40370	1.81890		1.32606					
	1.67730	2.17167	1.70551	1.42630							
		2.06032	1.81969	1.42621							
		1.91828	1.28319								
			1.41800								
Fe/Mn ratio	0.00098	0.00391	0.01562	0.0625	0.25	1.0	4.0	16.0	64.0	256	1024
Total	1.12616	3.22460	5.14743	7.06002	8.62463	8.52265	7.59266	6.38946	4.97085	3.04609	1.43746
Mean	1.12616	1.61230	1.71581	1.76500	1.72493	1.42044	1.51853	1.59736	1.65695	1.52304	1.43746

experiments the ratio effect was investigated by performing a regression analysis.

EXPERIMENT 7. A preliminary investigation of the effect of varying the iron:manganese ratio, over a wide range, on the growth and development of cultures of Penicillium notatum.

This experiment was designed primarily to investigate the effect of varying the iron:manganese balance in the culture solution on the growth and development of Penicillium notatum. As a subsidiary object the experiment was designed to investigate the effect of supplying iron without a chelating agent. The experiment was designed in the form of a grid (Table 27) to enable a wide range in iron:manganese balances to be investigated. The method used was as described for previous experiments: Concentrated solutions of iron and manganese were prepared, and from these, by consecutive dilution (1 volume solution to 3 volumes of purified water) the other solutions in the series were prepared. The concentration of the solutions was such that a 1 ml. sample in 50 ml. gave the required concentration. Stock solutions from which the first dilution was made were prepared as follows:

Iron: 0.65240 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in purified water in a 100 ml. volumetric flask. 1 ml. of this solution in 50 ml. gave an iron concentration of 26.2144 p.p.m.

Manganese: 0.53210 gms. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ were dissolved in purified water in a 100 ml. volumetric flask. 1 ml. of this solution in 50 ml. gave a manganese concentration of 26.2144 p.p.m.

TABLE 27. The plan of Experiment 7, showing the levels of iron and manganese supply and the resulting iron:manganese ratios.

Manganese supply in p.p.m.	Iron Supply in p.p.m.									
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144
0.0001	1	4	16	64	256	1024	4096	16384	65536	262144
0.0004	0.25	1	4	16	64	256	1024	4096	16384	65536
0.0016	0.0625	0.25	1	4	16	64	256	1024	4096	16384
0.0064	0.015625	0.0625	0.25	1	4	16	64	256	1024	4096
0.0256	0.003906	0.015625	0.0625	0.25	1	4	16	64	256	1024
0.1024	0.000977	0.003906	0.015625	0.0625	0.25	1	4	16	64	256
0.4096	0.000244	0.000977	0.003906	0.015625	0.0625	0.25	1	4	16	64
1.6384	0.000061	0.000244	0.000977	0.003906	0.015625	0.0625	0.25	1	4	16
6.5536	0.000015	0.000061	0.000244	0.000977	0.003906	0.015625	0.0625	0.25	1	4
26.2144	0.000004	0.000015	0.000061	0.000244	0.000977	0.003906	0.015625	0.0625	0.25	1

E.D.T.A.: When required, 0.87358 gms. were dissolved with 0.65240 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in purified water in a 100 ml. volumetric flask.

Results.

The dry weight yields are given in Table 28, when iron was supplied without a chelate, and Table 29, when an equivalent amount of E.D.T.A. was supplied with the iron. The results of statistical analysis (analysis of variance) are given in Tables 30 and 31 respectively. The pH values of the filtrate are given in Tables 8 and 9 of the appendix respectively. The results will be considered under the following three headings:

- (a) The overall effect of increasing the iron supply irrespective of the level of manganese in the medium.
- (b) The overall effect of increasing the manganese supply irrespective of the level of iron in the medium.
- (c) The relation between growth and development and the iron:manganese ratio in the medium.

TABLE 30. The results of analysis of variance performed on the dry weight yield data obtained with Penicillium when iron was supplied without a chelate.

Factor	D.F.	S.S.	M.S.	Ratio	Significance
Iron	9	.043776	.004864	360.08	Significant at 0.1%
Manganese	9	.016845	.00189	13.99	Significant at 0.1%
Error	81	.01094	.00013		

TABLE 28. The dry weight yield, in grams, obtained from cultures of Penicillium notatum in Experiment 7, when iron was supplied without a chelating agent.

Manganese supply in p.p.m.	Iron Supply in p.p.m.										Total for each Manganese Treatment	Mean of Total for each Mn Treatment
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144		
0.0001	0.23047	0.22843	0.20454	0.22267	0.25450	0.27829	0.30314	0.41406	0.33746	0.41538	2.88894	0.28889
0.0004	0.18625	0.23191	0.18234	0.19635	0.24322	0.27130	0.29571	0.32150	0.36839	0.34620	2.64317	0.26432
0.0016	0.18708	0.20285	0.17631	0.17279	0.19088	0.28450	0.35397	0.31970	0.45683	0.41527	2.76018	0.27602
0.0064	0.27318	0.17972	0.24167	0.23184	0.24111	0.26109	0.31978	0.34661	0.27457	0.27400	2.64267	0.26427
0.0256	0.17403	0.22755	0.15673	0.20072	0.23313	0.28318	0.28585	0.37130	0.31759	0.35925	2.60573	0.26057
0.1024	0.22820	0.17561	0.19245	0.25735	0.27343	0.28326	0.34294	0.43268	0.43279	0.27909	2.89780	0.28978
0.4096	0.23209	0.15194	0.20199	0.23031	0.21579	0.25639	0.30811	0.31361	0.39352	0.43086	2.73461	0.27346
1.6384	0.16106	0.19713	0.17998	0.22920	0.22125	0.28255	0.30049	0.29449	0.31562	0.34400	2.52577	0.25258
6.5536	0.16402	0.13690	0.21101	0.25820	0.24210	0.23291	0.34764	0.26413	0.33122	0.35421	2.54234	0.25423
26.2144	0.14374	0.18956	0.16566	0.20850	0.23800	0.23417	0.28719	0.30929	0.39121	0.36682	2.55414	0.25541
Total for each Fe Treatment	1.97652	1.92160	1.93268	2.20793	2.35341	2.66674	3.14482	3.38737	3.61920	3.58508		
Mean of Total for each Fe Treatment	0.19765	0.19216	0.19327	0.22079	0.23534	0.26667	0.31448	0.33874	0.36192	0.35851		

Least significant difference between means of each treatment at 0.05 probability level = ± 0.00998 grams.

TABLE 29. The dry weight yield, in grams, obtained from cultures of Penicillium notatum in Experiment 7, when the iron was supplied with an equivalent amount of E.D.T.A.

Manganese supply in p.p.m.	Iron Supply in p.p.m.										Total for each Manganese Treatment	Mean of Total for each Mn Treatment
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144		
0.0001	0.17325	0.13104	0.17390	0.23261	0.20286	0.25141	0.23949	0.22774	0.40067	0.61244	2.64541	0.26454
0.0004	0.20507	0.17727	0.15906	0.15067	0.19315	0.20535	0.25050	0.27384	0.47729	0.63849	2.73089	0.27309
0.0016	0.23549	0.23028	0.24303	0.17816	0.23592	0.21668	0.30333	0.30305	0.40774	0.60070	2.95438	0.29544
0.0064	0.23859	0.20046	0.14524	0.19110	0.23455	0.27014	0.33770	0.30143	0.39558	0.72325	3.03804	0.30380
0.0256	0.36425	0.20252	0.16060	0.21501	0.26578	0.31465	0.47932	0.57412	0.64109	0.70415	3.92149	0.39215
0.1024	0.19608	0.26097	0.26278	0.18324	0.28536	0.27904	0.40749	0.54359	0.56474	0.64800	3.63129	0.36313
0.4096	0.23055	0.16921	0.22396	0.21963	0.22873	0.28659	0.39727	0.58261	0.63293	0.54777	3.51925	0.35192
1.6384	0.19009	0.13962	0.20255	0.22332	0.22347	0.35045	0.37619	0.50613	0.64125	0.66110	3.51417	0.35142
6.5536	0.25755	0.19609	0.23439	0.25346	0.27705	0.27361	0.36162	0.44085	0.50603	0.67238	3.47303	0.34730
26.2144	0.22222	0.24874	0.28994	0.29908	0.26701	0.27900	0.33183	0.39326	0.60792	0.60588	3.54488	0.35449
Total for each Fe Treatment	2.31314	1.95620	2.09545	2.14628	2.41388	2.72692	3.48474	4.14662	5.27524	6.41436		
Mean of Total for each Fe Treatment	0.23131	0.19562	0.20954	0.21463	0.24139	0.27269	0.34847	0.41466	0.52752	0.64144		

Least significant difference between means of each treatment at 0.05 probability level = ± 0.005148 grams.

TABLE 31. The results of analysis of variance performed on the dry weight yield data obtained with Penicillium when iron was supplied with an equivalent amount of E.D.T.A.

Factor	D.F.	S.S.	M.S.	Ratio	Significance
Iron	9	2.10272	.23363	71.01	Significant at 0.1%
Manganese	9	0.16235	.01804	5.48	Significant at 0.1%
Error	81	0.26641	.00329		

- (a) The overall effect of increasing the iron supply, irrespective of the level of manganese in the medium.

At low levels of iron supply, 0.0001 - 0.0016 p.p.m., irrespective of the presence of a chelating agent, increasing the concentration of iron in the culture medium had little effect on the dry weight yield (Tables 28 and 29). However, subsequent increases resulted in a marked progressive increase in the yield. This effect was found, by analysis of variance, to be highly significant, (Tables 30 and 31). There is some evidence (Table 28) that, when the level of iron supply was increased above 12.80 p.p.m., in the absence of a chelating agent, there was no significant change in the yield. This would suggest that optimal iron concentration was between 12.8 and 26.2144 p.p.m. There was no evidence of an optimal level of iron supply when a chelating agent was present. Raising the iron concentration from 12.80 to 26.2144 p.p.m. resulted in a significant increase in the dry weight yield. However, this evidence, which implied that iron-toxicity might be reduced by the presence of a chelating agent is not conclusive.

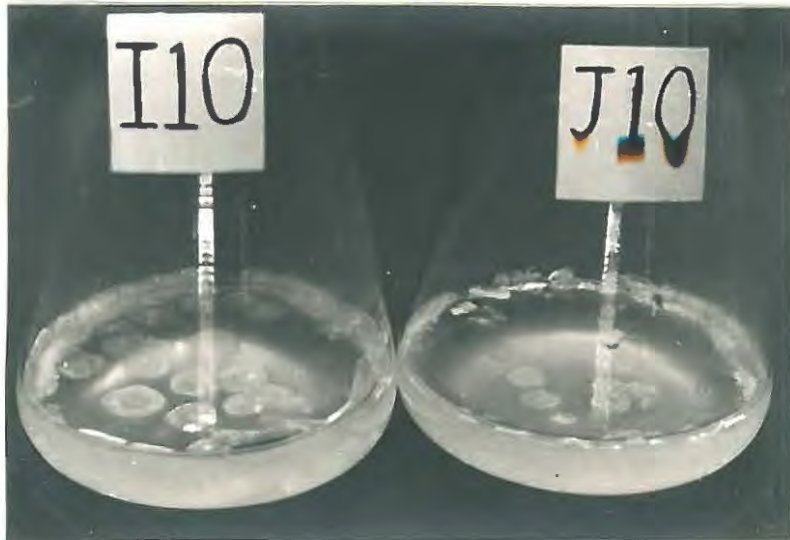
The level of iron supply exerted a marked effect on the form and sporulation of the felt. At low levels of iron supply (Plates 17 and 19) there was very little surface growth, the felts being thin and

cottony. As the level of iron supply was raised, the felts became progressively wrinkled and compact. Thus the felts produced at high levels of iron supply were compact and heavily wrinkled (Plates 18 and 20). There was no evidence of sporulation at low levels of iron supply (Plates 17 and 19) but at high levels, (Plates 18 and 20), sporulation was heavy. When the iron was supplied without a chelating agent, there was a tendency for the pH of the filtrate to decrease with increase in iron supply (Table 8 of Appendix). However raising the level of iron in the presence of E.D.T.A., resulted in slightly increased pH values, (Table 9 of Appendix).

- (b) The overall effect of increasing the manganese supply, irrespective of the level of iron in the culture medium.

In cultures receiving between 0.0001 and 0.1024 p.p.m. of manganese, there was no clear evidence that the level of manganese was important in determining the yield trends, when no chelating agent was present, (Table 28). However there is evidence that levels above 0.1024 resulted in diminished yields, due to manganese toxicity. When a chelating agent was present in the culture medium, increasing the level of manganese up to 0.2550 p.p.m. resulted in progressively increased dry weight yields. Further increases up to 26,2144 p.p.m. resulted in a significant decrease in the yield. The presence of a chelating agent did not therefore decrease the symptoms of manganese toxicity.

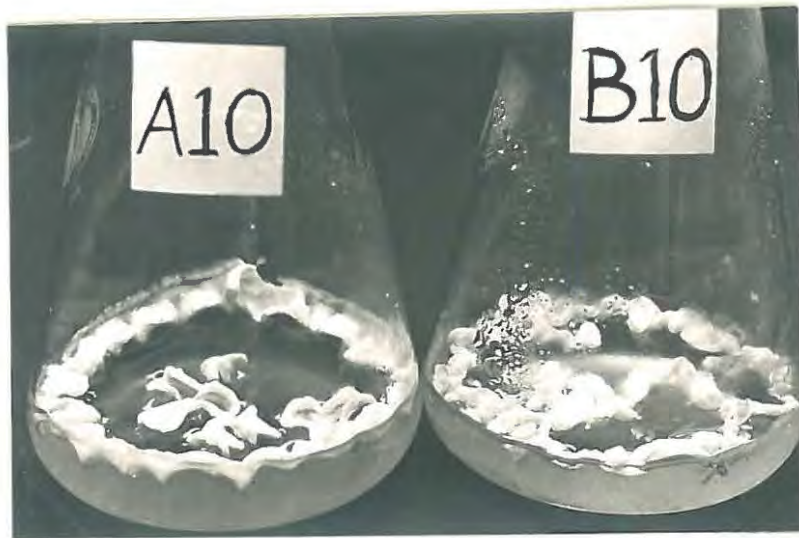
There was no evidence that the level of manganese supply was important in determining either the degree of wrinkling or sporulation of the felts. Raising the level of manganese in the culture medium, resulted in slightly increased pH of the filtrate when no chelating agent was present, (Table 8 of Appendix). However when E.D.T.A. was present in the culture medium, increasing the level of manganese supply resulted in slightly lowered pH values.



Fe. 0.0004 p.p.m.
 Mn. 0.0001 p.p.m.
 Fe/Mn 4.0

0.0001 p.p.m.
 0.0001 p.p.m.
 1.0

PLATE 19. The effect of low iron and manganese supplies on the growth of Penicillium notatum.



Fe. 26.2144 p.p.m.
 Mn. 0.0001 p.p.m.
 Fe/Mn 262144

6.5536 p.p.m.
 0.0001 p.p.m.
 65536

PLATE 20. The effect of high iron and low manganese supplies on the growth of Penicillium notatum.

- (c) The relation between growth and development and the iron:manganese ratio in the medium.

Increasing the iron:manganese ratio in the culture medium when:

- (i) no chelate was present (Table 32), resulted in progressively diminished dry weight yields,
 (ii) an equivalent amount of E.D.T.A. was supplied with the iron, resulted in progressively increased yields.

(Table 33.)

There was no evidence in either case of an optimal ratio. This would suggest that the chelating agent prevented iron and, or, manganese toxicity.

The iron:manganese balance did not have a noticeable effect on the degree of wrinkling or sporulation of the felts.

Conclusions.

There is some evidence that the presence of a chelating agent in the culture medium influences the yield trends. Thus, when no E.D.T.A. was supplied, there was a tendency for levels of iron of approximately 26.2144 p.p.m. to be toxic, but, when it was present in the medium, there were no signs of diminished dry weight yield. This was also evident when the iron:manganese balance was considered. In the absence of a chelating agent, increasing the ratio resulted in diminished yields, whereas the presence of E.D.T.A. resulted in progressively increased yield. Further experiments were, therefore, planned with a view to elucidating the effect of varying the iron:manganese balance, and also, as a subsidiary object, the effect of the presence of a chelating agent in the culture mediums.

62.

TABLE 32. The dry weight yield, in grams, obtained for each iron:manganese ratio in the culture medium with Penicillium notatum in Experiment 7, (no chelate supplied).

									0.41538										
									0.34620	0.33746									
								0.41527	0.36839	0.41406									
							0.27400	0.45683	0.32150	0.30314									
							0.35925	0.27457	0.31970	0.29571	0.27829								
							0.27909	0.31759	0.34661	0.35397	0.27130	0.25450							
							0.43086	0.43279	0.37130	0.31978	0.28450	0.24322	0.22267						
							0.34400	0.39352	0.43263	0.28585	0.26019	0.19088	0.19635	0.20454					
							0.35421	0.31562	0.31361	0.34294	0.28318	0.24111	0.17279	0.18234	0.22843				
							0.36682	0.33122	0.29449	0.30811	0.28326	0.23313	0.23184	0.17631	0.23191	0.23047			
							0.39121	0.26413	0.30049	0.25639	0.27343	0.23072	0.24167	0.20285	0.13625				
							0.30929	0.34764	0.28255	0.21579	0.25735	0.15673	0.17970	0.18708					
							0.28719	0.23291	0.22125	0.23031	0.19245	0.22755	0.27318						
							0.23417	0.24210	0.22920	0.20199	0.17561	0.17043							
							0.23800	0.25820	0.17998	0.15194	0.22820								
							0.20850	0.21101	0.19713	0.23209									
							0.18566	0.13690	0.16106										
							0.18956	0.16402											
							0.14374												
Fe/Mn ratio	0.00003	0.00012	0.00049	0.00024	0.00098	0.0039	0.0156	0.0625	0.25	1.0	4.0	16.0	64.0	256.0	1024	4096	16384	65536	262144
Total	0.36682	0.74542	0.98451	1.29780	1.54891	1.87705	2.06553	2.32558	2.56431	2.67079	2.32674	1.94155	1.56159	1.39172	0.97731	0.88104	0.62353	0.41463	0.23047
Mean	0.36682	0.37271	0.32817	0.32445	0.30978	0.30984	0.29508	0.29080	0.28492	0.26708	0.25853	0.24269	0.22308	0.23195	0.19546	0.22023	0.20784	0.20734	0.23047

TABLE 33. The dry weight yield, in grams, obtained for each iron:manganese ratio in the culture medium with Penicillium notatum in Experiment 7, (chelate supplied as L.D.F.A.).

									0.17325										
									0.20507		0.13104								
									0.23549		0.17727		0.17390						
									0.23859		0.23028		0.15906		0.23261				
									0.36425		0.20046		0.24303		0.15067		0.20286		
									0.19608		0.20252		0.14524		0.17816		0.19315		0.25141
									0.23055		0.26097		0.16060		0.19110		0.23592		0.20525
									0.19009		0.16921		0.26278		0.21501		0.23455		0.21668
									0.25755		0.13962		0.22396		0.18324		0.26578		0.27014
									0.22222		0.19609		0.20255		0.21963		0.28536		0.31465
									0.24874		0.23439		0.22332		0.22873		0.27904		0.47932
									0.28994		0.25346		0.22347		0.28659		0.40749		0.57412
									0.29908		0.27705		0.35045		0.39727		0.54359		0.64109
									0.26701		0.27361		0.37619		0.53261		0.56474		0.70415
									0.27900		0.36162		0.50613		0.63293		0.64300		
									0.33183		0.44085		0.64125		0.54777				
									0.39326		0.50603		0.66110						
									0.60792		0.67238								
									0.60588										
Fe/Mn ratio	0.000003	0.000012	0.000049	0.00024	0.00098	0.00391	0.01562	0.0625	0.25	1.0	4.0	16.0	64.0	256.0	1024	4096	16384	65536	262144
Total	0.22222	0.50629	0.67612	0.90364	1.08831	1.62855	1.75243	2.11385	2.79251	3.34478	3.32119	3.14757	2.66677	2.30196	1.90469	1.64432	1.30573	1.03936	0.61244
Mean	0.22222	0.25314	0.22534	0.22591	0.21766	0.27142	0.25035	0.26423	0.31028	0.33448	0.37758	0.39345	0.38097	0.38366	0.38094	0.41108	0.43524	0.56968	0.61244

EXPERIMENT 8. To determine the effect of varying iron: manganese balances in the culture medium, on the growth and development of Aspergillus niger and Penicillium glaucum.

The experiment was carried out in two parts:

- (i) Where the maximum levels of iron and manganese in the culture medium were 4.0960 p.p.m. and,
- (ii) where the maximum levels of iron and manganese in the culture medium were 128.0 p.p.m.

Thus, although a wide range of iron and manganese concentrations was investigated, the range in each part of the experiment was not so large as to preclude the replication of each treatment. There were three replications of each treatment.

Two methods of statistical analysis were performed on the dry weight yield data:

(i) Analysis of variance. This method was used to determine whether the level of iron and manganese, or the iron:manganese interaction were significant in determining yield trends. This method of analysis could not be used in an investigation of the iron:manganese ratio effect because there were not equal numbers of treatments for each iron:manganese ratio, i.e. the experiment was orthogonal with respect to the levels of iron and manganese supply, but not to ratio. An example of this method of analysis is given in Appendix II.

(ii) Regression analysis. This type of analysis is not a significant test for each effect, although it does supply information as to whether the experimental data are significantly different from purely chance data. The value of the method lies in its use to determine the response surface of the data, and thereby, the relative importance of each factor in determining the yield. The contours on

the response surface are obtained by solving the regression equation. In this investigation the equation was solved by substituting the coded values for either x_1 or x_2 and using an arbitrary value for yield (y).

E.g. Regression equation $y = 0.1 + 0.2x_1 + 0.3x_2 - 0.4x_3 + 0.5x_4 - 0.6x_5$

Substitute $y = 1.42$

$$x_1 = 1.0 \quad (x_3 = x_1^2 = 1.0, \quad x_5 = x_1x_2 = -0.6x_2)$$

$$x_4 = x_2^2$$

$$\therefore 1.42 = 0.1 + 0.2 + 0.3x_2 - 0.4 + 0.5x_2^2 - 0.6x_2$$

$$= 0.5x_2^2 - 0.3x_2 - 1.5z$$

$$\text{Solve } x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

Thus for a particular yield value, and different values of x_1 the corresponding x_2 values can be calculated, and the graph plotted.

An example of regression analysis is given in Appendix II.

The yield data were analysed for the iron and manganese, and the iron and iron:manganese balance response surfaces.

Part 1.

The plan of part 1 of the experiment is given in Table 34.

A series of stock solutions of iron and manganese were prepared, by 1:1 (1 volume solution to 1 volume of purified water) dilution of a concentrated solution, from which a 1 ml. sample in 50 ml. gave the maximum concentration required. The concentrated iron and manganese solutions, from which the other solutions were prepared, were made up as follows.

Iron: 1.01941 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in purified water in a 1 litre volumetric flask. 1 ml. of this solution in 50 ml. gave an iron concentration of 4.0960 p.p.m.

TABLE 34. The plan of Experiment 8, part 1, showing the levels of iron and manganese supply and the resulting iron:manganese ratios.

Level of Mn supply in p.p.m.	0.064	0.128	0.256	0.512	1.024	2.048	4.096
0.064	1.0	2.0	4.0	8.0	16.0	32.0	64.0
0.128	0.5	1.0	2.0	4.0	8.0	16.0	32.0
0.256	0.25	0.5	1.0	2.0	4.0	8.0	16.0
0.512	0.125	0.25	0.5	1.0	2.0	4.0	8.0
1.024	0.0625	0.125	0.25	0.5	1.0	2.0	4.0
2.048	0.03125	0.0625	0.125	0.25	0.5	1.0	2.0
4.096	0.015625	0.03125	0.0625	0.125	0.25	0.5	1.0

Manganese: 0.83143 gms. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was dissolved in purified water in a 1 litre volumetric flask. 1 ml. of this solution in 50 ml. gives a manganese concentration of 4.0960 p.p.m.

E.D.T.A.: Ethylene-diamine-tetra-acetic acid was used in the preparation of the iron solution for the investigation with Aspergillus niger. 1.36502 gms. E.D.T.A. were dissolved with 1.01941 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in purified water in a 1 litre volumetric flask.

Results. The results are given in the following Tables;

	<u>Aspergillus.</u>	<u>Penicillium</u>
Dry weight yield	35	36
pH of filtrate (in Appendix)	10	11
Analysis of variance	37	38
Regression Analysis for Fe and Mn effect	39	40
Regression Analysis for Fe/Mn ratio effect	41	42

The results will be considered under the following headings:-

- (a) The effect of increasing the level of iron supply, irrespective of the level of manganese in the medium.
- (b) The effect of increasing the level of manganese supply irrespective of the level of iron in the medium.
- (c) The iron:manganese interaction effect on the yield.
- (d) The relation between growth and development and the iron:manganese ratio in the medium.

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TABLE 35. The dry weight yield, in grams, obtained with *Aspergillus niger* in Experiment 8, part 2.

Manganese supply in p.p.m.	Iron Supply in p.p.m.							Totals for each Manganese Treatment.	Mean of Total for each Mn Treatment
	0.064	0.128	0.256	0.512	1.024	2.048	4.096		
	0.53130	0.56915	0.77314	0.63416	0.43356	0.54536	0.71014		
0.064	0.60029	0.36086	0.49464	0.53443	0.55015	0.45927	0.57416	12.01737	1.71677
	0.42766	0.60151	0.59739	0.64345	0.81931	0.44586	0.71158		
	0.51324	0.45333	0.54627	0.45486	0.49451	0.71934	0.33272		
0.128	0.52923	0.65713	0.44450	0.57061	0.61750	0.84501	0.68451	11.89527	1.69932
	0.52956	0.63169	0.61446	0.36743	0.71004	0.30976	0.86956		
	0.52036	0.60020	0.62812	0.59711	0.66925	0.69961	0.82543		
0.256	0.55987	0.40697	0.57727	0.67780	0.70290	0.30789	0.28652	11.89181	1.69883
	0.43994	0.37490	0.52677	0.59666	0.44589	0.69936	0.74899		
	0.55700	0.69457	0.51191	0.63191	0.73478	0.81964	0.32768		
0.512	0.62471	0.71112	0.38144	0.74987	0.74450	0.51652	0.74577	13.03713	1.86248
	0.49762	0.37680	0.61908	0.47196	0.71427	0.82336	0.78262		
	0.52316	0.78436	0.39760	0.63186	0.63293	0.46640	0.76436		
1.024	0.50411	0.47001	0.45943	0.54057	0.57711	0.78536	0.66365	12.27171	1.75310
	0.50641	0.50169	0.55490	0.50984	0.53746	0.65166	0.80884		
	0.74386	0.44929	0.96626	0.48767	0.48087	0.81232	0.68816		
2.048	0.67491	0.70111	0.63743	0.80473	0.70369	0.60475	0.76679	13.82476	1.97497
	0.52899	0.60704	0.60736	0.85864	0.76162	0.49287	0.44640		
	0.81367	0.72506	0.50366	0.67944	0.61007	0.25611	0.77129		
4.096	0.55841	0.47067	0.44394	0.82439	0.62997	0.87958	0.75441	13.28394	1.89771
	0.57503	0.48492	0.68597	0.55239	0.76554	0.62004	0.67938		
Totals for each Fe Treatment	11.75933	11.63238	11.97154	12.81973	13.33592	12.76007	13.94297		
Mean of Total for each Fe Treatment	1.67990	1.66177	1.71022	1.83140	1.90513	1.82287	1.99185		

Least significant difference between means at 0.05 probability level = ± 0.13035 grams.

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TABLE 36. The dry weight yield, in grams, obtained with Penicillium glaucum in Experiment 8, part 2.

Manganese supply in p.p.m.	Iron Supply in p.p.m.							Totals for each Manganese Treatment	Mean of total for each Mn Treatment
	0.064	0.128	0.256	0.512	1.024	2.048	4.096		
	0.34094	0.39754	0.70294	0.71422	0.71886	0.77864	0.65793		
0.064	0.31629	0.46049	0.68396	0.68245	0.69615	0.73207	0.78130	13.29020	1.89860
	0.48934	0.47051	0.67152	0.66789	0.77432	0.78184	0.77100		
	0.45124	0.47854	0.68268	0.68245	0.72537	0.77267	0.72408		
0.128	0.52776	0.46243	0.66469	0.73031	0.70142	0.70224	0.75156	13.40685	1.91527
	0.28349	0.37133	0.75142	0.72658	0.74071	0.76224	0.71364		
	0.35339	0.39045	0.61417	0.76068	0.69269	0.78335	0.78976		
0.256	0.32176	0.43697	0.69525	0.72624	0.68515	0.80390	0.77196	13.03492	1.86213
	0.35092	0.40683	0.56461	0.68550	0.73337	0.73378	0.73419		
	0.28911	0.38083	0.62703	0.72629	0.71862	0.76933	0.74122		
0.512	0.33702	0.57189	0.59073	0.71950	0.71771	0.76862	0.82133	13.02060	1.86009
	0.26057	0.42049	0.53562	0.73851	0.76059	0.76450	0.76109		
	0.31480	0.39049	0.52766	0.74495	0.73368	0.71578	0.74264		
1.024	0.35094	0.34550	0.56893	0.70091	0.71813	0.75866	0.73298	12.91244	1.84463
	0.38151	0.46287	0.67849	0.75370	0.75297	0.65907	0.79778		
	0.33702	0.34993	0.59811	0.63980	0.70230	0.78140	0.74438		
2.048	0.28626	0.42821	0.56145	0.70575	0.74993	0.75078	0.73506	12.80950	1.82993
	0.31996	0.46189	0.69730	0.70561	0.76160	0.72278	0.76998		
	0.34485	0.37391	0.46040	0.77692	0.74482	0.72744	0.75168		
4.096	0.35027	0.39629	0.52874	0.66307	0.71693	0.76128	0.75100	12.48388	1.78341
	0.28546	0.34034	0.65064	0.64797	0.74904	0.76106	0.70177		
Totals for each Fe Treatment	7.29290	8.79773	13.05634	14.89930	15.29436	15.87143	15.74633		
Mean of Totals for each Fe Treatment	1.04184	1.25682	1.86519	2.12847	2.18491	2.26735	2.24948		

Least significant difference between means at .05 probability level = ± 0.05115 grams.

TABLE 37. The results of an analysis of variance performed on the dry weight yield data of Aspergillus in Experiment 8, part 1.

Factor	Degrees Freedom	Sum Squares	Mean sum squares	F value	Significance
Iron	6	0.21102	0.03517	1.57	Not significant
Manganese	6	0.17041	0.02840	1.27	Not significant
Interaction	36	0.40789	0.01133	0.51	Not significant
Replication	2	0.00110	0.00055	0.03	Not significant
Error	96	2.14625	0.02236		

TABLE 38. The results of an analysis of variance performed on the dry weight yield data of Penicillium in Experiment 8, part 1.

Factor	Degrees Freedom	Sum Squares	Mean sum Squares	F value	Significance
Iron	6	3.56627	0.56105	164.95	Significant at 0.1%
Manganese	6	0.02672	0.00448	1.31	Not significant
Interaction	36	0.35020	0.00972	2.85	Significant at 0.1%
Replication	2	0.00318	0.00159	0.47	Not significant
Error	96	0.32652	0.00340		

TABLE 39. The results of a regression analysis of the dry weight yield data of Aspergillus in Experiment 8, part 1, for the iron and manganese effects.

Factor	Degrees Freedom	Sum squares	Mean Sum squares	F value	Significance
Regression	5	0.53816	0.10763	2.52	Not significant
Residual	43	1.83342	0.04264		

$$y = 1.820 - 0.002x_1 + 0.052x_2 + 0.003x_3 + 0.003x_4 - 0.001x_5 \quad (\text{Graph 1.})$$

TABLE 40. The results of a regression analysis of the dry weight yield data of Penicillium in Experiment 8, part 1, for the iron and manganese effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	10.73012	2.14602	300.14	Significant at 1%
Residual	43	0.37311	0.00715		

$$y = 1.648 - 0.019x_1 + 0.213x_2 - 0.002x_3 - 0.050x_4 + 0.022x_5 \quad (\text{Graph 2.})$$

TABLE 41. The results of a regression analysis, of the dry weight yield data of Aspergillus in Experiment 8, part 1, for the iron and iron:manganese ratio effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.87086	0.17417	4.99	Significant at 5%
Residual	43	1.50072	0.03490		

$$y = 1.649 + 0.090x_1 - 0.040x_2 + 0.004x_3 + 0.003x_4 - 0.004x_5 \quad (\text{Graph 4.})$$

TABLE 42. The results of a regression analysis of the dry weight yield data of Penicillium in Experiment 8, part 1, for the iron and iron:manganese ratio effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	7.79611	1.55922	20.27	Significant at 01%
Residual	43	3.30722	0.07691		

$$y = 1.648 - 0.194x_1 + 0.019x_2 - 0.043x_3 - 0.002x_4 - 0.005x_5 \quad (\text{Graph 5.})$$

- (a) The effect of increasing the iron supply, irrespective of the level of manganese in the medium.

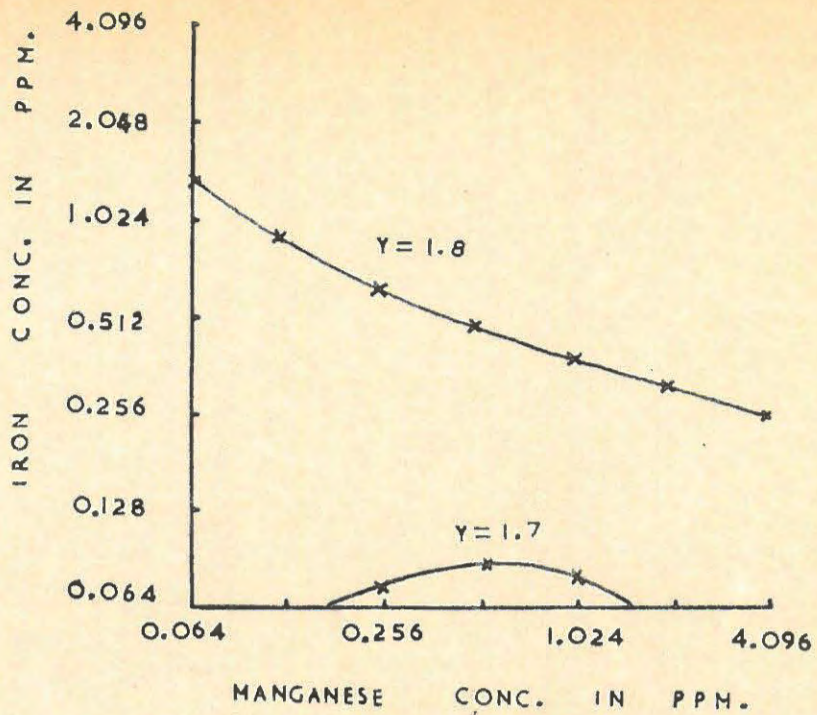
There is evidence (Tables 35 and 36) that in cultures of Aspergillus and Penicillium, increasing the level of iron supply, irrespective of the level of manganese in the medium, resulted in increased dry weight yields. In Aspergillus, the yields obtained from cultures receiving 0.064 p.p.m. were not significantly different from those receiving 0.256 p.p.m., and similarly the yields obtained from cultures receiving 0.512 and 4.096 p.p.m. were only barely significantly different. Thus although the yields obtained at high levels of iron supply were significantly different from those at low levels, the overall iron effect was not very marked, and was shown by an analysis of variance to be not significant, (Table 37). In cultures of Penicillium, however, the iron effect was shown to be highly significant (Table 38). It is clearly evident from Table 36 that, with the exception of the difference in the yield between cultures receiving 2.048 and 4.096 p.p.m., which was less than the least significant difference, for each increase in the level of iron supply the resulting increase in the dry weight yield was highly significant. These observations are confirmed by the regression analysis results.

From graphs 1 and 2 it can be seen that:

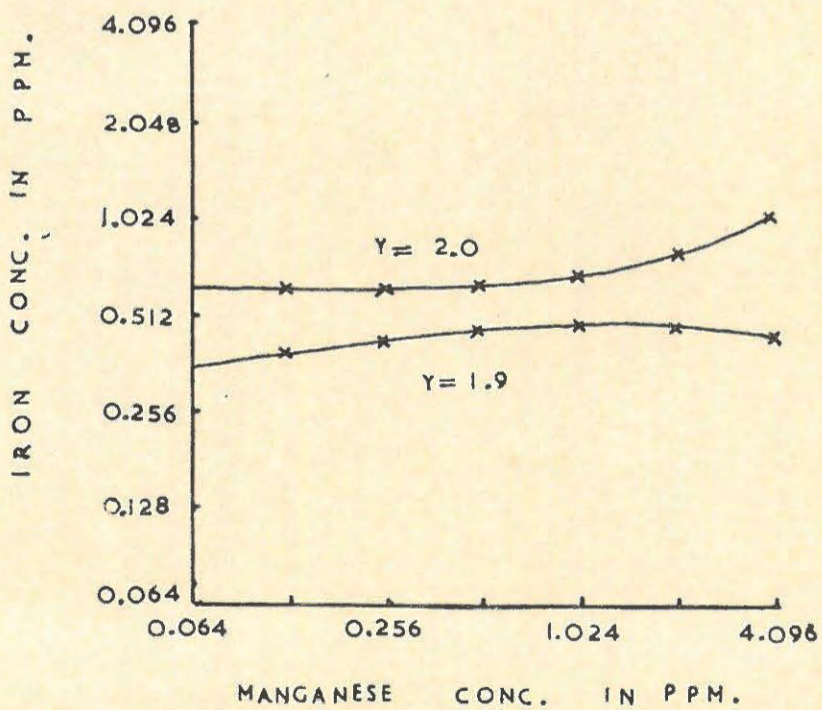
(i) The contours of the response surface, given by the yield values (y), are determined primarily by the level of iron supply, i.e. the contours are almost at right angles, rather than parallel to the axis for iron concentration.

(ii) The yield value (y) for each contour increases progressively with increased iron supply. Thus as the level of iron in the medium is raised, the dry weight yield increases.

There is some evidence that the level of iron supply in the culture medium was important in determining the degree of wrinkling



GRAPH 1. The effect of iron and manganese on the growth of Aspergillus niger.



GRAPH 2. The effect of iron and manganese on the growth of Penicillium glaucum.

and of sporulation of the felt in cultures of Aspergillus (Table 43). Increasing the concentration of iron resulted in a progressive increase in the wrinkling and sporulation.

- (b) The effect of increasing the manganese supply, irrespective of the level of iron in the medium.

In cultures of Aspergillus, increasing the concentration of manganese in the culture medium to 0.256 p.p.m. had little effect on the dry weight yield (Table 35). A further increase to 0.512 p.p.m., however, resulted in a significantly increased yield. There was no evidence that, in cultures receiving between 0.512 p.p.m. and 4.096 p.p.m. of manganese, the level of supply was important in determining the yield. Thus, although the yields obtained at high levels of manganese were significantly different from those at low levels of manganese supply, the manganese effect as a whole was found to be not significant (Table 38). There was evidence in Table 36 that increasing the level of manganese supply up to 0.128 p.p.m. had little effect on the dry weight yield in cultures of Penicillium but that subsequent increases resulted in progressively diminished yields. However, this effect was also found to be not significant (Table 33).

That the concentration of manganese in the culture medium was not important in the determination of the dry weight yield trends, was confirmed by the regression analysis results (graphs 1 and 2). From graphs 1 and 2 it can be seen that:

- (i) the contours of the response surface given by the yield values (y) are not markedly influenced by the level of manganese supply, since they tend to be parallel to the manganese concentration axis. In Aspergillus, however, the level of manganese was of some importance in determining the yield, when the yield was low. Thus for low yield values, approximately 1.7 gms., there was a tendency

for the yield to be depressed at intermediate manganese concentrations, and increased at high and low levels of manganese supply. At higher yield values (1.8 gms.), however, the level of manganese had a less marked effect on the yield trend.

There was some evidence, that, in cultures of Aspergillus, increasing the level of manganese supply resulted in a tendency for increased wrinkling of the felt (Table 43). However, the concentration of manganese in the medium did not markedly affect the sporulation, although there was some indication of depressed sporulation when the concentration of manganese in the culture medium was above 1.024 p.p.m.

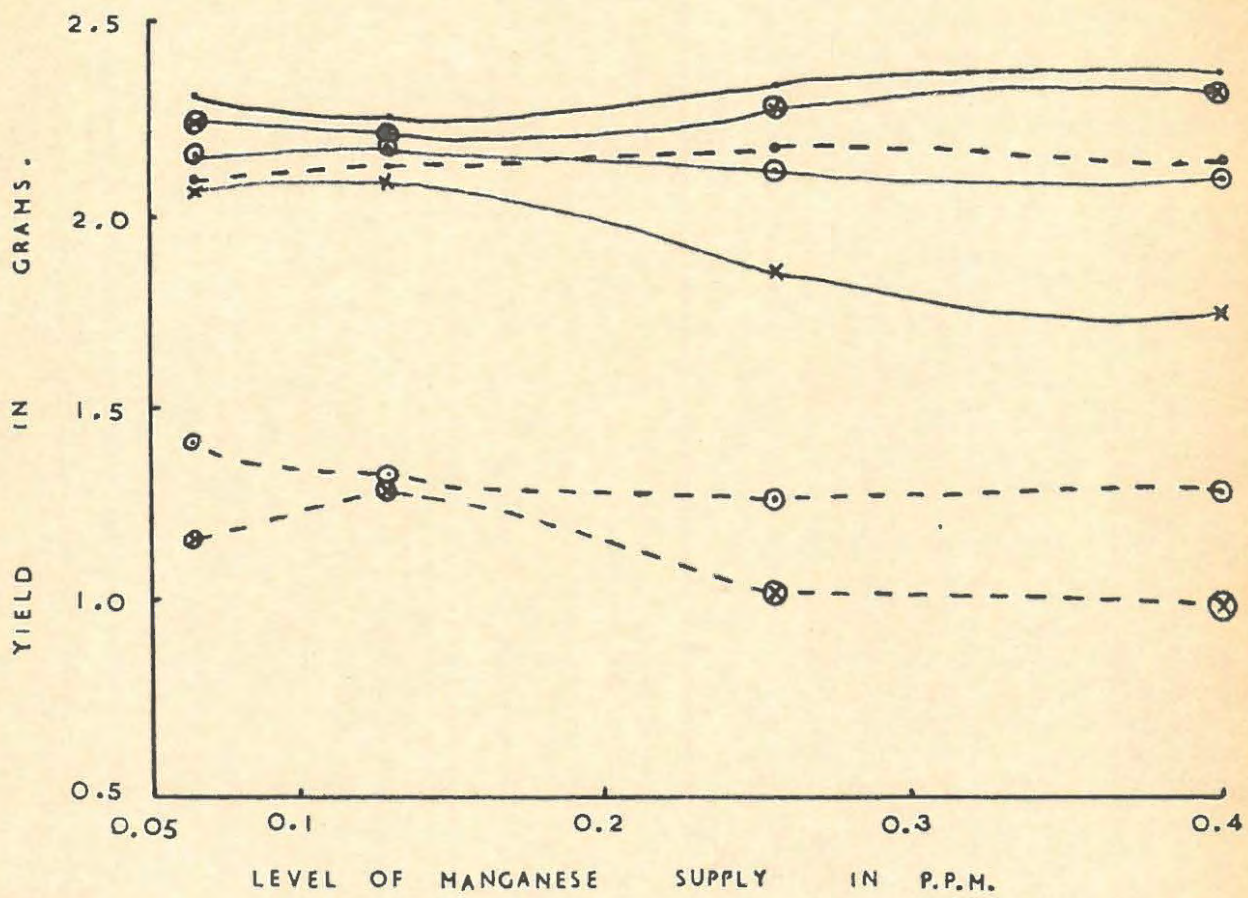
(c) The iron:manganese interaction effect on the yield.

The iron:manganese interaction effect was found to be significant in the yield data for Penicillium (Table 38) but not significant for Aspergillus (Table 37). Thus in cultures of Penicillium the iron and manganese effects, in determining the dry weight, were not independent. From graph 3 it can be seen that at low levels of iron, there was a tendency for the yield to increase with increased manganese supply up to 0.125 p.p.m., above which, raising the level of manganese resulted in slightly diminished yields. At high levels of iron supply, increasing the concentration of manganese in the culture medium had little effect on the yield.

(d) The relation between growth and development and the iron:manganese ratio in the medium.

From the yield data (Table 44) it was evident that varying the iron:manganese balance in the culture medium had little effect on the dry weight yield obtained from cultures of Aspergillus. The regression analysis results (graph 4) indicated that:

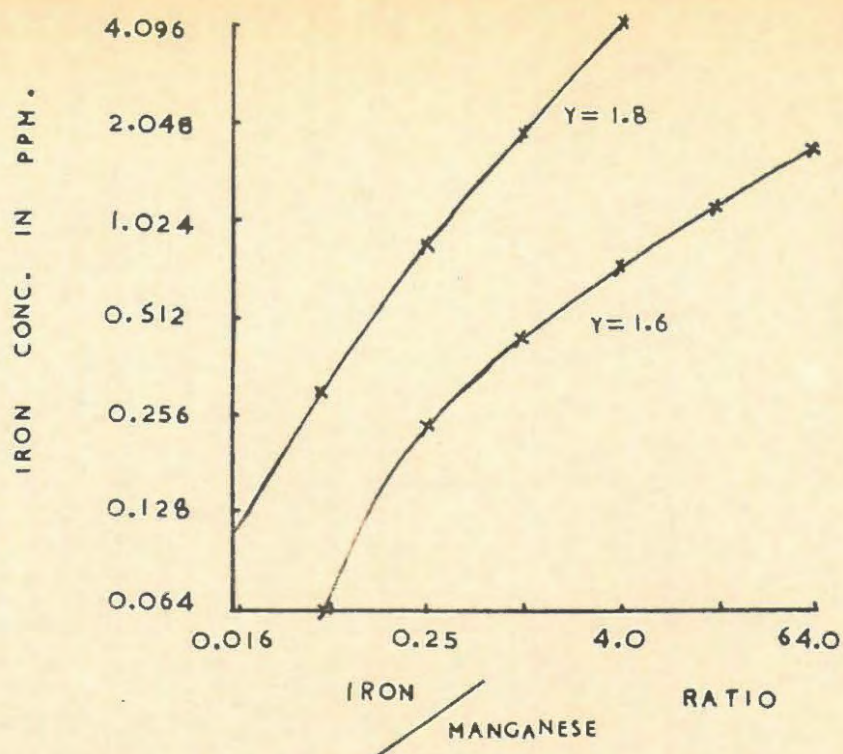
(i) the iron concentration and the iron:manganese balance in



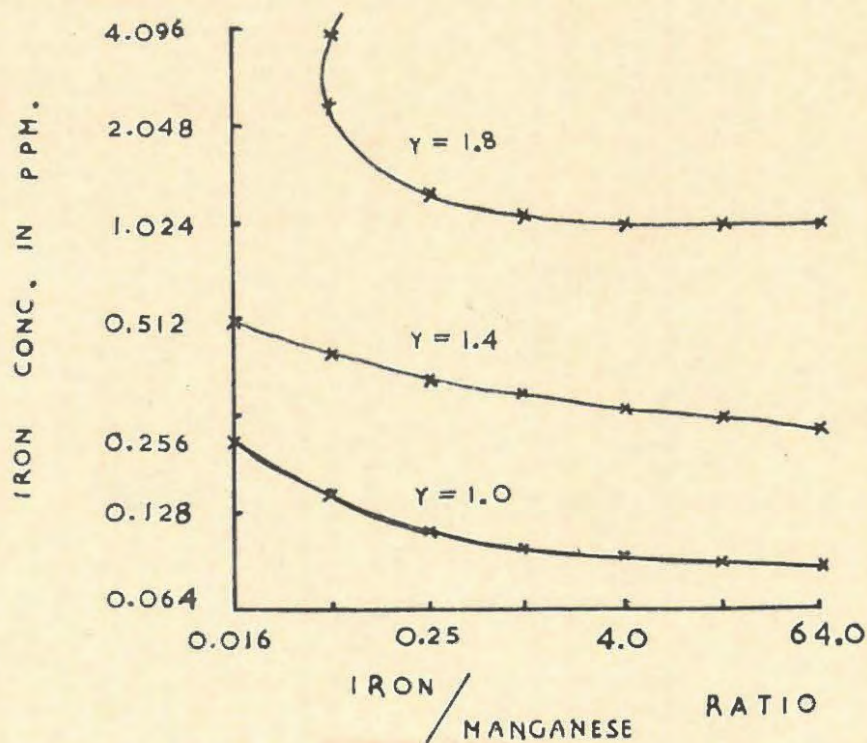
LEVEL OF Fe.
IN P.P.M.

⊗ --- ⊗ 0.064
 ⊖ --- ⊖ 0.128
 × ——— × 0.256
 • --- • 0.512
 ⊙ ——— ⊙ 1.024
 ⊗ ——— ⊗ 2.048
 ——— • 4.096

GRAPH 3. The iron:manganese interaction in Penicillium,
Experiment 8, part 1.



GRAPH 4. The effect of iron and the iron:manganese balance on the growth of Aspergillus niger.



GRAPH 5. The effect of iron and the iron:manganese balance on the growth of Penicillium glaucum.

TABLE 43. The effect of increasing the iron and manganese supply on the form and sporulation of cultures of Aspergillus niger.

Manganese supply in p.p.m.	Iron Supply in p.p.m.						
	0.064	0.128	0.256	0.512	1.024	2.048	4.096
0.064	SM. 2,6,4	S.W. 5,3,4	F.W. 1,2,6	H.W. 4,4,5	S.W. 7,4,2	H.W. 9,7,4	V.H.W. 4,4,7
0.128	SM. 5,6,5	S.W. 7,7,3	V.S.W. 3,3,2	H.W. 3,4,5	M.W. 6,5,6	S.M. 3,4,7	H.W. 9,8,3
0.256	SM. 2,4,4	V.S.W. 3,2,5	V.S.W. 4,5,3	M.W. 7,6,4	M.W. 7,7,5	S.M. 9,2,7	M.W. 9,2,9
0.512	S.M. 2,5,5	M.W. 7,4,6	S.W. 5,5,4	M.W. 3,6,6	H.W. 7,7,5	H.W. 9,9,4	F.W. 9,9,2
1.024	V.S.W. 3,2,4	S.M. 4,3,6	S.M. 6,3,3	S.W. 2,5,7	H.W. 4,4,3	V.H.W. 2,9,2	S.W. 6,6,9
2.048	V.S.W. 3,5,5	S.M. 5,8,3	H.W. 4,6,9	M.W. 6,6,4	V.H.W. 5,6,8	H.W. 5,2,3	V.H.W. 2,9,2
4.096	V.S.W. 4,5,4	S.M. 5,2,7	H.W. 1,3,6	M.W. 1,6,6	V.H.W. 1,5,7	F.W. 2,10,5	H.W. 8,7,8

KEY.

SM.	SMOOTH.
V.S.W.	VERY SLIGHTLY WRINKLED
S.W.	SLIGHTLY WRINKLED
M.W.	MODERATELY WRINKLED
H.W.	HIGHLY WRINKLED
V.H.W.	VERY HIGHLY WRINKLED

Index of Sporulation:

1 = Very sparse

10 = Very dense

the medium were both equally important in the determination of the yield trends. i.e. the line for each contour is diagonal, rather than parallel (predominantly iron effect) or vertical (predominantly ratio effect) to the iron:manganese ratio axis. However, the iron effect was found, by an analysis of variance, to be not significant.

(ii) The yield value for each contour decreased with increase in the iron:manganese ratio. Thus as the iron:manganese balance in the culture medium increased there was a tendency for the dry weight yield to decrease.

In Penicillium (Table 45) there was some evidence that the yield increased with increase in the iron:manganese ratio up to 32, but a further increase to 64 had little effect. These results were to a certain extent confirmed by the regression analysis results. From graph 5 it can be seen that:

(i) the contour lines for different yield values tended to be parallel to the iron:manganese ratio axis, thereby indicating that the level of iron supply was the major factor determining the yield. However, since the contour lines for each yield value tended to curve upwards at low iron:manganese ratios, the iron:manganese ratio was of some importance in the determination of the yield trends.

(ii) There was a tendency for the yield to increase with increase in the level of iron supply and iron:manganese ratio. Thus maximum yield was obtained when the concentration of iron in the medium was between 2.048 and 4.096 p.p.m. and the iron:manganese ratio was approximately 4.0.

There was no evidence that the iron:manganese balance was important in determining the degree of wrinkling or sporulation of the felts produced in cultures of Aspergillus or Penicillium.

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TABLE 44. The dry weight yields, in grams, obtained for each iron:manganese ratio, with Aspergillus niger in Experiment 8, part 1.

				1.55925									
				1.57203		1.53152							
			1.52017		1.74215		1.86517						
		1.67933		1.38207		1.60523		1.81204					
	1.53368		1.78249		1.73216		1.39290		1.80302				
	1.94776		1.75606		1.51243		1.87157		1.82205		1.45049		
1.94711		1.75744		1.41193		1.85374		1.81804		1.87411		1.99588	
	1.68065		2.21105		1.68227		2.19355		1.70686		1.88680		
		1.63357		2.15104		1.74750		2.15952		1.86094			
			2.05622		1.94618		1.90342		1.85607				
				2.00558		1.90994		2.23685					
					1.75573		1.90135						
						2.20508							
Total	1.94711	3.62841	4.92469	7.70266	8.87121	9.85971	12.74982	11.00664	9.47648	7.19702	5.53807	3.3379	1.99885
Mean of total	1.94711	1.81420	1.64156	1.92566	1.77424	1.65995	1.82140	1.83444	1.89509	1.79925	1.84602	1.66864	1.99585
Fe/Mn ratio	0.016	0.031	0.062	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0

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TABLE 45. The dry weight yields, in grams, obtained for each iron:manganese ratio, with Penicillium glaucum in Experiment 8, part 1.

						1.14657							
					1.26249		1.32854						
				1.02607		1.31230		2.05842					
		0.88670			1.23425		2.10879		2.06456				
	1.04725			1.37321		1.87403		2.13934		2.18933			
0.94324		1.19886			1.75338		2.17242		2.16750		2.29255		
0.98058		1.24003		1.77508		2.18430		2.11121		2.23715		2.21023	
	1.11054		1.85686		2.19956		2.19692		2.32103		2.18928		
		1.63978		2.05116		2.20478		2.30245		2.29591			
			2.03796		2.21383		2.21351		2.32364				
				2.21079		2.25496		2.27340					
					2.24978		2.24942						
						2.20445							
Total	0.98058	2.05378	3.92706	6.03038	8.43631	10.91329	13.18139	12.26960	10.88482	8.87673	6.72239	4.48183	2.21023
Mean of Totals	0.98058	1.02689	1.30902	1.50759	1.68726	1.81888	1.88306	2.04493	2.17696	2.21919	2.24079	2.24091	2.21023
Fe/Mn ratio	0.016	0.031	0.062	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0

Part 2.

The plan of part 2 of Experiment 8 is given in Table 46.

A series of stock solutions of iron and manganese were prepared, by serial 1:1 (1 volume solution to 1 volume of purified water) dilutions of a concentrated solution, from which a 1 ml. sample in 50 ml. gave the maximum concentration required. The concentrated iron and manganese solutions, from which the other solutions were prepared, were made up as follows.

Iron: (i) Prepared without a chelating agent.

6.371 gms. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in purified water in a 200 ml. volumetric flask.

1 ml. of this solution in 50 ml. gives an iron concentration of 128 p.p.m.

(ii) Citric acid as chelating agent.

6.371 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved with 4.8154 gms. citric acid in purified water in a 200 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives an iron concentration of 128 p.p.m.

(iii) E.D.T.A. as chelating agent.

6.371 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved with 8.53095 gms. E.D.T.A. in purified water in a 200 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives an iron concentration of 128 p.p.m.

Manganese: (i) Prepared without a chelating agent.

5.1964 gms. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was dissolved in purified water in a 200 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives a manganese concentration of 128 p.p.m.

TABLE 46. The plan of Experiment 8, part 2, showing the levels of iron and manganese supply, and the resulting iron:manganese ratios.

Level of Mn supply in p.p.m.	Level of Iron Supply in p.p.m.						
	2.0	4.0	8.0	16.0	32.0	64.0	128.0
2.0	1.0	2.0	4.0	8.0	16.0	32.0	64.0
4.0	0.5	1.0	2.0	4.0	8.0	16.0	32.0
8.0	0.25	0.5	1.0	2.0	4.0	8.0	16.0
16.0	0.125	0.25	0.5	1.0	2.0	4.0	8.0
32.0	0.0625	0.125	0.25	0.5	1.0	2.0	4.0
64.0	0.03125	0.0625	0.125	0.25	0.5	1.0	2.0
128.0	0.015625	0.03125	0.0625	0.125	0.25	0.5	1.0

(ii) E.D.T.A. as chelating agent.

5.1964 gms. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was dissolved with 8.67426 gms. E.D.T.A. in purified water in a 200 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives a manganese concentration of 128 p.p.m.

Results.

The results are given as shown in Table 47.

TABLE 47. The results of Experiment 8, part 2. Table giving the aspect considered and the number of the table in which the results for each factor are given.

Aspect considered	Aspergillus (No chelating agent)	Aspergillus (Citric acid as chelating agent)	Penicillium (E.D.T.A. as chelating agent)
Dry weight yield	48	49	50
pH of filtrate (No. of table in Appendix)	12	13	14
Analysis of variance	51	52	53
Regression analysis iron and manganese effects.	54	55	56
Regression analysis iron and iron:manganese ratio effects.	57	58	59

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TABLE 48. The dry weight yield in grams, obtained with Aspergillus niger in Experiment 8, part 2.
(No chelating agent supplied.)

Manganese supply in p.p.m.	Iron Supply in p.p.m.							Total for each manganese treatment.	Mean of total for each Mn treatment.
	2	4	8	16	32	64	128		
	.11442	.15032	.44411	.32515	.48084	.36994	.34305		
2	.48379	.23295	.20523	.28260	.46262	.12831	.51185	7.27052	1.03865
	.38917	.53849	.25405	.49449	.51182	.26192	.28540		
	.15840	.35600	.28265	.28057	.27870	.14539	.34852		
4	.58035	.37764	.21985	.36185	.43627	2.5878	.27405	6.49123	0.92732
	.17930	.59741	.31985	.21705	.29512	2.5300	.27048		
	.34262	.53457	.29908	.52430	.40710	.36251	.31985		
8	.27927	.46906	.30400	.60358	.57425	.35371	.23305	8.30287	1.18670
	.27549	.46335	.37158	.39495	.30171	.46290	.42594		
	.28715	.49802	.49792	.24828	.21497	.46926	.26080		
16	.38634	.37666	.53805	.36430	.39821	.30265	.23015	7.18504	1.02643
	.34801	.27185	.42980	.33017	.27212	.16416	.29617		
	.19817	.13419	.41239	.42661	.44279	.19320	.31292		
32	.29731	.19869	.18925	.37260	.28345	.35552	.44717	7.01070	1.00153
	.36830	.38205	.38976	.45346	.43848	.26764	.44675		
	.12780	.21009	.27200	.41399	.18887	.43535	.34600		
64	.37158	.16520	.33130	.27995	.27915	.45627	.29544	6.17831	0.88262
	.29677	.21904	.21927	.22670	.40534	.32305	.31515		
	.34924	.30387	.21270	.36045	.41745	.37490	.43684		
128	.25407	.13935	.33988	.19395	.16102	.34956	.39085	6.40636	0.91519
	.23503	.26979	.28269	.36181	.34887	.20373	.42031		
Total for each Fe treatment	7.21074	6.49175	7.59915	7.51681	6.81541	6.88859	6.32258		
Mean of total for each Fe treatment	1.03011	0.92739	1.08570	1.07383	0.97363	0.98408	0.90323		

Least significant difference between means at 0.05 probability level = ± 0.08250 gms.

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TABLE 49. The dry weight yield in grams, obtained with Aspergillus niger in Experiment 8, part 2.
(Citric acid as chelating agent.)

Manganese supply in p.p.m.	Iron Supply in p.p.m.							Total for each manganese treatment	Mean of total for each Mn treatment
	2	4	8	16	32	64	128		
2	.24496	.19150	.36786	.24226	.08306	.35078	.40105	7.20487	1.02927
	.36467	.36241	.47725	.31812	.20932	.39766	.44356		
	.43081	.33818	.49604	.31781	.42868	.33997	.39842		
4	.31929	.36978	.41997	.22970	.13710	.24336	.23153	6.77693	0.96813
	.36778	.31826	.24225	.32701	.26179	.39606	.44532		
	.38065	.31621	.31105	.42130	.22877	.33580	.47295		
8	.24776	.37570	.36468	.44497	.19760	.27109	.29085	6.82304	0.97472
	.43156	.35025	.35125	.23782	.22160	.35473	.40087		
	.35809	.35740	.35555	.10965	.42847	.40423	.26392		
16	.49017	.29285	.36001	.35295	.36788	.23023	.24218	6.68890	0.95556
	.36734	.36762	.43089	.43587	.26807	.26392	.22444		
	.31723	.47240	.31149	.29690	.17661	.24096	.17589		
32	.38866	.35626	.37510	.34438	.33297	.38038	.20025	7.23491	1.03356
	.23335	.34126	.37336	.28251	.22050	.22612	.19868		
	.48956	.38507	.37618	.40254	.32230	.25508	.45040		
64	.41186	.34863	.32540	.38363	.10811	.37932	.37750	7.65833	1.09405
	.46223	.31041	.42147	.31917	.38013	.49529	.40190		
	.22196	.38105	.40227	.14665	.33839	.39081	.35215		
128	.43563	.21195	.40634	.51365	.43863	.14048	.41078	7.81607	1.11658
	.28692	.48998	.39407	.34501	.23276	.47067	.46881		
	.51454	.54074	.39342	.13825	.27776	.40056	.29967		
Total for each Fe treatment	7.15612	6.97050	6.26205	6.61015	7.95590	7.47791	7.76502		
Mean of total for each Fe treatment	1.02230	0.99579	0.89458	0.94431	1.13656	1.06826	1.10929		

Least significant difference between means at 0.05 probability level = \pm 0.09322 gms.

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TABLE 50. The dry weight yield in grams, obtained with *Penicillium glaucum* in Experiment 8, part 2.

Manganese supply in p.p.m.	Iron Supply in p.p.m.							Total for each manganese treatment	Mean of total for each Mn treatment
	2	4	8	16	32	64	128		
2	.54088	.56267	.63211	.63361	.82750	.50873	.78375	13.39474	1.91353
	.55176	.76333	.66545	.57851	.57227	.71350	.54738		
	.57214	.62799	.57283	.59540	.86944	.45736	.81813		
4	.59235	.55022	.73679	.65732	.80183	.66769	.72055	13.40317	1.91474
	.48675	.55236	.66039	.48265	.71256	.55439	.67796		
	.53423	.55729	.65158	.66493	.65973	.67787	.80370		
8	.60850	.60437	.74417	.70513	.67382	.61496	.81952	13.58113	1.95445
	.56143	.63514	.67682	.50075	.74617	.60979	.69569		
	.55359	.64318	.61293	.72330	.63585	.59115	.62487		
16	.53763	.52811	.59196	.64321	.53565	.67797	.63677	12.63735	1.80534
	.54016	.57527	.59265	.51228	.62435	.65110	.69445		
	.59463	.63137	.52447	.71299	.58431	.53514	.71288		
32	.51354	.60034	.69476	.64872	.77976	.61760	.74688	13.22932	1.83847
	.61400	.63850	.60379	.68534	.54556	.57017	.67698		
	.49787	.60616	.60104	.61456	.69178	.64496	.62791		
64	.49333	.57095	.45695	.56752	.72022	.72073	.77372	13.33718	1.90531
	.51390	.45804	.66499	.60748	.78421	.59345	.76559		
	.73236	.50452	.81144	.51245	.54756	.75131	.78636		
128	.53675	.59840	.61156	.66054	.62184	.66414	.84365	13.08064	1.86866
	.55975	.62989	.57388	.60889	.55908	.62539	.51574		
	.53504	.56181	.58105	.74055	.62226	.61625	.81418		
Total for each Fe treatment	11.67057	12.39991	13.27071	13.05613	14.11580	13.06375	15.08666		
Mean of total for each Fe treatment	1.66722	1.77142	1.89582	1.86516	2.01654	1.86625	2.15524		

Least significant difference between means at 0.05 probability level = \pm 0.03960 gms.

TABLE 51. The results of an analysis of variance performed on the dry weight yield data of Aspergillus, (No chelate supplied) in Experiment 8, part 2.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Iron	6	0.06815	0.01139	1.287	Not significant
Manganese	6	0.14707	0.02451	2.769	Not significant
Interaction	36	0.55723	0.01548	1.749	Significant at 5%
Replication	2	0.00445	0.00224	0.25	Not significant
Error	96	0.84964	0.00885		

TABLE 52. The results of an analysis of variance performed on the dry weight yield data of Aspergillus, (Citric acid as chelating agent), in Experiment 8, part 2.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Iron	6	0.10689	0.1781	1.580	Not significant
Manganese	6	0.05927	0.00988	0.088	Not significant
Interaction	36	0.02217	0.000616	0.005	Not significant
Replication	2	0.02079	0.01048	0.930	Not significant
Error	96	1.07724	0.01122		

TABLE 53. The results of an analysis of variance performed on the dry weight yield data of *Penicillium*, in Experiment 8, part 2.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Iron	6	0.35361	0.05893	29.62	Significant at 0.1%
Manganese	6	0.02757	0.00489	2.46	Not significant
Interaction	36	0.18911	0.00525	2.64	Significant at 0.1%
Replication	2	0.01221	0.00610	3.00	Not significant
Error	96	0.21112	0.00199		

TABLE 54. The results of a regression analysis of the dry weight yield data of *Aspergillus* (no chelating agent) in Experiment 8, part 2, for the iron and manganese effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.87696	0.17539	5.24	Significant at 5%
Residual	43	1.41038	0.03350		

$$y = 0.913 + 0.013x_1 - 0.036x_2 - 0.010x_3 - 0.011x_4 - 0.028x_5 \quad (\text{Graph 6}).$$

TABLE 55. The results of a regression analysis of the dry weight yield data of *Aspergillus*, (citric acid as chelating agent), in Experiment 8, part 2, for the iron and manganese effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.31371	0.06274	10.74	Significant at 1%
Residual	43	0.25118	0.00584		

$$y = 1.004 + 0.020x_1 + 0.023x_2 + 0.010x_3 + 0.009x_4 - 0.004x_5 \quad (\text{Graph 7}).$$

TABLE 56. The results of a regression analysis of the dry weight yield data of Penicillium in Experiment 8, part 2, for the iron and manganese effect.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.81836	0.16367	7.89	Significant at 5%
Residual	43	0.89243	0.02075		

$$y = 1.976 - 0.007x_1 + 0.063x_2 + 0.002x_3 - 0.001x_4 + 0.005x_5 \quad (\text{Graph 8}).$$

TABLE 57. The results of a regression analysis of the dry weight yield data of Aspergillus (no chelating agent), in Experiment 8, part 2, for the iron and iron:manganese balance effect.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.36647	0.07329	1.61	Not significant
Residual	43	1.95107	0.04537		

$$y = 0.962 - 0.023x_1 + 0.014x_2 - 0.003x_3 - 0.006x_4 + 0.014x_5 \quad (\text{Graph 11}).$$

TABLE 58. The results of a regression analysis of the dry weight yield data of Aspergillus (citric acid as chelating agent) in Experiment 8, part 2, for the iron and iron:manganese balance effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.41103	0.08220	23.09	Significant at 1%
Residual	43	0.15319	0.00356		

$$y = 1.128 - 0.001x_1 - 0.019x_2 + 0.027x_3 + 0.020x_4 - 0.014x_5 \quad (\text{Graph 12}).$$

TABLE 59. The results of a regression analysis of the dry weight yield data of Penicillium in Experiment 8, part 2, for iron and iron:manganese balance effects.

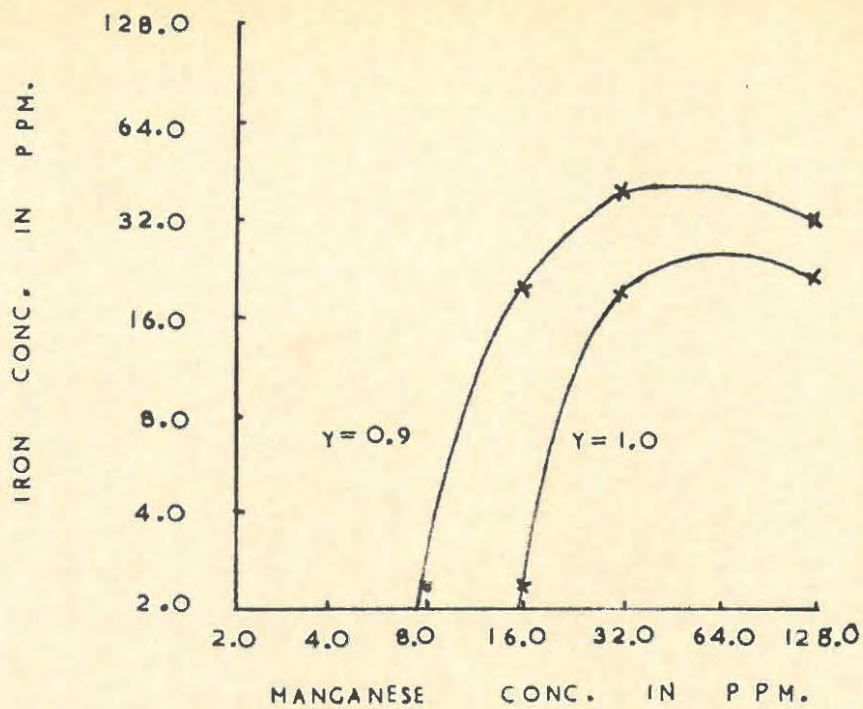
Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.84902	0.16980	8.47	Significant at 5%
Residual	43	0.86187	0.02004		

$$y = 1.891 + 0.048x_1 + 0.015x_2 + 0.004x_3 + 0.002x_4 - 0.009x_5 \quad (\text{Graph 13}).$$

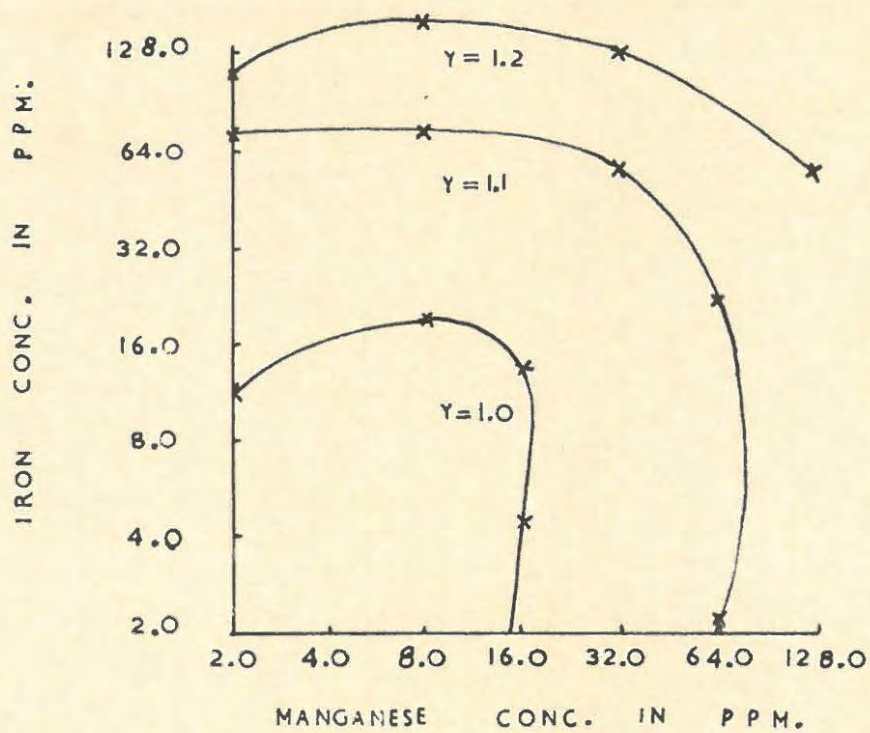
The results will be considered under the following headings:

- (a) The effect of increasing the level of iron supply, irrespective of the level of manganese in the medium.
 - (b) The effect of increasing the level of manganese supply, irrespective of the level of iron in the medium.
 - (c) The iron:manganese interaction effect on the yield.
 - (d) The relation between growth and development and the iron:manganese ratio in the medium.
- (a) The effect of increasing the level of iron supply, irrespective of the level of manganese in the medium.

The effect of raising the level of iron supply in the culture medium on cultures of Aspergillus was not marked, irrespective of the presence of a chelating agent (Tables 48 and 49). When no citric acid was present in the culture medium, there was some evidence of slightly diminished yields at high levels of iron supply (Table 48), but the effect was not significant. From the graph of the regression analysis (graph 6) it was evident that there was a tendency for the yield to be maximal at low levels of iron supply, indicating that high levels of iron were slightly toxic. Since the contours for each yield tended to be parallel (predominant manganese effect), rather



GRAPH 6. The effect of iron and manganese on the growth of Aspergillus niger. (No chelating agent.)

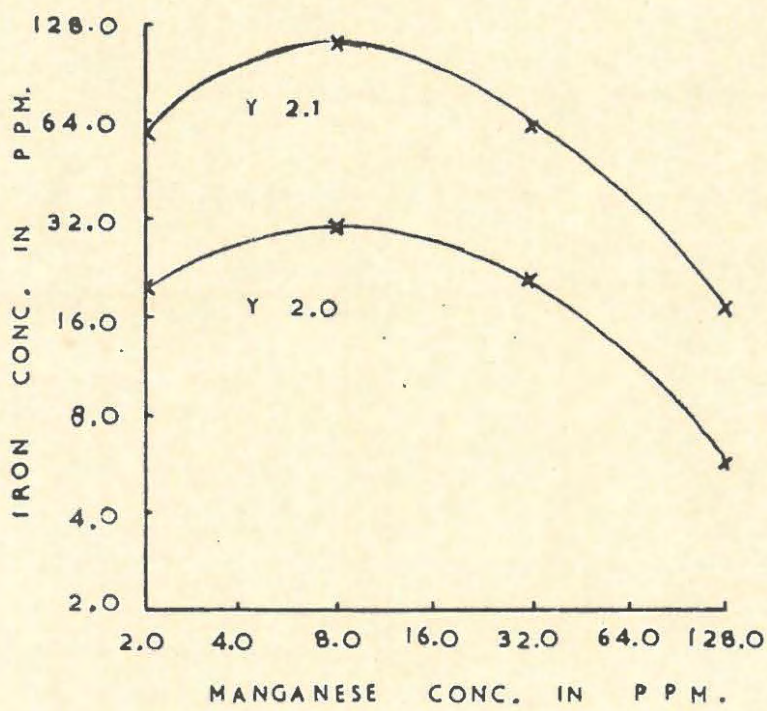


GRAPH 7. The effect of iron and manganese on the growth of Aspergillus niger. (Citric acid as chelating agent.)

than at right angles (predominant iron effect), to the iron concentration axis, it is evident that the concentration of iron was not the major factor determining the yield trends. Raising the level of iron supply in the presence of citric acid resulted in a slight increase in the yield, although the effect was not significant, (Table 51), however, the regression analysis results (graph 7) indicate that the levels of iron and manganese supply are equally important in determining the yield trends.

In cultures of Penicillium the overall effect of increasing the level of iron supply in the culture medium was shown to be highly significant in determining the yield trends, (Table 53). The yield data (Table 50) shows clearly that with each increase in the concentration of iron in the culture medium, there was a significant change in the yield, and that the overall trend was for progressively increased dry weight yield with increased iron supply. These observations were clearly illustrated by the graph (graph 8) of the regression analysis results. Thus the yield values for each contour increase with increase in the iron concentration, and the contours tend to be orientated at right angles to the iron concentration, indicating a predominant iron effect. There is some evidence, therefore, that high levels of iron were slightly toxic when no chelating agent was present in the culture medium, but in the presence of citric acid or E.D.T.A. there was no evidence of diminished yields at high iron levels.

Raising the level of iron supply in cultures of Aspergillus and Penicillium, irrespective of the presence of a chelating agent, had little effect on the pH of the filtrate or the degree of sporulation and wrinkling of the felts.



GRAPH 8. The effect of iron and manganese on the growth of Penicillium glaucum.

- (b) The effect of increasing the level of manganese supply, irrespective of the level of iron in the medium.

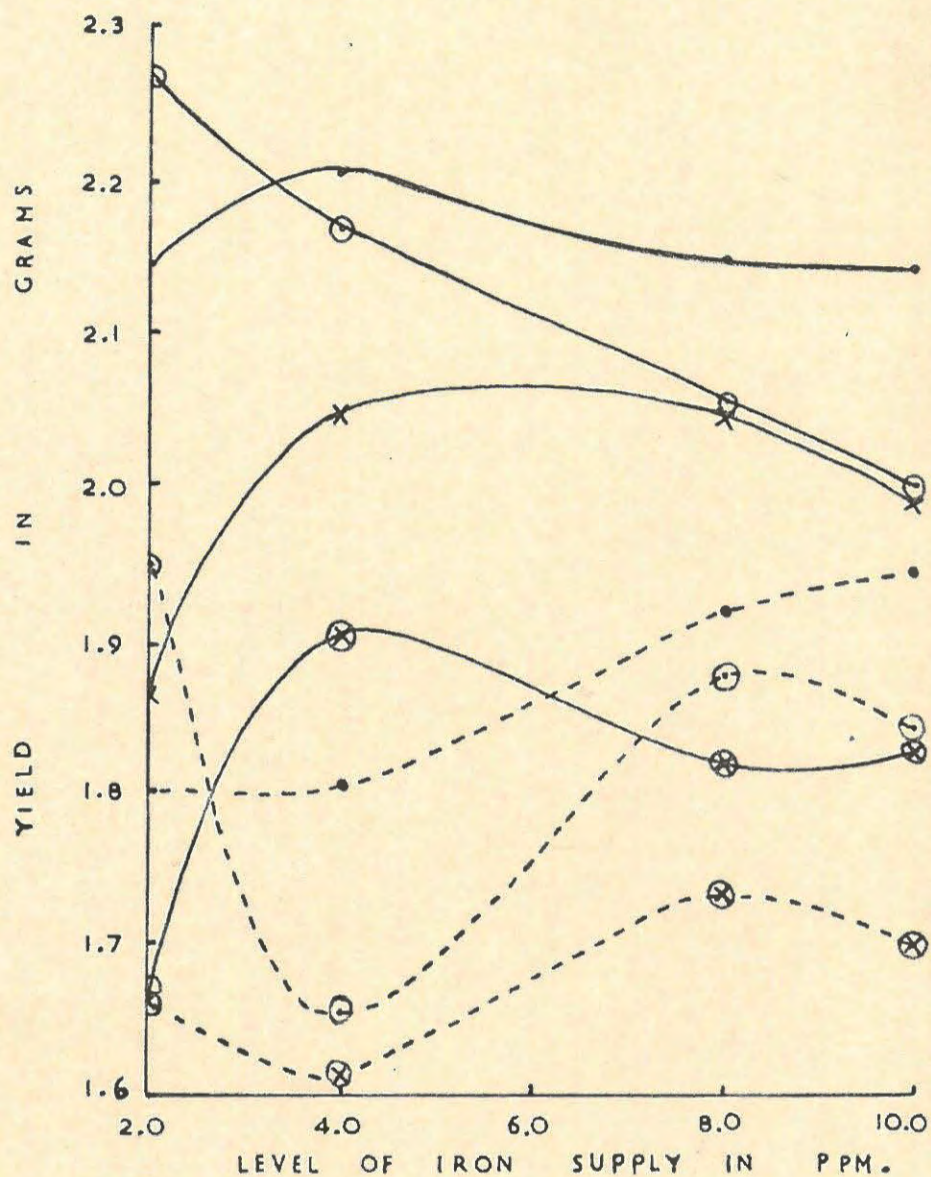
In cultures of Aspergillus and Penicillium, irrespective of the presence of a chelating agent, raising the level of manganese in the medium did not have a marked effect, and was found, by an analysis of variance to be not significant (Tables 51, 52 and 53). With Aspergillus when no chelating agent was present in the medium, raising the level of manganese supply to 8.0 p.p.m. resulted in a barely significant increase in the yield, (Table 48). Subsequent increases, up to 128.0 p.p.m., resulted in significantly decreased yields. The results of the regression analysis (graph 6) show clearly that the level of manganese supply was the major factor determining the yield and that maximal yield was obtained at low levels of iron supply and high levels of manganese. When citric acid or E.D.T.A., were supplied as chelating agents there was no evidence of diminished yields at high levels of manganese (Tables 49 and 50). The yield trends were determined jointly by the levels of iron and manganese supplies, in Aspergillus, but mainly by the level of iron supply in Penicillium.

The level of manganese supply had no effect on the pH of the filtrate at the degree of sporulation and wrinkling of the felts.

- (c) The iron:manganese interaction effect on the yield.

The iron:manganese interaction effect was found by analysis of variance (Tables 51 and 53) to be significant in Penicillium and in Aspergillus when no chelating agent was supplied. In Penicillium (graph 9) raising the level of iron supply to 4.0 p.p.m., at low levels of manganese in the medium resulted in diminished yield.

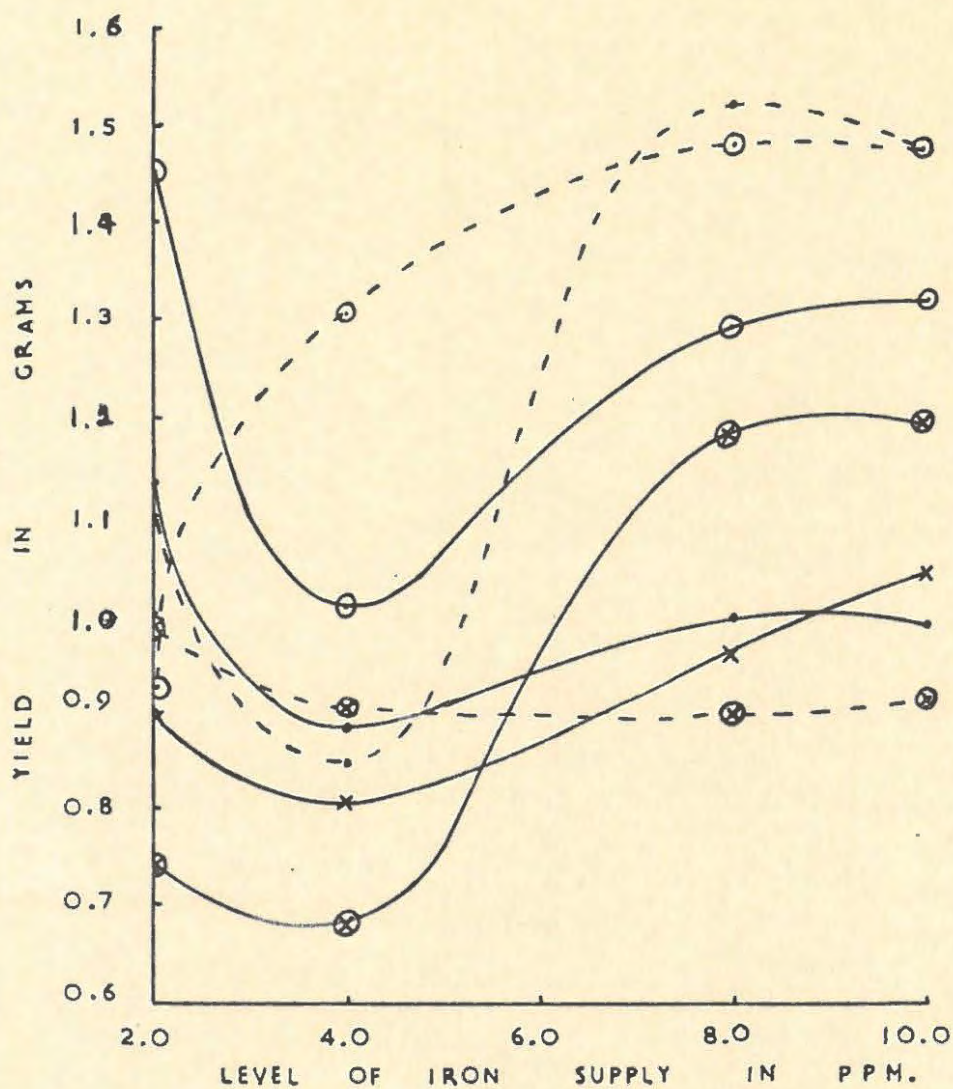
Further increases to 8.0 p.p.m. resulted in increased yield. At high levels of manganese in the medium there was a tendency for the yield to increase when the level of iron supply was raised to 4.0 p.p.m. Subsequent increases in the iron supply to 8.0 p.p.m.



LEVEL OF Mn.
IN P.P.M.

- ⊗-----⊗ 2.0
- ⊕-----⊕ 4.0
- x-----x 8.0
- 16.0
- ⊖-----⊖ 32.0
- ⊗-----⊗ 64.0
- 128.0

GRAPH 9. The interaction effect of iron and manganese in Penicillium glaucum, in Experiment 8, part 2.



LEVEL OF Fe.
IN P.P.M.

- ⊗ --- ⊗ 2.0
- ⊙ --- ⊙ 4.0
- × — × 8.0
- - - · 16.0
- ⊕ — ⊕ 32.0
- ⊗ — ⊗ 64.0
- — 128.0

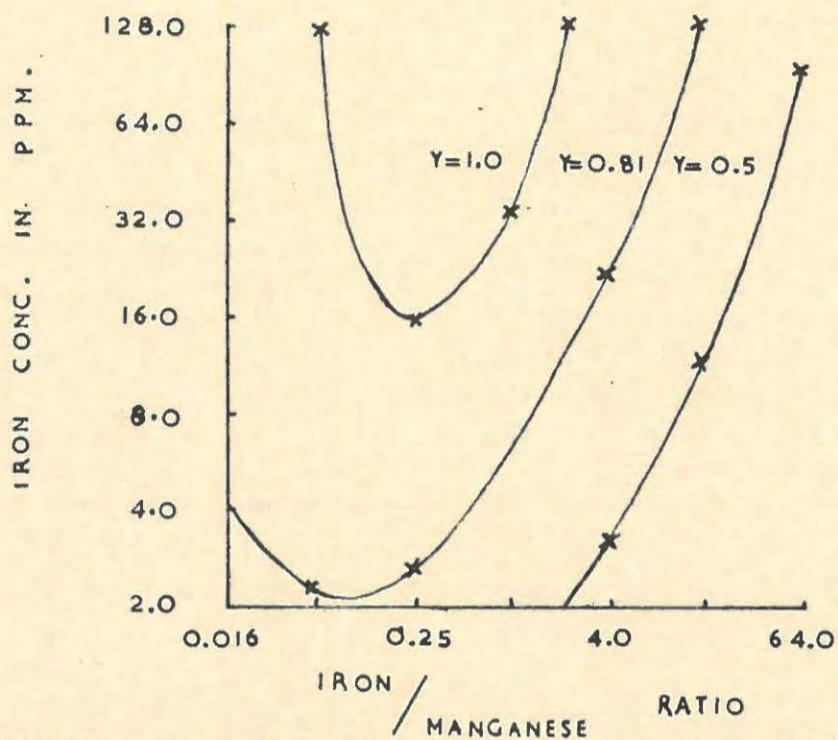
GRAPH 10. The interaction effect of iron and manganese in Aspergillus niger, in Experiment 8, part 2.

resulted in gradually diminished yield. Thus there was evidence that the supplies of iron and manganese were not independent in determining the yield.

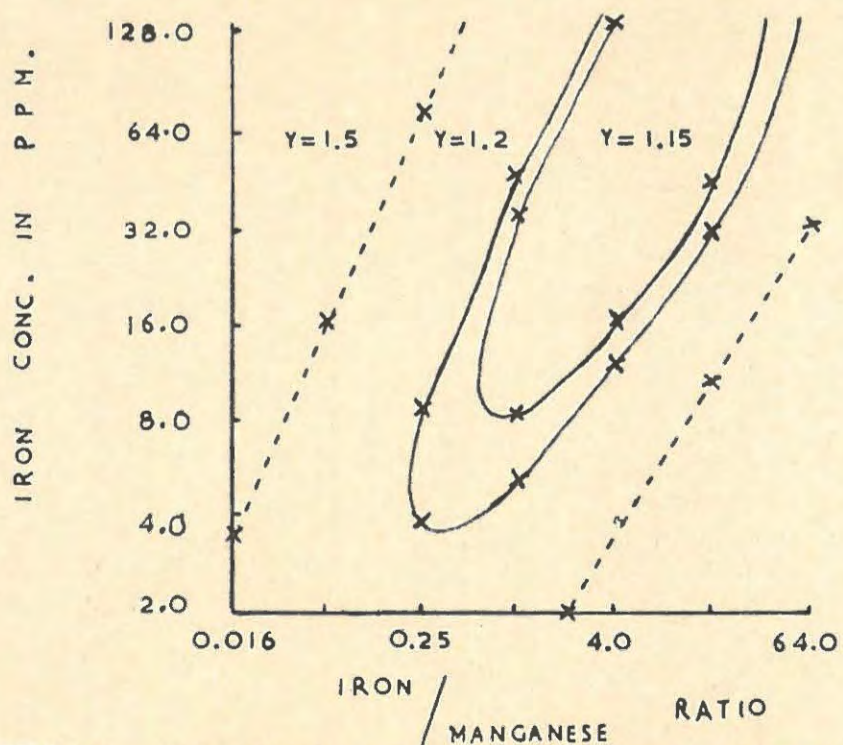
In Aspergillus the interaction effect was not very significant. In graph 10, there is evidence that at all levels of manganese supply above 4.0 p.p.m., increasing the level of iron to 4.0 p.p.m. resulted in a marked tendency for diminished yields. At low levels of manganese, the trends were markedly different. When the manganese level was 2.0 p.p.m. increasing the iron supply to 4.0 p.p.m. resulted in diminished yields, but further increases had little effect. At the 4.0 p.p.m. level of manganese supply, increasing the level of iron supply to 8.0 p.p.m. resulted in a marked progressive increase in the yield. Thus in both Aspergillus and Penicillium the iron:manganese interaction is most evident when the concentrations of iron and manganese in the medium are 4.0 p.p.m.

- (d) The relation between growth and development and the iron:manganese ratio in the medium.

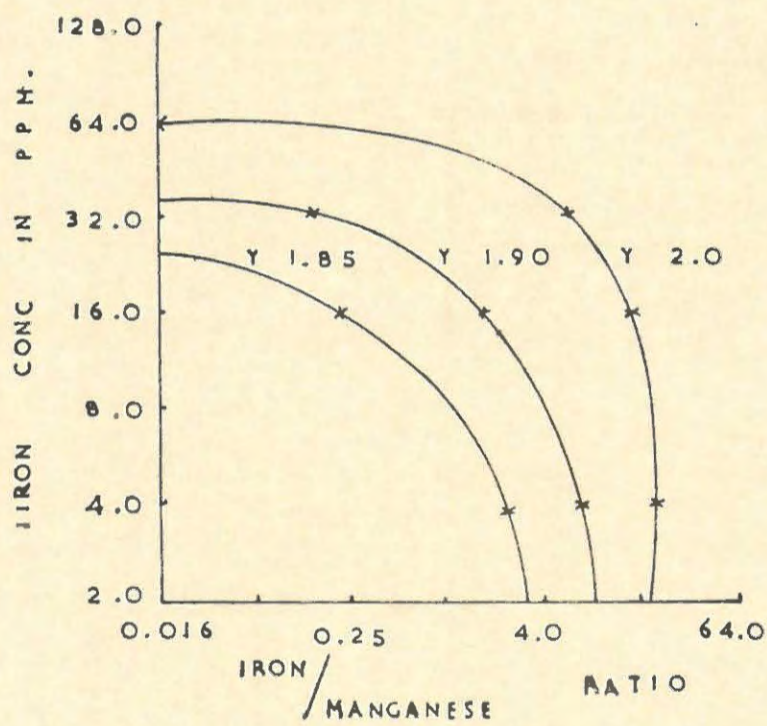
There is some evidence (Table 60) that raising the iron:manganese ratio to approximately 0.5 resulted in increased dry weight yield in cultures of Aspergillus when no chelating agent was present in the culture medium. Subsequent increases resulted in slightly diminished yields. These observations were clearly evident in the graph of the regression analysis results, (graph 11). The yield values for the contour lines increase with the iron:manganese ratio, to 0.5 and then decrease with subsequent raising of the ratio to 64.0. Since the yield was maximal at high levels of iron supply, both the level of iron supply and the iron:manganese balance were important in determining the yield trends. However, since the contours are orientated vertically with respect to the iron:manganese ratio effect, the ratio and not the level of iron supply was the major factor determining the yield trends.



GRAPH 11. The effect of iron and the iron:manganese balance on the growth of Aspergillus niger. (No chelating agent.)



GRAPH 12. The effect of iron and the iron:manganese balance on the growth of Aspergillus niger. (Citric acid as chelating agent.)



GRAPH 13. The effect of iron and the iron:manganese balance on the growth of Penicillium glaucum.

In cultures of Aspergillus where citric acid was supplied as a chelating agent, there was evidence that the yield tended to diminish as the iron:manganese ratio approached 1.0. Thus maximum yields were obtained at high and low iron:manganese ratios (Table 61). This trend is clearly evident in the graph of the regression analysis (graph 12). Since the contours for the yield values were orientated diagonally, the iron:manganese ratio and iron concentration in the medium were equally important in determining the yield trends. By virtue of the plan of the experiment (Table 46), low iron:manganese ratios were only obtained with very high levels of manganese supply and low levels of iron, and similarly high ratios were obtained with high levels of iron supply and low levels of manganese. There was some evidence in previous experiments (Experiments 7 and 3) that high levels of iron and manganese were toxic when no chelating agent was present. Thus the absence of diminished yields, at the low ratios (high manganese levels) and the high ratios (high iron levels), can be explained by the presence of citric acid. At high levels of iron and manganese, symptoms of toxicity are not evident because the presence of citric acid tends to keep the iron and manganese in solution in the fungus, thereby preventing toxicity.

In cultures of Penicillium there is also evidence that the presence of a chelating agent reduces symptoms of toxicity (Table 50). Increasing the iron:manganese ratio resulted in increased yield (Table 60) and there was no evidence of an optimal ratio. The graph of the regression analysis results (graph 13), however, indicates that the yield trends were determined equally by the level of iron supply and the iron:manganese balance. This would suggest that the increasing yield obtained with increasing the iron:manganese ratio may be due partially to the increased iron supply in order to increase the iron:manganese ratio (Table 46)

TABLE 60. The dry weight yield, in grams, obtained for each iron:manganese ratio with Aspergillus niger in Experiment 3, part 2. . (No chelating agent.)

				0.98738		
			0.91805	0.92176		
		0.89738	1.33195	0.90339		
	1.02150	1.46698	0.82235	1.10224		
	0.86378	1.14653	0.97486	0.85947	1.45528	
0.79615	0.71493	1.46577	1.52283	1.01009	0.76017	
0.83834	0.59433	0.99140	0.94275	1.28306	0.65717	1.14030
	0.71301	0.82257	1.25267	0.88530	1.17912	0.89305
	0.83527	0.92064	1.16472	0.93607	0.97884	
		0.91621	0.87336	0.81636	0.78712	
		0.92734	1.21467	1.20684		
			0.92819	0.95695		
			1.24300			

Fe/Mn ratio	0.016	0.031	0.062	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0
Total	0.83834	1.50916	2.29338	3.47521	4.88329	6.90502	7.86321	5.92555	5.18883	4.07857	3.09129	1.65322	1.14030
Mean of total	0.83834	0.75458	0.76446	0.86880	0.97660	1.15084	1.12317	0.98759	1.03777	1.01964	1.03043	0.87661	1.14030

TABLE 61. The dry weight yield in grams, obtained for each iron:manganese ratio, with Aspergillus niger in Experiment 8, part 2. (Citric acid as chelating agent.)

				1.04044									
				1.06772		0.39209							
			1.03741		1.00425		1.34115						
		1.17474		1.08335		0.97327		0.87819					
	0.90157		1.13287		1.07148		0.97801		0.72516				
	1.09605		1.08259		1.10239		0.79244		0.62866		1.08841		
1.23709		1.04009		1.12464		1.08572		0.84767		0.97522		1.24303	
	1.24267		1.14914		1.02943		0.81256		1.03005		1.14980		
		1.19383		0.84945		0.11577		0.73811		0.96064			
			0.99691		1.12663		0.86158		0.64251				
				0.94920		0.126512		0.84933					
					1.01171		1.13155						
						1.17926							
Fe/Mn ratio	0.016	0.031	0.062	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0
Total	1.23709	2.33872	3.35249	4.40338	5.09357	6.62123	7.82334	5.43659	4.75527	3.18141	2.65742	2.23821	1.24303
Mean of total	1.23709	1.16936	1.11750	1.10084	1.01871	1.12020	1.11762	0.90610	0.95105	0.79535	0.88581	1.11910	1.24303

79c

TABLE 62. The dry weight yield, in grams, obtained for each iron:manganese ratio with Penicillium glaucum in Experiment 8, part 2.

				1.66478			
			1.61331	1.95399			
		1.72352	1.65987	1.87039			
	1.67242	1.88269	2.04876	1.80752			
	1.52541	1.73475	2.03392	1.80490	2.26921		
1.73959	1.84500	1.70908	1.92918	2.17417	1.67959		
1.63154	1.53351	1.90869	1.86848	2.05584	1.89995	2.14926	
1.79010	1.93338	1.94862	1.74431	1.81590	2.20221		
	1.76549	1.68745	2.01710	1.86421	2.14008		
	2.00998	2.05199	1.83273	2.04410			
		1.80318	2.06559	2.05177			
		1.90578	2.32567				
			2.17357				

Fe/Mn ratio	0.016	0.031	0.062	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0
Total	1.63154	3.52969	4.92541	7.46078	8.85759	11.11147	13.48331	11.83464	9.64711	7.84169	6.30924	3.88180	2.14926
Mean of total	1.63154	1.76484	1.64180	1.86519	1.77152	1.85191	1.92619	1.80577	1.92942	1.95834	2.10308	1.94090	2.14926

Conclusions.

There was some evidence, in both parts of this experiment, that the iron:manganese balance may be important in the determination of the yield trends. Since the number of yield values obtained for ratio decreased progressively towards the extremities of the range of ratios, (plan of experiments Tables 34 and 46), the effect of the iron:manganese balances on the yield could not be clearly separated from the separate effects of iron and manganese. There was also some evidence that the presence of a chelating agent tended to reduce the symptoms of iron and manganese toxicity.

Thus it was decided to design a further experiment, in which equal numbers of treatments were obtained for each iron:manganese ratio.

EXPERIMENT 9. To determine the effect of varying the absolute supply of iron and manganese, at fixed iron:manganese ratios, in the culture medium, on the growth and development of cultures of Aspergillus niger and Penicillium glaucum.

The plan of the experiment is given in Table 63. From this Table it is evident that the experiment was designed so that the numbers of treatments were orthogonal with respect to iron concentration, and the iron:manganese ratio in the culture solution. This arrangement permitted a test of significance, by analysis of variance, to be performed on the dry weight yield data, for the effects of both the level of iron supply, and the iron:manganese ratio.

The iron:manganese ratio in the culture solution was varied by adding 1 ml. samples of different concentrations of iron and manganese to the culture solution. The different concentrations of the stock solutions were prepared by dilution of a concentrated solution of each, iron and manganese. In the case of iron, the dilution factor was 2 (1 volume solution in 1 volume of purified water), but for

manganese, several dilution factors were used. The concentrated solutions of iron and manganese were prepared as follows:

Iron: 1.24440 gms. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved with 1.66741 gms. E.D.T.A. in purified water in a 500 ml. volumetric flask. 1 ml. of this solution in 50 ml. gave an iron concentration of 10.0 p.p.m.

Manganese: 20.27986 gms. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ was dissolved with 30.87550 gms. E.D.T.A. in purified water in a 500 ml. volumetric flask. 1 ml. of this solution in 50 ml. gave a manganese concentration of 200 p.p.m.

Results. The results are given in the following tables:

	<u>Aspergillus niger.</u>	<u>Penicillium glaucum.</u>
Dry weight yield	64	65
pH of filtrate (in Appendix)	15	16
Analysis of variance	66	67
Regression analysis	68	69

TABLE 63. The plan of Experiment 9, showing the levels of supply of iron, manganese and the iron:manganese ratio.

Concentration of iron in p.p.m.	2.5	5.0	10.0
Iron:Manganese Ratio	Concentration of Manganese in p.p.m.		
500	0.005	0.01	0.02
50	0.05	0.10	0.20
5.0	0.5	1.0	2.0
2.5	1.0	2.0	4.0
1.0	2.5	5.0	10.0
0.5	5.0	10.0	20.0
0.2	12.5	25.0	50.0
0.1	25.0	50.0	100.0
0.066	37.5	75.0	150.0
0.050	50.0	100.0	200.0

TABLE 66. The results of an analysis of variance performed on the dry weight yield obtained from cultures of Aspergillus niger in Experiment 9.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Ratio	9	0.08924	0.00991	1.03	Not significant
Iron	2	0.06284	0.03142	3.26	Significant at 5%
Replication	4	0.01874	0.00468	0.46	Not significant
Error	135	1.30020	0.00963		

TABLE 64. The dry weight yield, in grams, obtained from cultures of *Aspergillus niger* in Experiment 9.

Iron: Manganese ratio	Iron Supply in p.p.m.															Yield totals for each ratio treatment	Mean for each ratio treatment
	2.5					5.0					10.0						
500	.44094	.54392	.66297	.44719	.69755	.71929	.56978	.33785	.43919	.67662	.63904	.54477	.70479	.68563	.52414	8.63367	0.57558
50	.65025	.44706	.57490	.49827	.61417	.43081	.40351	.70331	.30847	.68836	.49784	.45176	.51467	.39097	.70900	7.38335	0.52556
5	.59225	.36441	.39898	.59861	.54264	.46106	.41317	.58002	.40581	.55213	.43048	.55865	.59785	.44290	.39272	7.33168	0.43378
2.5	.54911	.61144	.60368	.48095	.59518	.51220	.51990	.48693	.48509	.26970	.50327	.41948	.71696	.49041	.45926	7.70356	0.51357
1.0	.61930	.52116	.58882	.62898	.60904	.47250	.41436	.67072	.50920	.51642	.71320	.58417	.52036	.56194	.47917	8.40934	0.56062
0.5	.38626	.56567	.41097	.62617	.47999	.71103	.64399	.45352	.62366	.49361	.52484	.65085	.56347	.51930	.46982	8.12315	0.54154
0.2	.42818	.56768	.43313	.53395	.42740	.49746	.49835	.48138	.50252	.56192	.48679	.62476	.53720	.66483	.58651	7.83206	0.52214
0.1	.53160	.55291	.43656	.46918	.65424	.56473	.60556	.34457	.47478	.42767	.65942	.63261	.63339	.75406	.58663	8.32791	0.55519
0.066	.57371	.57905	.37876	.67316	.51867	.6343	.49379	.60514	.46326	.52459	.56446	.67520	.57853	.62033	.57371	8.29079	0.55272
0.050	.71181	.32582	.38662	.46467	.59370	.54463	.57112	.56928	.37598	.66355	.50101	.47775	.62843	.49655	.61975	7.93037	0.52871
Yield totals for each Fe treatment	26.59163					25.71092					28.16363						
Mean for each Fe treatment	0.53183					0.51439					0.56327						

Least significant difference between means at 0.05 probability level for Fe treatments = ± 0.03845 gms.

Least significant difference between means at 0.05 probability level for ratio treatments = ± 0.07013 gms.

TABLE 65. The dry weight yield, in grams, obtained from cultures of Penicillium glaucum in Experiment 9.

Iron: Manganese ratio	Iron Supply in p.p.m.															Total for each ratio treatment	Mean for each ratio treatment.
	2.5					5.0					10.0						
500	.71644	.34834	.47629	.38666	.73779	.41047	.58957	.60693	.64025	.41927	.46209	.38326	.58496	.31009	.59711	7.66953	0.51130
50	.39683	.48629	.34782	.35964	.53576	.44435	.46196	.26401	.30214	.49214	.60212	.36383	.63320	.49790	.50417	6.49216	0.43281
5	.46593	.33136	.38095	.39390	.34337	.53005	.48293	.56847	.47602	.40547	.44785	.48607	.64560	.45185	.43089	6.89171	0.45945
2.5	.41364	.41837	.32685	.36948	.40324	.43638	.52122	.50496	.50665	.44495	.55884	.39156	.37458	.39644	.43167	6.49383	0.43325
1.0	.37655	.30436	.43206	.40637	.37150	.33714	.35596	.44959	.52903	.35957	.45462	.39294	.37154	.38486	.46954	5.99563	0.39971
0.5	.37173	.34053	.44236	.34130	.37458	.33902	.36009	.45906	.30058	.48697	.42593	.48663	.48150	.54698	.45665	6.35601	0.42373
0.2	.39617	.30567	.26123	.38665	.37506	.45887	.37225	.52728	.44741	.53250	.41831	.49125	.40907	.50644	.42628	6.31449	0.42097
0.1	.35482	.37231	.39693	.28417	.35235	.50676	.45845	.46909	.45383	.49197	.45004	.39805	.51187	.33953	.49341	6.33358	0.42224
0.066	.38723	.37194	.33976	.41728	.37637	.44186	.34908	.44087	.50062	.42087	.49252	.33130	.38669	.46636	.46961	6.29236	0.41949
0.050	.41590	.41373	.34357	.35920	.32649	.41843	.43315	.43977	.44415	.53360	.45461	.41781	.42022	.43606	.19595	6.10264	0.40684
Total for each iron treatment	19.53723					22.81801					22.59170						
Mean for each iron treatment	0.39075					0.45636					0.45183						

Least significant difference between means at 0.05 probability level for Fe treatments = ± 0.09294 grams.

Least significant difference between means at 0.05 probability level for ratio treatments = ± 0.16964 grams.

TABLE 67. The results of an analysis of variance performed on the dry weight yield obtained from cultures of Penicillium glaucum in Experiment 9.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Ratio	9	0.13723	0.01525	0.27	Not significant
Iron	2	0.13433	0.06717	1.19	Not significant
Replication	4	0.02502	0.00626	0.11	Not significant
Error	135	7.58738	0.05620		

TABLE 68. The results of a regression analysis of the dry weight yields obtained from cultures of Aspergillus niger in Experiment 9.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	1.45790	0.29158	50.18	Significant at 0.1%
Residual	24	0.13759	0.00573		

$$y = 2.699 - 0.042x_1 + 0.330x_2 + 0.004x_3 + 0.167x_4 + 0.054x_5 \quad (\text{Graph 14}).$$

TABLE 69. The results of a regression analysis of the dry weight yields obtained from cultures of Penicillium glaucum in Experiment 9.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.80939	0.16188	3.12	Significant at 10%
Residual	24	1.24670	0.05194		

$$y = 2.545 - 0.140x_1 + 0.128x_2 + 0.011x_3 - 0.018x_4 + 0.005x_5 \quad (\text{Graph 15}).$$

The results are considered under the following headings:

- (a) The effect of varying the level of iron supply, irrespective of the iron:manganese ratio in the medium.
- (b) The effect of varying the iron:manganese ratio, irrespective of the levels of iron and manganese supply in the medium.
- (a) The effect of varying the level of iron supply, irrespective of the iron:manganese ratio in the medium.

In cultures of Penicillium glaucum, raising the level of iron supply had little effect on the dry weight yield (Table 65). The overall iron effect was shown by analysis of variance to be not significant. In Aspergillus niger increasing the concentration of iron in the medium resulted in a significant increase in the dry weight yield (Table 65). However, a study of the dry weight yield data (Table 64) shows the iron effect as not being very marked. Raising the concentration of iron from 2.5 to 5.0 p.p.m., resulted in slightly diminished yields but the decrease was not significant. Subsequent increases of the iron concentration from 5.0 to 10.0 p.p.m. resulted in a barely significant increase in the dry weight yield. A comparison of the mean yield for the 2.5 p.p.m. iron treatment, with that of the 10.0 p.p.m. iron treatment, indicated an increase which is less than the value for the least significant difference. Thus in cultures of both Penicillium and Aspergillus the level of iron supply did not exert a marked effect on the determination of the dry weight yield.

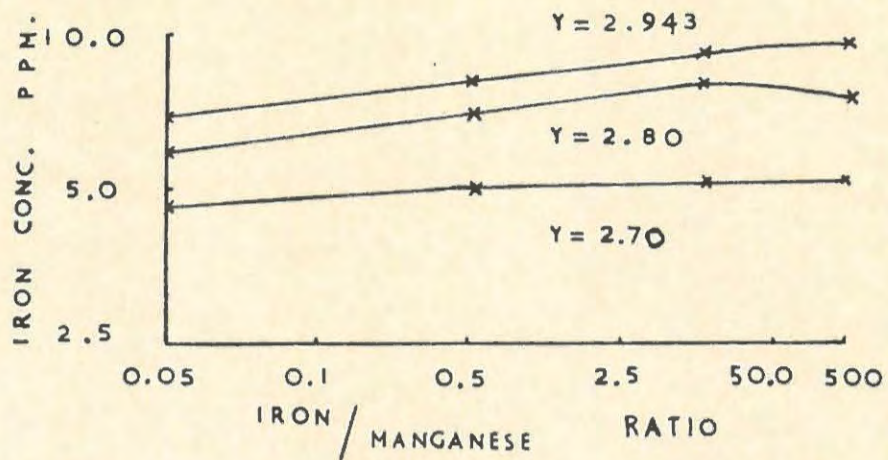
There was no evidence that the level of iron supply had any effect on the wrinkling and sporulation of the felts in cultures of Aspergillus and Penicillium. Marked differences in the sporulation between replications of the same treatment made the observation of trends difficult.

These results suggest that the presence of a chelating agent tends to reduce the marked increase in dry weight yield, which was observed in the preliminary experiments (Tables 6, 7, 16 and 17), when the level of iron supply was increased.

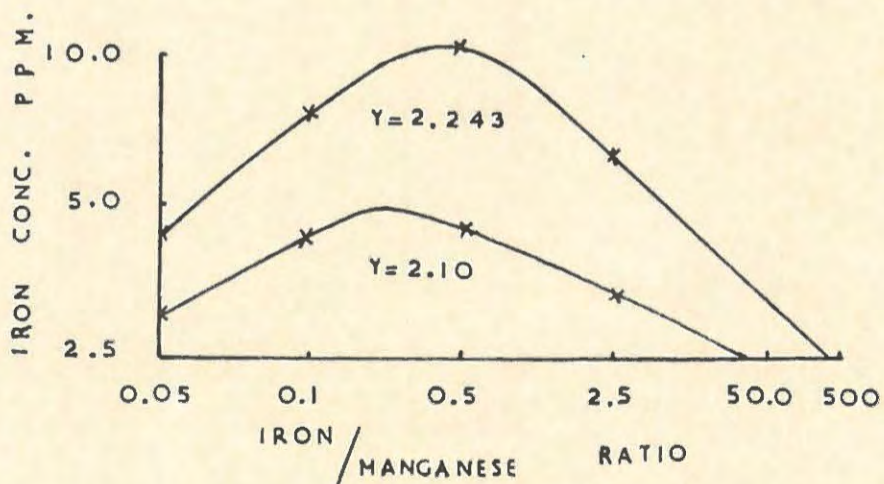
- (b) The effect of varying the iron:manganese ratio, irrespective of the levels of iron and manganese supply in the medium.

The effect of raising the iron:manganese ratio in the culture medium, on the dry weight yield of cultures of Aspergillus niger and Penicillium glaucum was not marked (Tables 64 and 65). The results of the test of significance (analysis of variance) performed on the dry weight yield data showed the ratio effect to be not significant, (Tables 66 and 67). The graphs of the regression analysis shows that, whilst in Aspergillus, the iron:manganese ratio in the culture medium played little or no part in the determination of the dry weight yield trends, (graph 14), in Penicillium, there is some evidence of a ratio effect, (graph 15). It is evident in graph 15 that there was a tendency for the dry weight yield to increase as the iron:manganese ratio increased or decreased from 0.5, i.e. the yield was lowest when the iron:manganese ratio was of the order of 0.5. A similar depression of the yield, when the ratio was 0.4, was observed in Experiment 8, part 2 (graph 11). These results suggest that the presence of a chelating agent prevents the appearance of toxic symptoms, which would normally appear at high and low ratios, because the high iron:manganese ratios in the culture medium were obtained by using high concentrations of iron, and the low ratios by using high concentrations of manganese.

The increased yields even with very high concentrations would suggest that the presence of a chelating agent does not necessarily reduce the availability of the heavy metal ions, but rather prevents



GRAPH 14. The effect of the level of iron supply and the iron:manganese ratio on the dry weight yield of Aspergillus niger.



GRAPH 15. The effect of the level of iron supply and the iron:manganese ratio on the dry weight yield of Penicillium glaucum.

precipitation, and thereby accumulation in toxic amounts.

There was no evidence that the iron:manganese ratio in the culture medium had any effect on the wrinkling and sporulation of the felts. Considerable variation between replications of the same treatment hampered observation of trends.

Conclusions.

The results of this experiment suggest that the iron:manganese ratio in the culture solution plays little or no part in the determination of the growth and development of cultures of Aspergillus and Penicillium. However there is some evidence that the presence of a chelating agent, may prevent precipitation of the heavy metal ions and thereby prevent toxicity. This could prevent the appearance of a ratio effect, if such an effect exists.

CHAPTER V.

Included in this chapter are three experiments dealing with the effect of varying the Zinc:Copper ratio in the culture medium, on the growth and development of cultures of Aspergillus niger and Penicillium glaucum.

Experiment 10, was essentially preliminary in nature, since it was designed primarily, to investigate a wide range of Zinc:Copper ratios in the culture medium. The experiment was orthogonal with respect to copper and zinc treatments, and thus the effect of varying the levels of supply of copper and zinc could be investigated statistically by performing an analysis of variance. The effect of varying the Zinc:Copper ratio was not analysed statistically.

In Experiments 11 and 12, the effect of varying the Zinc:Copper ratio on the dry weight yield of cultures of Aspergillus niger and Penicillium glaucum was investigated statistically by regression analysis. In Experiment 12 which was designed so that it was orthogonal with respect to level of copper supply and Copper:Zinc ratio, the effect of the Copper:Zinc ratio in the medium was analysed by analysis of variance.

EXPERIMENT 10. A preliminary investigation of the effect of varying the Zinc:Copper ratio, over a wide range, on the growth and development of cultures of Aspergillus niger and Penicillium glaucum.

The experiment was designed in the form of a grid (Table 70), so that a wide range of Zinc:Copper ratios could be investigated.

A series of stock solutions of copper and zinc were prepared by serial dilution of a concentrated solution of each copper, and zinc. The dilution factor was 4 (1 volume solution to 3 volumes of purified

TABLE 71. The dry weight yield, in grams, obtained with Aspergillus niger in Experiment 10.

Level of Copper supply in p.p.m.	Level of Zinc Supply in p.p.m.										Total for each copper treatment	Mean of total for each copper treatment
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144		
0.0001	0.53342	0.30946	0.50345	0.49053	0.54663	0.63589	0.66975	0.77313	0.32654	0.69644	5.48524	0.54852
0.0004	0.53331	0.50932	0.39014	0.51685	0.31587	0.57264	0.83622	0.90369	0.89018	0.92024	6.38846	0.63885
0.0016	0.46385	0.41759	0.39063	0.46025	0.53585	0.55517	0.79482	0.85598	0.56500	0.85775	5.89639	0.58969
0.0064	0.47924	0.47497	0.55156	0.45813	0.56384	0.71627	0.86929	0.46273	0.85142	0.88654	6.31339	0.63134
0.0256	0.38431	0.48853	0.52634	0.44589	0.49122	0.63970	0.76150	0.88037	0.79369	0.84482	6.25637	0.62564
0.1024	0.42687	0.45633	0.50060	0.48542	0.52881	0.60544	0.80185	0.84942	0.81372	0.85874	6.32720	0.63272
0.4096	0.51329	0.52124	0.42170	0.34061	0.58066	0.72738	0.70260	0.84216	0.74352	0.67154	6.06470	0.60647
1.6384	0.43133	0.49618	0.22937	0.34867	0.52541	0.74595	0.75546	0.74837	0.76986	0.44741	5.49801	0.54980
6.5536	0.44356	0.52872	0.47321	0.58592	0.51632	0.77764	0.76962	0.78735	0.71656	0.82670	6.42560	0.64256
26.2144	0.29737	0.27303	0.18757	0.28464	0.30498	0.24109	0.47761	0.83919	0.80910	0.51867	4.23325	0.42332
Total for each zinc treatment	4.50655	4.47537	4.17457	4.41691	4.90959	6.21717	7.43872	7.94239	7.27959	7.52885		
Means of total for each Zinc treatment	0.45065	0.44754	0.41746	0.44169	0.49096	0.62172	0.74387	0.79424	0.72796	0.75288		

Least significant difference between means of each treatment at 0.05 probability level ± 0.09671 grams.

TABLE 72. The dry weight yield, in grams, obtained with Penicillium glaucum in Experiment 10.

Level of copper supply in p.p.m.	Level of Zinc Supply in p.p.m.										Total for each copper treatment	Mean of total for each copper treatment
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144		
0.0001	0.38364	0.37043	0.32354	0.35409	0.32079	0.30792	0.36018	0.41774	0.38764	0.33880	3.56477	0.35648
0.0004	0.39420	0.32632	0.26576	0.37919	0.41956	0.37262	0.38855	0.37570	0.37566	0.46162	3.75918	0.37592
0.0016	0.23507	0.36410	0.26572	0.39182	0.36522	0.33805	0.46827	0.47241	0.47712	0.43120	3.30898	0.38090
0.0064	0.36665	0.25865	0.36922	0.33005	0.34922	0.49973	0.42764	0.53220	0.48605	0.55276	4.17217	0.41722
0.0256	0.39849	0.39301	0.30178	0.37239	0.28436	0.38903	0.66797	0.71821	0.51755	0.33559	4.37832	0.43783
0.1024	0.27282	0.24608	0.38848	0.36022	0.46606	0.46626	0.68098	0.70502	0.75355	0.74132	5.08079	0.50808
0.4096	0.37764	0.38599	0.24790	0.33872	0.46389	0.77225	0.78192	0.85788	0.95810	0.57851	5.76280	0.57628
1.6384	0.35092	0.24637	0.35825	0.31212	0.29555	0.38845	0.54952	0.90676	0.60066	0.92700	4.93560	0.49356
6.5536	0.26869	0.11159	0.32204	0.25190	0.36879	0.40882	0.47635	0.63442	0.70936	0.57423	4.12619	0.41262
26.2144	0.19783	0.18649	0.30290	0.22906	0.25917	0.36660	0.38838	0.36762	0.31898	0.40238	3.01941	0.30194
Total for each zinc treatment	3.24595	2.88903	3.14559	3.31956	3.59261	4.30973	5.18976	5.98796	5.58467	5.34341		
Means of total for each Zinc treatment	0.32459	0.28890	0.31456	0.33196	0.35926	0.43097	0.51898	0.59880	0.55847	0.53434		

Least significant difference between means of each treatment at 0.05 probability level \pm 0.09831 grams.

TABLE 70. The plan of Experiment 10 showing the levels of zinc and copper supply and the zinc:copper ratios.

Level of copper supply in p.p.m.	Level of Zinc Supply in p.p.m.									
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144
0.0001	1	4	16	24	256	1024	4096	16384	15536	26.2144
0.0004	0.25	1	4	16	24	256	1024	4096	16384	65536
0.0016	0.0625	0.25	1	4	16	24	256	1024	4096	16384
0.0064	0.0156	0.0625	0.25	1	4	16	24	256	1024	4096
0.0256	0.0039	0.0156	0.0625	0.25	1	4	16	24	256	1024
0.1024	0.00097	0.0039	0.0156	0.0625	0.25	1	4	16	24	256
0.4096	0.00024	0.00097	0.0039	0.0156	0.0625	0.25	1	4	16	24
1.6384	0.00006	0.00024	0.00097	0.0039	0.0156	0.0625	0.25	1	4	16
6.5536	0.000015	0.00006	0.00024	0.00097	0.0039	0.0156	0.0625	0.25	1	4
26.2144	0.000004	0.000015	0.00006	0.00024	0.00097	0.0039	0.0156	0.0625	0.25	1

water). The concentrated solutions were prepared so that a 1 ml. sample in 50 ml. gave the maximum concentration required. They were prepared as follows.

Zinc: 0.35965 gms. $ZnSO_4 \cdot H_2O$ was dissolved in purified water in a 100 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives a Zn concentration of 26.2144 p.p.m.

Copper: 0.51484 gms. $CuSO_4 \cdot 5H_2O$ was dissolved in purified water in a 100 ml. volumetric flask. 1 ml. of this solution in 50 ml. gives a Cu concentration of 26.2144 p.p.m.

Results: The results are given in the following tables.

	<u>Aspergillus niger.</u>	<u>Penicillium glaucum.</u>
Dry weight yield	71	72
pH of filtrate (in Appendix)	-	17
Analysis of variance	73	74

The results will be dealt with under the following headings.

- (a) The overall effect of increasing the level of zinc supply, irrespective of the concentration of copper in the medium.
- (b) The overall effect of increasing the level of copper supply, irrespective of the concentration of zinc in the medium.
- (c) The relation between growth and development and the Zinc:Copper ratio in the medium.
- (a) The overall effect of increasing the level of zinc supply, irrespective of the concentration of copper in the medium.

Increasing the concentration of zinc in the culture medium from

TABLE 73. The results of analysis of variance on the dry weight yield data from Aspergillus niger in Experiment 10.

Factor	$^{\circ}F$	S.S.	M.S.	F value	Significance
Zinc	9	2.132019	.23689	20.007	Significant at 0.1%
Copper	9	.413037	.04589	3.87	Significant at 0.1%
Error	81	.959202	.01184		

TABLE 74. The results of analysis of variance on the dry weight yield data from Penicillium glaucum in Experiment 10.

Factor	$^{\circ}F$	S.S.	M.S.	F value	Significance
Zinc	9	1.226196	.13624	11.10	Significant at 0.1%
Copper	9	.590499	.06561	5.35	Significant at 0.1%
Error	81	.994032	.01227		

0.0001 to 0.0256 p.p.m. had little effect on the dry weight yield obtained from cultures of Aspergillus niger (Table 71). However, subsequent increases to 0.1024 p.p.m. and from 0.1024 to 0.4096 p.p.m., resulted in a marked increase in the yield. These increases were considerably greater than the value for the least significant difference, and therefore contributed to the highly significant zinc effect, obtained by analysis of variance (Table 73). Raising the level of zinc from 0.4096 to 26.2144 p.p.m. had no significant effect on the yield (Table 71).

In cultures of Penicillium, increasing the level of zinc supply from 0.0001 to 0.0256 p.p.m., had no significant effect on the dry weight yield (Table 72). Each subsequent increase, 0.0256 - 0.1024 - 0.4096 - 1.6384 p.p.m. zinc, resulted in marked increases in the yield. This effect was found, by analysis of variance, to be highly significant (Table 74). Raising the concentration of zinc in the medium from 1.6384 - 26.2144 p.p.m. resulted in slightly diminished yields, but the overall decrease was not significant (Table 72).

The level of zinc supply had little effect on the sporulation, but raising the concentration resulted in increased wrinkling of the felts in both Aspergillus and Penicillium. In Penicillium there was a tendency for the pH of the filtrate to increase as the concentration of zinc in the medium was raised (Table 17 of Appendix).

- (b) The overall effect of increasing the level of copper supply, irrespective of the concentration of zinc in the medium.

In cultures of Aspergillus, raising the level of copper supply from 0.0001 to 6.5536 p.p.m. resulted in a slight increase in the dry weight yield (Table 72), but the effect was not statistically significant. However, a further increase in the copper supply, to 26.2144 p.p.m., resulted in a very marked decrease in the yield, due

to copper toxicity. This is the major factor determining the highly significant copper effect obtained by analysis of variance (Table 74).

Increasing the concentration of copper in the medium, from 0.0001 to 0.0256 p.p.m., had little effect on the yield in cultures of Penicillium (Table 72). Subsequent increases, 0.0256 - 0.1024 - 0.4096 p.p.m., resulted in quite a marked increase in the yield, although each individual increase was not significant. Raising the level of copper supply, further resulted in markedly reduced yields. This was particularly evident when the concentration was increased from 6.5536 to 26.2144 p.p.m. (Table 72). The marked reduction in the yield at high levels of copper supply, due to copper toxicity, is the major factor responsible for the highly significant copper effect obtained by analysis of variance (Table 74).

Raising the level of copper supply in the medium had little effect on sporulation and wrinkling of the felts, in cultures of Aspergillus and Penicillium. However there is some evidence that sporulation was reduced at the high levels (26.2144 p.p.m.) of copper supply.

- (c) The relation between growth and development and the Zinc:Copper ratio in the medium.

Varying the Zinc:Copper ratio in the medium had little effect on the dry weight yield obtained from cultures of Aspergillus and Penicillium. In Aspergillus there is some evidence of a slight increase in the yield when the ratio was raised to 16384, but subsequent increases to 262144 resulted in a slight decrease in the yield (Table 75). In Penicillium the yield increased slightly as the ratio was raised to 16, above which there was a tendency for reduced yields (Table 76).

These results do not show a clear optimal Zinc:Copper ratio for yield. The low yield values obtained at low ratios are probably expressions of copper toxicity, since the low ratios were obtained by using high concentrations of copper and low concentrations of zinc in

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TABLE 75. The dry weight yield, in grams, obtained for each zinc:copper ratio, with Aspergillus niger in Experiment 10.

									0.53342									
								0.53331		0.30946								
							0.46385		0.50932		0.50345							
						0.47924		0.41759		0.39014		0.49053						
						0.38431		0.47497		0.39063		0.51685		0.54603				
						0.42687		0.48853		0.55156		0.46025		0.31587		0.63589		
						0.51329		0.45633		0.52634		0.45813		0.53585		0.57264		0.66975
						0.43133		0.52124		0.50060		0.44589		0.56384		0.55517		0.83622
						0.44356		0.49618		0.42170		0.48542		0.49122		0.71627		0.79482
0.29737						0.52872		0.22937		0.34061		0.48542		0.49122		0.71627		0.79482
						0.27303		0.47321		0.34867		0.58066		0.60544		0.76150		0.46273
						0.18757		0.58592		0.52541		0.72738		0.80185		0.88037		0.85142
						0.28464		0.51632		0.74595		0.70260		0.84942		0.79369		0.88654
						0.30498		0.77764		0.75546		0.84216		0.81372		0.84482		
						0.24109		0.76962		0.74837		0.74352		0.85874				
						0.47761		0.78735		0.76986		0.67154						
						0.83919		0.71656		0.44741								
						0.80910		0.82670										
						0.51867												

Zn/Cu ratio	0.00004	0.00015	0.0006	0.0024	0.0097	0.0039	0.0156	0.0625	0.25	1.0	4.0	16.0	64.0	256	1024	4096	16384	65536	262144
Total	0.29737	0.71659	1.14762	1.76732	2.06838	2.36842	3.58964	4.88600	5.55645	5.67436	5.60396	5.07427	4.59649	4.02925	4.02433	3.02498	2.52106	1.24678	0.69644
Mean	0.29737	0.35829	0.34927	0.44183	0.41368	0.39474	0.51281	0.61075	0.61738	0.56744	0.62266	0.63428	0.65664	0.67154	0.80487	0.75624	0.84035	0.62339	0.69644

TABLE 76. The dry weight yield, in grams, obtained for each zinc:copper ratio, with *Penicillium glaucum* in Experiment 10.

									0.38364									
								0.39420		0.37043								
							0.23507		0.32632		0.32354							
							0.36665		0.36410		0.26576		0.35409					
							0.39849		0.25365		0.26572		0.37919		0.32079			
							0.27282		0.39301		0.35922		0.39182		0.41956		0.30792	
							0.37764		0.24608		0.30178		0.33005		0.36522		0.41956	
							0.35092		0.38599		0.38848		0.37239		0.34922		0.33805	
							0.26869		0.24637		0.24790		0.36022		0.28436		0.49973	
0.19783							0.11159		0.35825		0.33872		0.46606		0.38903		0.42764	
							0.18649		0.32204		0.31212		0.45389		0.46626		0.66797	
							0.30290		0.25190		0.29555		0.77225		0.68098		0.71821	
							0.22906		0.36879		0.33845		0.78192		0.70502		0.51755	
							0.25917		0.40882		0.54952		0.85788		0.75355		0.33559	
							0.36660		0.47635		0.90676		0.95810		0.74132			
							0.38838		0.63442		0.60066		0.57851					
							0.36762		0.70936		0.92700							
							0.31898		0.57423									
							0.40238											

Zn/Cu ratio	0.000004	0.000015	0.00006	0.00024	0.00097	0.0039	0.0156	0.0625	0.25	1.0	4.0	16.0	64.0	256	1024	4096	16384	65536	262144
Total	0.19783	0.45518	0.76541	1.17511	1.52813	1.93998	2.57961	2.85203	4.24114	4.85677	4.48001	4.82577	3.58961	2.95275	1.99052	1.76576	1.22460	0.84926	0.33880
Mean	0.19783	0.22759	0.25514	0.29378	0.30543	0.32335	0.36852	0.35650	0.47346	0.48568	0.49778	0.60322	0.51280	0.49212	0.39310	0.44144	0.40820	0.42463	0.33880

the culture solution. Similarly, the slightly diminished yields at high ratios may represent slight zinc toxicity.

There is no evidence that the Zinc:Copper ratio played any role in determining sporulation, wrinkling, or the pH of the filtrate.

Conclusions.

The responses of Aspergillus and Penicillium to varying concentrations of copper or zinc in the medium are similar. Raising the level of zinc supply to approximately 1.6384 p.p.m. resulted in a marked increase in the yield, but further increases had little effect. Thus no zinc toxicity was evident, and therefore no optimal zinc concentration was apparent. With copper the response was different. Raising the level of supply to approximately 6.5536 p.p.m. had little effect on the yield, but further increases resulted in markedly reduced yields due to copper toxicity. Thus an optimal range of copper concentrations was evident.

There is no evidence that the Zinc:Copper ratio is important in determining the yield trends or the general condition of the cultures. The experiment was however somewhat limited, because there were not equal numbers of treatments for each ratio.

It was decided to conduct further experiments in which the treatments could be replicated and in which the effect of the Zinc:Copper ratio could be studied statistically.

EXPERIMENT 11. To determine the effect of varying Zinc:Copper ratios, in the culture medium, on the growth and development of cultures of Aspergillus niger and Penicillium glaucum.

The experiment was carried out in two parts;

1. where the maximum levels of zinc and copper supply in the culture medium were 4.096 p.p.m., and

2. where the maximum levels of zinc and copper supply in the culture medium were 128.0 p.p.m.

Thus although a wide range of zinc and copper concentrations was investigated, the range in each experiment was not so large as to preclude the use of replications of treatments. There were three replications of each treatment.

Two methods of statistical analysis were used to analyse the dry weight yield data. A brief description of each method is given on page 63, and detailed examples are given in Appendix II.

Part 1.

The plan of Part 1 of the experiment is given in Table 77.

A series of stock solutions of copper and zinc were prepared by 1:1 dilution, (1 volume solution to 1 volume of purified water), of a concentrated solution, from which a 1 ml. sample in 50 ml. gave the maximum concentration required. The concentrated solutions of copper and zinc, from which the other solutions were prepared, were made up as follows:

Zinc: 0.56215 gms. $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ was dissolved with 1.16610 gms. E.D.T.A. in purified water in a 1 litre volumetric flask. 1 ml. of this solution in 50 ml. gives a Zn concentration of 4.096 p.p.m.

Copper: 0.80441 gms. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was dissolved with 1.19930 gms. E.D.T.A., in purified water in a 1 litre volumetric flask. 1 ml. of this solution in 50 ml. gives a Cu concentration of 4.096 p.p.m.

TABLE 77. The plan of Experiment 11, part 1, showing the levels of copper and zinc supply and the zinc:copper ratios.

Level of copper supply in p.p.m.	Level of Zinc Supply in p.p.m.						
	0.064	0.128	0.256	0.512	1.024	2.048	4.096
0.064	1.0	2.0	4.0	8.0	16.0	32.0	64.0
0.128	0.5	1.0	2.0	4.0	8.0	16.0	32.0
0.256	0.25	0.5	1.0	2.0	4.0	8.0	16.0
0.512	0.120	0.25	0.5	1.0	2.0	4.0	8.0
1.024	0.0625	0.120	0.25	0.5	1.0	2.0	4.0
2.048	0.03125	0.0625	0.120	0.25	0.5	1.0	2.0
4.096	0.01562	0.03125	0.0625	0.120	0.25	0.5	1.0

Results. The results are given in the following tables.

	<u>Aspergillus niger.</u>	<u>Penicillium glaucum.</u>
Dry weight yield	73	79
pH of filtrate (in Appendix)	18	19
Analysis of variance	80	81
Regression analysis for Copper and Zinc effects	82	83
Regression analysis for Zinc:Copper ratio effect	84	85

TABLE 80. The results of analysis of variance performed on the dry weight yield data from Aspergillus niger in Experiment 11.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Zinc	6	0.68193	0.11366	7.45	Significant at 1%
Copper	6	0.42844	0.07141	4.68	Significant at 5%
Interaction	36	0.66325	0.01842	1.21	Not significant
Replication	2	0.01839	0.00969	0.63	Not significant
Error	96	1.46545	0.01526		

TABLE 81. The results of analysis of variance performed on the dry weight yield data from Penicillium glaucum in Experiment 11.

Factor	Degrees Freedom	Sum Squares	Mean sum squares	F value	Significance
Zinc	6	0.28089	0.04681	8.59	Significant at 1%
Copper	6	0.03940	0.00656	1.20	Not significant
Interaction	36	0.59243	0.01645	3.02	Significant at 1%
Replication	2	0.01588	0.00794	1.45	Not significant
Error	96	0.52313	0.00545		

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TABLE 78. The dry weight yield, in grams, obtained with *Aspergillus niger* in experiment 11.

Level of Copper supply in p.p.m.	Level of Zinc Supply in p.p.m.							Total for each copper treatment	Mean of each copper treatment
	0.064	0.128	0.256	0.512	1.024	2.048	4.096		
0.064	.52454	.58629	.73698	.90000	.74145	.99400	.90439	15.16327	2.16618
	.43791	.74053	.82275	.63447	.70326	.69489	.85642		
	.36780	.55990	.63277	.90669	.65301	.86215	.32307		
0.128	.35438	.64763	.63798	.84024	.65499	.75408	.52437	13.53437	1.93348
	.36482	.61112	.72734	.68137	.47012	.88290	.94634		
	.34575	.46215	.78473	.90011	.65475	.69340	.63580		
0.256	.38632	.54911	.79098	.42236	.80098	.61126	.76035	12.66039	1.80863
	.53160	.47740	.53161	.66240	.73389	.91251	.45347		
	.39674	.51417	.54826	.30010	.51409	.62938	.63341		
0.512	.50226	.70508	.61321	.71682	.54367	.76032	.65407	12.97281	1.85326
	.62777	.66778	.42049	.63945	.65287	.57417	.64190		
	.45370	.55394	.72390	.58555	.53332	.62142	.78112		
1.024	.61289	.78571	.60437	.70923	.71117	.35035	.53408	13.97547	1.99650
	.60285	.69677	.84822	.72838	.53967	.76843	.48629		
	.53148	.67107	.84515	.73337	.59001	.48454	.64144		
2.048	.43785	.69738	.78738	.51717	.43993	.59183	.56175	12.11609	1.73087
	.61732	.75008	.61500	.79802	.68767	.42772	.55528		
	.36439	.39815	.44672	.70691	.58101	.49091	.64362		
4.096	.52487	.52383	.49963	.39467	.90606	.53781	.52519	11.48707	1.64101
	.49062	.61022	.52929	.50403	.64718	.48034	.89541		
	.41083	.62212	.36220	.40132	.48243	.55821	.58026		
Total for each Zn treatment	9.88669	12.83048	13.50896	14.22266	13.24153	14.18112	14.03803		
Mean for each Zn treatment	1.41238	1.83293	1.92985	2.03181	1.89165	2.02587	2.00543		

Least significant difference between means at 0.05 probability level \pm 0.07929 grams.

TABLE 79. The dry weight yield, in grams, obtained with Penicillium glaucum in Experiment 11.

Level of copper supply in p.p.m.	Level of Zinc Supply in p.p.m.							Total for each copper treatment	Mean for each copper treatment
	0.064	0.128	0.256	0.512	1.024	2.048	4.096		
	0.9934	.42223	.53425	.59091	.49035	.54501	.45601		
0.064	.41702	.42205	.57563	.51894	.6056	.40957	.37093	10.29014	1.47002
	.33985	.50191	.59925	.66943	.45079	.52224	.39387		
	.41651	.47191	.65087	.52221	.54413	.62676	.45191		
0.128	.47175	.50689	.50723	.54103	.57559	.53219	.54892	10.88651	1.55522
	.45049	.47301	.53460	.63728	.37893	.55867	.33498		
	.30645	.47602	.69938	.45508	.59371	.62033	.41224		
0.256	.47213	.45749	.54577	.67524	.62034	.53691	.32805	11.27402	1.61057
	.47622	.57031	.54037	.55692	.76734	.51917	.48355		
	.41976	.48599	.50043	.59496	.57389	.42661	.42501		
0.512	.37582	.41764	.50091	.52997	.62098	.46998	.57894	10.21825	1.45975
	.36335	.40670	.53147	.51809	.65656	.39358	.42761		
	.37286	.47451	.63285	.55377	.59793	.43437	.36051		
1.024	.48116	.48880	.61151	.44632	.44142	.49979	.39946	10.64223	1.52032
	.52755	.48736	.62357	.62274	.73608	.47739	.37178		
	.39406	.42965	.59064	.57209	.46931	.43798	.56071		
2.048	.39565	.37187	.52849	.70397	.49370	.55025	.42881	10.68886	1.52698
	.40931	.43930	.62676	.66921	.65294	.42621	.53295		
	.39708	.45993	.77688	.47947	.48911	.42529	.47696		
4.096	.40367	.46743	.57952	.66114	.73474	.62804	.40083	11.34968	1.62138
	.43324	.45959	.58791	.71659	.49432	.77491	.50303		
Total for each Zn treatment	8.78327	9.69059	12.37929	12.34101	12.04272	10.81575	9.29706		
Mean for each Zn Treatment	1.25475	1.38437	1.76847	1.76300	1.72039	1.54511	1.3282		

Least significant difference between means at 0.05 probability level \pm 0.04738 grams.

TABLE 82. The results of a regression analysis of the dry weight yield data obtained from cultures of Aspergillus niger in Experiment 11, for the copper and zinc effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	3.16270	0.63254	11.20	Significant at 1%
Residual	43	2.42817	0.05647		

$$y = 1.780 - 0.064x_1 + 0.076x_2 - 0.002x_3 - 0.030x_4 - 0.035x_5 \quad (\text{Graph 16}).$$

TABLE 83. The results of a regression analysis of the dry weight yield data obtained from cultures of Penicillium glaucum in Experiment 11, for the zinc and copper effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	1.85658	0.37132	17.20	Significant at 1%
Residual	43	0.92828	0.02159		

$$y = 1.311 + 0.006x_1 + 0.018x_2 - 0.097x_3 + 0.075x_4 + 0.004x_5 \quad (\text{Graph 17}).$$

TABLE 84. The results of a regression analysis of the dry weight yield data, from Aspergillus niger in Experiment 11, for the zinc and Zinc:Copper ratio effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	2.15903	0.43181	5.41	Significant at 5%
Residual	43	3.43184	0.07981		

$$y = 1.779 + 0.017x_1 + 0.059x_2 - 0.010x_3 + 0.015x_4 + 0.042x_5 \quad (\text{Graph 19}).$$

TABLE 85. The results of a regression analysis of the dry weight yield data, from Penicillium glaucum in Experiment 11, for the zinc and Zinc:Copper ratio effects.

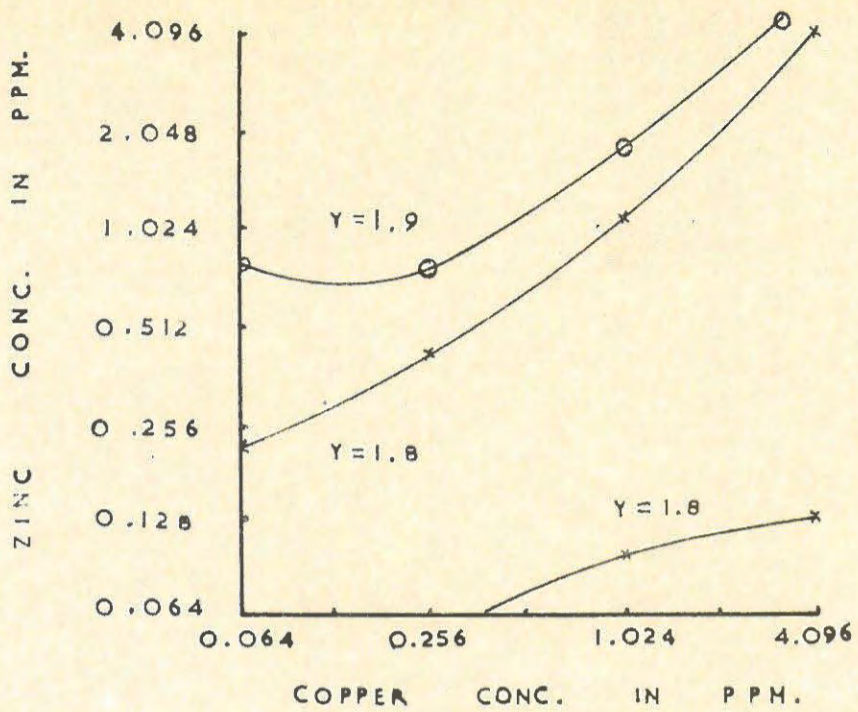
Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	1.86375	0.33275	15.54	Significant at 1%
Residual	43	0.92111	0.02142		

$$y = 1.496 + 0.029x_1 - 0.011x_2 - 0.049x_3 + 0.003x_4 - 0.009x_5 \quad (\text{Graph 20})$$

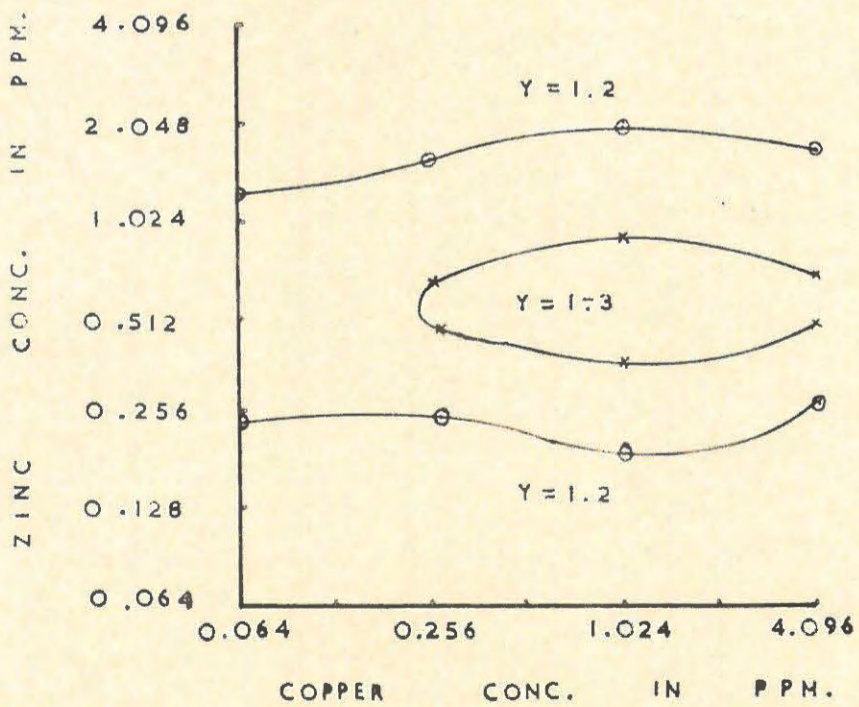
The results will be considered under the following headings.

- (a) The overall effect of increasing the level of zinc supply, irrespective of the concentration of copper in the medium.
- (b) The overall effect of increasing the level of copper supply, irrespective of the concentration of zinc in the medium.
- (c) The Copper:Zinc interaction effect on the yield.
- (d) The relation between growth and development, and the Zinc:Copper ratio in the medium.
- (e) The overall effect of increasing the level of zinc supply, irrespective of the concentration of copper in the medium.

In cultures of Aspergillus niger, increasing the concentration of zinc in the culture medium from 0.064 to 0.512 p.p.m., resulted in a marked increase in the dry weight yield, (Table 78). This effect is responsible for the significance of the zinc effect as shown by analysis of variance, (Table 80). The overall effect of subsequent increases in the level of zinc supply to 4.096 p.p.m., was not marked. The significant decrease in the yield, when the concentration of zinc



GRAPH 16. The effect of copper and zinc on the growth of Aspergillus niger.



GRAPH 17. The effect of zinc and copper on the growth of Penicillium glaucum.

was increased from 0.512 to 1.024 p.p.m., is considered to be atypical and due to some error, because a further increase in the level of supply to 2.048 p.p.m. resulted in a significant increase in the yield, and also because the difference between the yields obtained at the 0.512 and 2.048 p.p.m. levels of supply is less than the value obtained for the least significant difference. There was no evidence of zinc toxicity.

Increasing the concentration of zinc in the culture medium from 0.064 to 0.256 p.p.m., in cultures of Penicillium glaucum, resulted in a significant increase in the dry weight yield (Table 81). A subsequent increase to 0.512 p.p.m. had no effect, but when the level was raised further to 4.096 p.p.m. there was a highly significant decrease in the yield (Table 79). The overall zinc effect was found by analysis of variance to be significant (Table 81).

Raising the level of zinc supply resulted in increased wrinkling of the felts, but there was no evidence that the level of zinc supply was important in determining trends in the sporulation or the pH of the filtrate, obtained from cultures of Aspergillus and Penicillium.

- (b) The overall effect of raising the level of copper supply irrespective of the level of zinc in the medium.

In cultures of Aspergillus increasing the concentration of copper in the medium from 0.064 to 1.024 p.p.m. resulted in a marked increase in the dry weight yield (Table 78). Subsequent increases to 4.096 p.p.m. resulted in significantly diminished yields. The overall copper effect was found by analysis of variance to be significant (Table 80). The effect of raising the level of copper supply in cultures of Penicillium was shown to be highly significant (Table 81). The overall tendency was for the dry weight yield to increase as the level of supply was raised to 4.096 p.p.m. (Table 79). The significant decrease in the yield when the concentration was increased from

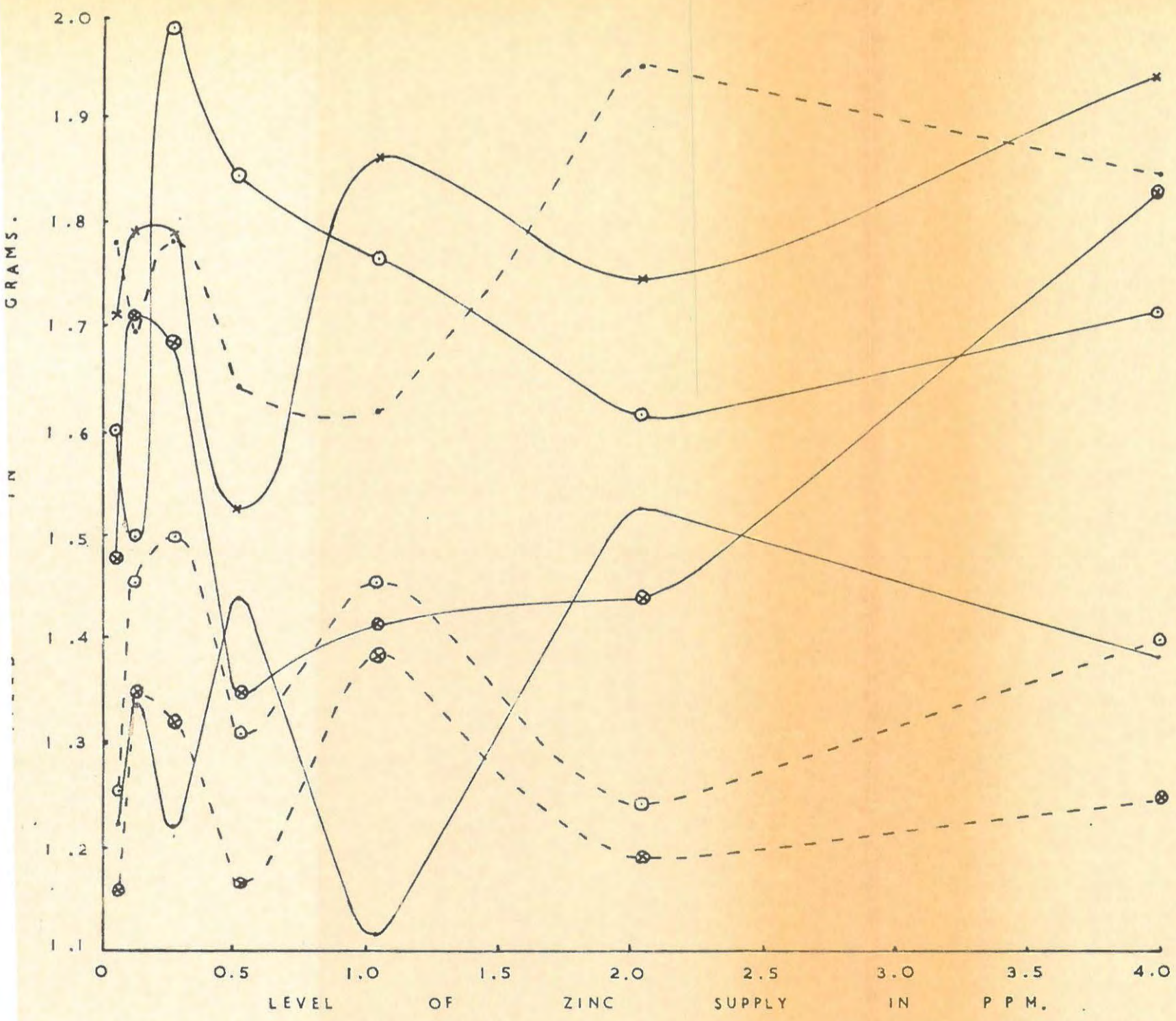
0.256 to 0.512 p.p.m., may be due to experimental error.

The concentration of copper in the culture medium had little effect on the appearance and sporulation of the felts of Aspergillus and Penicillium. There was some indication that sporulation was slightly suppressed at high levels of copper supply, but this was not nearly as marked as in the previous experiment (page 91).

(c) The Copper:Zinc interaction effect on the yield.

The Copper:Zinc interaction effect was found by analysis of variance to be highly significant in determining the dry weight yield trends in cultures of Penicillium (Table 81), but not in cultures of Aspergillus (Table 80). From graph 18, it can be seen that at zinc concentrations of up to 0.512 p.p.m., raising the concentration of copper to 4.096 p.p.m. had a fairly uniform effect on the yield. Raising the level of copper supply to 0.256 p.p.m. results in increased yield, while a further increase to 0.512 p.p.m. results in diminished yield. Similarly a subsequent increase in the copper concentration to 1.024 p.p.m. raises the yield, but a further increase to 2.048 reduces the yield. A final increase to 4.096 p.p.m. results in slightly increased yield.

At higher levels of zinc supply, the yield trends were somewhat different. When the zinc concentration in the medium was high (4.096 p.p.m.), the yield trends were almost the opposite of those observed at low zinc levels. Raising the copper concentration to 0.128 p.p.m. increased the yield, but a further increase to 0.256 p.p.m. reduced the yield. Similarly a subsequent increase to 0.512 p.p.m. raised the yield, and a further increase in the copper concentration to 1.024 p.p.m. reduced the yield. When the level of copper supply was increased further, to 2.048 p.p.m., the yield diminished. A subsequent increase in copper concentration to 4.096 p.p.m. resulted in slightly increased yield.



LEVEL OF COPPER
IN P P M.

⊖	-----	⊗	0.064
○	-----	⊙	0.128
×	-----	×	0.256
•	-----	•	0.512
○	-----	○	1.024
⊗	-----	⊗	2.048
—	-----	—	4.096

GRAPH 18. The copper:zinc interaction effect in Experiment 11 with Penicillium glaucum.

This evidence shows clearly that in cultures of Penicillium there is an interaction effect between copper and zinc in the determination of the dry weight yield trends.

- (d) The relation between growth and development and the Zinc:Copper ratio in the medium.

In cultures of Aspergillus niger raising the Zinc:Copper ratio in the culture medium between 0.01562 and 64.00 resulted in a marked increase in the dry weight yield, (Table 86). There is no evidence of an optimal Zinc:Copper ratio. It is clearly evident from the regression analysis results (Table 84, graph 19), that at high levels of zinc supply in particular, the Zinc:Copper ratio was the major factor determining the yield trends. At high levels of zinc supply the contours, for each yield value, are orientated vertically with respect to the Zinc:Copper ratio effect, but they tend towards a parallel orientation as the level of zinc supply in the culture medium decreases. Thus at low levels of zinc supply, the concentration of zinc in the medium is the major factor determining yield trends.

In cultures of Penicillium glaucum, raising the Zinc:Copper ratio to 0.0625 resulted in increased dry weight yield (Table 87). Subsequent increases to 16.0 had little effect, but there is some evidence that further increases to 64.0 resulted in diminished yields. The trend for increased yield at low Zinc:Copper ratios (0.01562 - 0.0625) and for decreased yield at high ratios (16.0 - 64.0) must be regarded with suspicion since the number of yield values obtained for these ratios were small. The graph of the regression equation (graph 20) indicates that the Zinc:Copper ratio was important in determining the yield trends, when the level of zinc supply was low, and that, as the concentration of zinc was increased, the Zinc:Copper ratio effect becomes less marked.

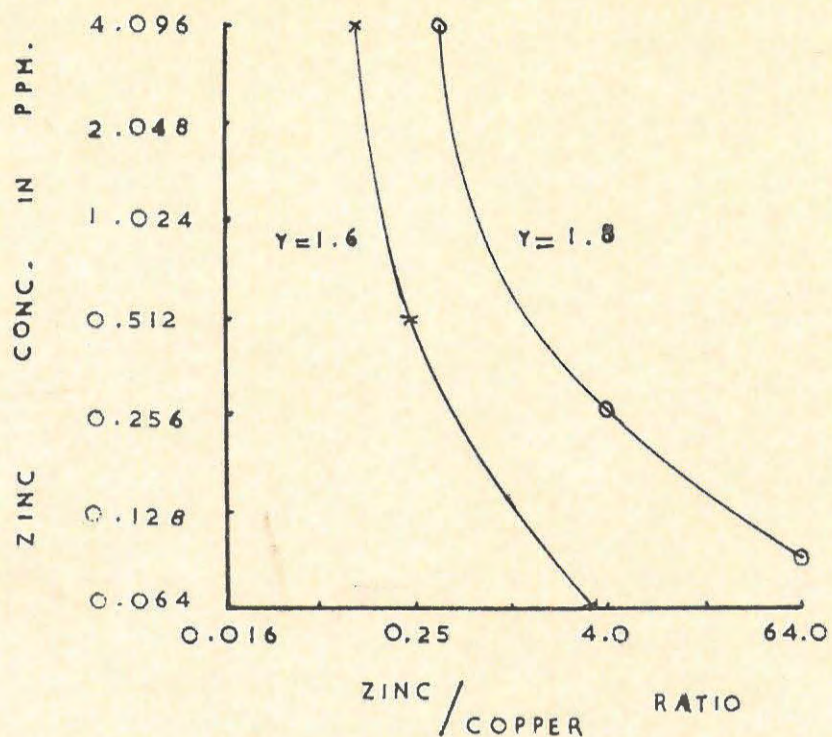
TABLE 86. The dry weight yield, in grams, obtained for each zinc:copper ratio, with Penicillium glaucum in Experiment 11.

				1.15621									
					1.33875		1.34619						
			1.31480		1.45181		1.70913						
		1.15893		1.50382		1.79270		1.77928					
	1.38157		1.31033		1.78652		1.70117		1.60170		s.		
	1.19902		1.45067		1.53281		1.73724		1.49865		1.47682		
1.23399		1.24082		1.86793		1.64302		1.93139		1.71762		1.22081	
	1.38695		1.74589		1.62283		1.85143		1.67641		1.33581		
		1.94431		1.95027		1.77543		1.29017		1.22384			
			1.85720		1.61595		1.41205		1.43156				
				1.71817		1.41444		1.13175					
					1.82824		1.52247						
						1.38082							
Zn/Cu ratio	0.01562	0.03125	0.0625	0.1250	0.250	0.50	1.0	2.0	4.0	8.0	16.0	32.0	64.0
Total	1.23399	2.58597	4.56670	6.21269	3.16150	9.44240	10.60825	9.71208	7.81361	6.38590	4.54316	2.86263	1.22081
Mean	1.23399	1.29298	1.52223	1.55254	1.63230	1.57373	1.51546	1.61868	1.56272	1.59647	1.54772	1.43131	1.22081

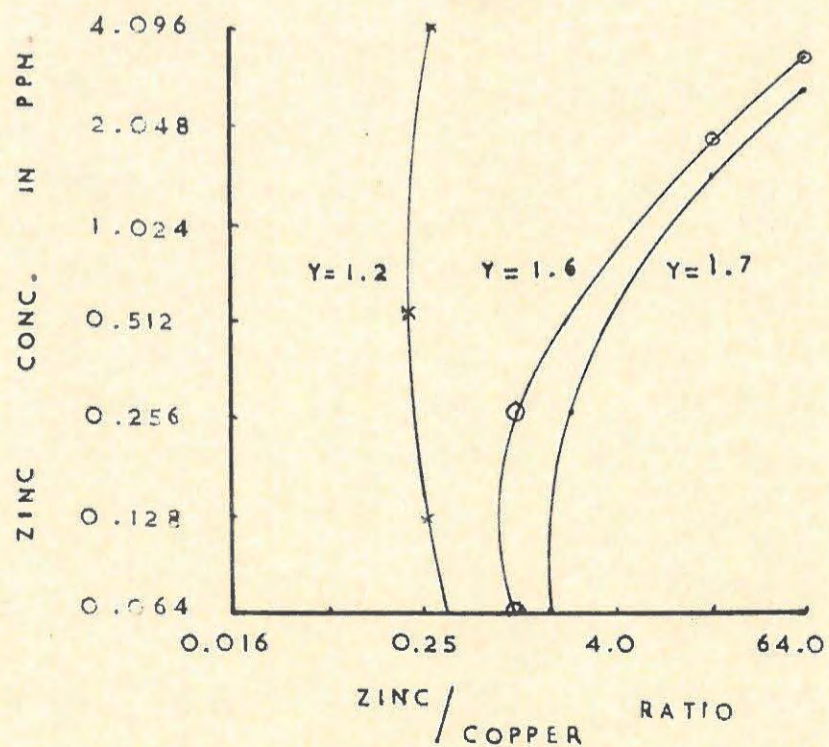
TABLE 87. The dry weight yield, in grams, obtained for each zinc:copper ratio with Aspergillus niger in Experiment 11.

				1.33025								
				1.06495		1.33672						
			1.31466		1.72090		2.12950					
		1.58373		1.54068		2.51005		2.52116				
	1.74722		1.92680		1.87085		2.38172		2.09772			
1.41956		2.15355		1.75760		1.88436		1.77986		2.55104		
1.42632	1.84561		2.29774		1.94182		2.04896		2.33038		2.58388	
	1.75622		1.84910		2.17098		1.72986		2.15315		2.10651	
		1.39112		2.02210		1.84085		1.95591		1.84723		
			1.30002		1.70861		2.10332		2.07709			
				2.03567		1.51046		1.66181				
					1.57686		1.76065					
						2.00086						

Zn/Cu ratio	0.01562	0.03125	0.0625	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0
Total	1.42632	3.17578	4.98395	6.88640	9.59697	9.81953	12.21599	11.87546	10.17790	8.53126	6.27533	4.65755	2.58388
Mean	1.42632	1.58789	1.66132	1.72160	1.91939	1.63661	1.74514	1.74591	2.03558	2.13281	2.09178	2.32877	2.58388



GRAPH 19. The effect of zinc and the zinc:copper balance on the growth of Aspergillus niger.



GRAPH 20. The effect of zinc and the zinc:copper balance on the growth of Penicillium glaucum.

There was no evidence in cultures of Aspergillus or Penicillium that the Zinc:Copper ratio in the medium was important in determining trends in sporulation or wrinkling of the felts.

Part 2.

The plan of part 2 of Experiment 11 is given in Table 88.

A series of stock solutions of copper and zinc were prepared by 1:1 dilution, (1 volume solution to 1 volume of purified water), of a concentrated solution, from which a 1 ml. sample in 50 ml. gave the maximum concentration required. The concentrated solutions of copper and zinc, from which the other solutions were prepared, were made up as follows.

Zinc: 3.51544 gms. $\text{ZnSO}_4 \cdot \frac{1}{2} \text{H}_2\text{O}$ was dissolved with 7.28786 gms. of E.D.T.A. in purified water in a 200 ml. volumetric flask. 1 ml. of this solution in 50 ml. gave a Zn concentration of 128.0 p.p.m.

Copper: 5.02757 gms. $\text{CuSO}_4 \cdot \frac{1}{2} \text{H}_2\text{O}$ was dissolved with 7.45536 gms. E.D.T.A. in purified water in a 200 ml. volumetric flask. 1 ml. of this solution in 50 mls. gives a Cu concentration of 128.0 p.p.m.

Results. The results obtained with Penicillium glaucum are given as follows,

	<u>Table No.</u>
Dry weight yield	89
pH of filtrate	20 (Appendix)
Analysis of variance	90
Regression analysis for copper and zinc effects	91
Regression analysis for Zinc:Copper ratio effect	92

TABLE 88. The plan of Experiment 11, part 2, showing the levels of copper and zinc supply, and the zinc:copper ratios.

Level of copper supply in p.p.m.	Level of zinc supply in p.p.m.						
	2.0	4.0	8.0	16.0	32.0	64.0	128.0
2.0	1.0	2.0	4.0	8.0	16.0	32.0	64.0
4.0	0.5	1.0	2.0	4.0	8.0	16.0	32.0
8.0	0.25	0.5	1.0	2.0	4.0	8.0	16.0
16.0	0.125	0.25	0.5	1.0	2.0	4.0	8.0
32.0	0.0625	0.125	0.25	0.5	1.0	2.0	4.0
64.0	0.03125	0.0625	0.125	0.25	0.5	1.0	2.0
128.0	0.01562	0.03125	0.0625	0.125	0.25	0.5	1.0

TABLE 89. The dry weight yield, in grams, obtained with Penicillium glaucum in Experiment 11, part 2.

Level of Copper supply in p.p.m.	Level of Zinc Supply in p.p.m.							Total for each Cu treatment	Mean
	2.0	4.0	8.0	16.0	32.0	64.0	128.0		
	.41201	.43186	.45996	.53512	.42795	.43276	.46001		
2.0	.43620	.47416	.37800	.56011	.36554	.45371	.50418	9.22035	1.31719
	.36702	.49268	.42170	.42506	.34673	.44214	.40345		
	.43094	.41170	.43368	.45324	.41835	.48166	.38102		
4.0	.55476	.50782	.34751	.44786	.36044	.42138	.43637	8.95427	1.27918
	.34537	.37914	.45051	.39936	.40471	.42631	.46324		
	.38406	.43754	.49108	.50975	.50238	.31848	.39112		
8.0	.46739	.47681	.33661	.49827	.40958	.42860	.50540	9.27554	1.32508
	.46692	.40238	.56217	.42456	.40170	.42339	.43835		
	.37518	.48051	.40669	.44504	.43834	.35634	.42356		
16.0	.41993	.35245	.45314	.48090	.41956	.48893	.42712	8.98616	1.28374
	.55077	.47344	.52993	.45971	.42255	.39685	.19522		
	.33058	.38443	.37353	.36523	.39219	.61443	.46516		
32.0	.39243	.35594	.35527	.43526	.45125	.37948	.52705	8.86663	1.26666
	.35809	.34204	.35474	.39333	.45065	.52436	.56329		
	.42738	.37249	.47834	.53857	.41270	.48061	.37364		
64.0	.32650	.36537	.42109	.60629	.45889	.47512	.42312	9.12432	1.30347
	.33444	.39155	.36976	.63897	.41148	.44722	.42079		
	.36479	.38271	.43813	.41356	.50860	.48002	.38901		
128.0	.45258	.42861	.41384	.41281	.36991	.38991	.37470	8.76308	1.25187
	.45281	.37190	.45541	.49511	.32681	.44214	.40472		
Total for each Zn treatment	8.70005	8.61553	8.93799	9.97411	8.70031	9.29184	8.97052		
Mean	1.24286	1.23079	1.27686	1.42487	1.24290	1.32741	1.28150		

Least significant difference between means at 0.05 probability level \pm 0.06648 grams.

TABLE 90. The results of analysis of variance, performed on the dry weight yield data from Penicillium glaucum in Experiment 11, part 2.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Zinc	6	0.07151	0.01192	1.11	Not significant
Copper	6	0.01035	0.00172	0.16	Not significant
Interaction	36	0.21361	0.00593	0.55	Not significant
Replication	2	0.01939	0.00969	0.90	Not significant
Error	96	1.02920	0.01072		

TABLE 91. The results of a regression analysis of the dry weight yield data from Penicillium glaucum in Experiment 11, part 2, for the zinc and copper effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.09109	0.01822	4.99	Significant at 5%
Residual	43	0.15706	0.00365		

$$y = 1.278 - 0.007x_1 + 0.010x_2 - 0.001x_3 - 0.007x_4 + 0.007x_5 \quad (\text{Graph 21}).$$

TABLE 92. The results of a regression analysis of the dry weight yield data, from Penicillium glaucum in Experiment 11, part 2, for the Zinc:Copper ratio, and zinc effects.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	0.19035	0.03807	28.41	Significant at .1%
Residual	43	0.05780	0.00134		

$$y = 1.256 - 0.032x_1 + 0.024x_2 - 0.003x_3 - 0.008x_4 + 0.010x_5 \quad (\text{Graph 22}).$$

The results will be considered under the following headings.

- (a) The overall effect of increasing the level of zinc supply, irrespective of the concentration of copper in the medium.
- (b) The overall effect of increasing the level of copper supply, irrespective of the concentration of zinc in the medium.
- (c) The relation between growth and development and the Zinc:Copper ratio in the medium.
- (a) The overall effect of increasing the level of zinc supply, irrespective of the concentration of copper in the medium.

The overall effect of increasing the concentration of zinc in the medium, in cultures of Penicillium glaucum, was found by analysis of variance to be not significant in the determination of the dry weight yield trends, (Table 90). However it is clearly evident from the dry weight yield data, (Table 89) and the regression analysis graph (graph 21), that there was a slight tendency for the dry weight yield to increase when the concentration of zinc in the medium was raised to 16.0 p.p.m. Subsequent increases resulted in diminished yields. That this trend was shown to be not significant, may, in part, be due to the fact that the reduction of the yield with increasing zinc concentration above 16.0 p.p.m. was not a uniform trend. Raising the level to 32.0 p.p.m. resulted in a significant decrease, but a further increase in the zinc concentration to 64.0 p.p.m. resulted in a significant increase in the yield. However, a comparison of the yields obtained at the 16.0 and 64.0 p.p.m. levels of zinc supply, shows the latter to be significantly less than the former. It is possible that there was some experimental error

at the 32.0 p.p.m. level of zinc supply.

There was no evidence that the level of zinc supply was important in determining sporulation and wrinkling of the felts, or the pH of the filtrate (Table 20 of Appendix).

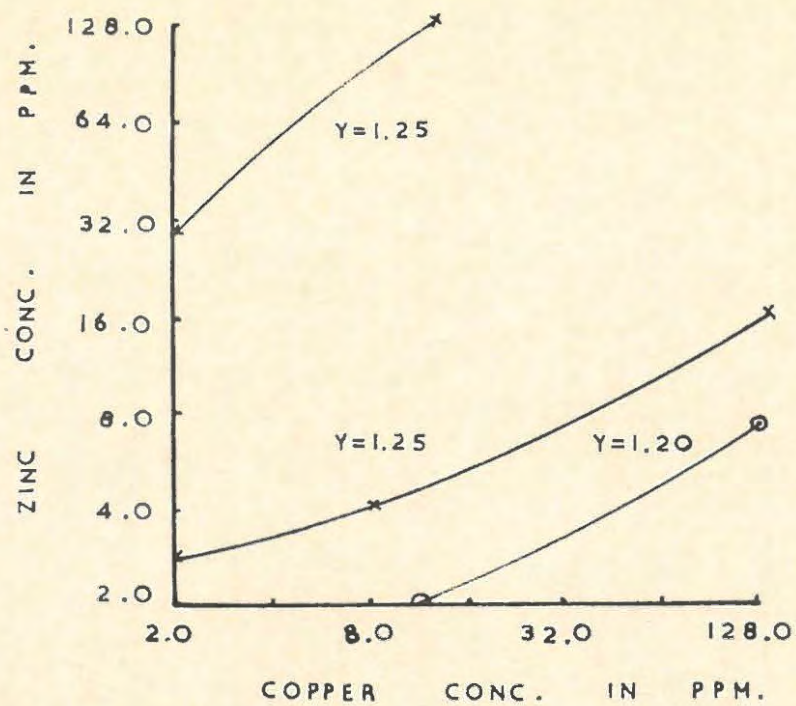
- (b) The overall effect of increasing the level of copper supply, irrespective of the concentration of zinc in the medium.

The overall effect of increasing the level of copper supply, from 2.0 to 128 p.p.m., in cultures of Penicillium glaucum, had little effect on the dry weight yield (Table 89), and was found by analysis of variance to be not significant (Table 90). There is some evidence, however, of copper toxicity resulting in reduced yields, at the high levels of copper supply, particularly when the concentration of zinc in the culture medium was low (graph 21).

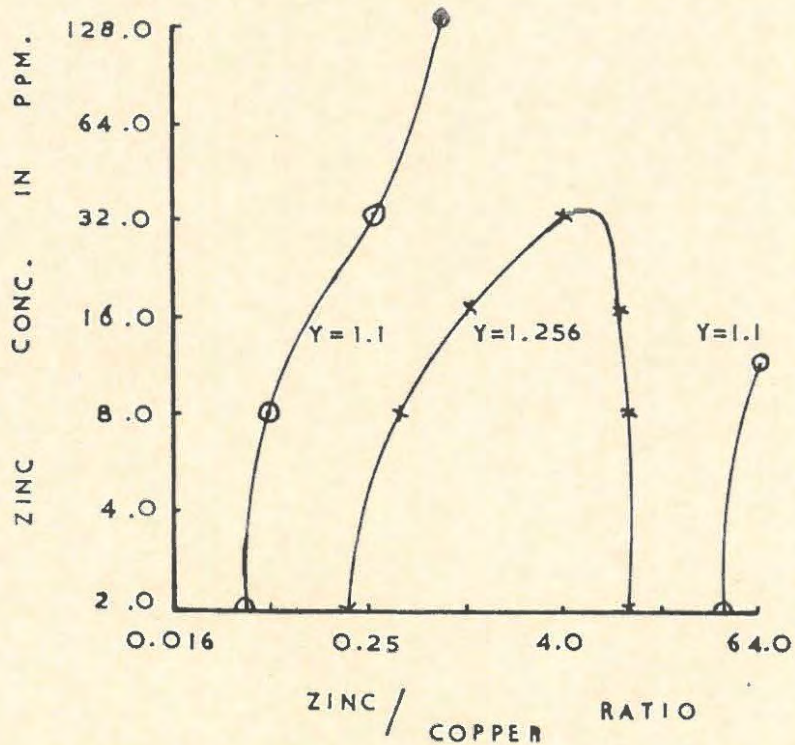
There is no evidence that the level of copper supply was important in determining sporulation and wrinkling of the felts, or the pH of the filtrate (Table 20 of Appendix).

- (c) The relation between growth and development and the Zinc:Copper ratio in the medium.

That the Zinc:Copper ratio in the culture medium was the major factor determining the dry weight yield trends, in cultures of Penicillium glaucum, is clearly evident from the regression analysis graph (graph 22). From this graph it is apparent that, at low levels of zinc supply (2.0 p.p.m.), raising the Zinc:Copper ratio from 0.0156 to 1.0 resulted in increased yield, but a subsequent increase to 64.0 p.p.m. resulted in diminished yield. At higher levels of zinc supply (32.0 p.p.m.) the optimum Zinc:Copper ratio for yield was 4.0, and at 128.0 p.p.m., approximately 16. The ratio



GRAPH 21. The effect of copper and zinc on the growth of Penicillium glaucum.



GRAPH 22. The effect of the zinc:copper balance on the growth of Penicillium glaucum.

effect becomes less marked with increase in concentration of zinc in the medium. Thus, while the Zinc:Copper ratio in the medium was important in the determination of the dry weight yield trends, no single optimal Zinc:Copper ratio was apparent. However Zinc:Copper ratios within the range 1.0 to 16.0 were optimal for dry weight yield.

There was no evidence that the Zinc:Copper ratio was important in determining the wrinkling or sporulation of the felts, or the pH of the filtrate (Table 20 of Appendix), in cultures of Penicillium glaucum.

Conclusions.

The results of Experiment 11, supply some evidence that, whilst in Aspergillus niger the Zinc:Copper ratio in the culture medium appears to have little direct effect on the determination of yield trends, in Penicillium glaucum a marked Zinc:Copper ratio effect is evident. In part 1 of the experiment, the evidence suggested that the optimal Zinc:Copper ratio lay within the range 0.0625 to 16.0. In part 2 the optimal range was found to be 1.0 to 16.0. It was also evident that, as the level of zinc in the medium increased, the Zinc:Copper ratio had to be raised to attain optimal conditions for dry weight yield.

The design of the experiment (Tables 77 and 88), was such that the number of yield values obtained for ratio decreased progressively towards the extremities of the range of ratios. It was therefore decided to carry out a further experiment in which equal numbers of treatments were obtained, for each Zinc:Copper ratio.

EXPERIMENT 12. To determine the effect of varying the absolute supply of copper and zinc, at fixed Copper:Zinc ratios in the culture medium, on the growth and development of cultures of Aspergillus niger and Penicillium glaucum.

The plan of the experiment is given in Table 94. From this Table it is evident that the experiment was designed so that the numbers of treatments were orthogonal with respect to the copper concentration and the Copper:Zinc ratio in the culture solution. This arrangement permitted a test of significance, by analysis of variance, to be performed on the dry weight yield data, for the effects of both the level of copper supply, and the Copper:Zinc ratio.

The Copper:Zinc ratio in the culture solution was varied by adding 1 ml. samples of different concentrations of copper and zinc to the culture solution. The different concentrations of the stock solutions were prepared by dilution of a concentrated solution of each, copper and zinc. In the case of copper, the dilution factor was 2 (1 volume solution in 1 volume of purified water), but for zinc several dilution factors were used. The concentrated solutions of copper and zinc were prepared as follows.

Copper: 0.98195 gms. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was dissolved with 1.46395 gms. E.D.T.A. in purified water in a 500 ml. volumetric flask. 1 ml. of this solution in 50 ml. gave a copper concentration of 10.0 p.p.m.

Zinc: 2.74488 gms. $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ was dissolved with 5.69364 gms. E.D.T.A. in a 100 ml. volumetric flask. 1 ml. of this solution in 50 ml. gave a zinc concentration of 200 p.p.m.

Results. The results are given in the following tables.

	<u>Aspergillus niger.</u>	<u>Penicillium glaucum.</u>
Dry weight yield.	95	96
pH of filtrate (in Appendix)	21	22
Analysis of variance	97	98
Regression analysis	99	100

TABLE 94. The plan of Experiment 12, showing the levels of supply of copper and zinc and the copper: zinc ratio.

Concentration of copper in p.p.m.	2.5	5.0	10.0
Copper:Zinc ratio	Concentration of zinc in p.p.m.		
500	0.005	0.01	0.02
50	0.05	0.10	0.20
5.0	0.50	1.0	2.0
2.5	1.0	2.0	4.0
1.0	2.5	5.0	10.0
0.5	5.0	10.0	20.0
0.2	12.5	25.0	50.0
0.1	25.0	50.0	100.0
0.066	37.5	75.0	150.0
0.05	50.0	100.0	200.0

TABLE 95. The dry weight yield, in grams, obtained from cultures of *Aspergillus niger* in Experiment 12.

Copper: Zinc Ratio	Level of Copper Supply in p.p.m.															Total for each ratio treatment	Mean
	2.5	5.0	10.0	15.0	20.0	25.0	30.0	35.0	40.0	45.0	50.0	55.0	60.0	65.0	70.0		
500	.24451	.45956	.29664	.27430	.36913	.53669	.33870	.41004	.43883	.62659	.95401	.78086	.56386	.63100	.65332	7.42804	0.49520
50.0	.58385	.61529	.49353	.50132	.30593	.50392	.74382	.59530	.61137	.72993	.98518	.69928	.99964	.89018	.93732	10.24586	0.68306
5.0	.80234	.87256	.54476	.92592	.89427	.90572	.66488	.54380	.59458	.80548	.99349	.78490	.44237	.98340	.93593	11.59440	0.77962
2.5	.97187	.73638	.78782	.65609	.66636	.93204	.77501	.66340	.82360	.79814	.92899	.86535	.47523	.91542	1.02601	12.02671	0.80178
1.0	.51794	.84827	.80181	.67567	.73303	.44843	.71500	.70819	.61430	.60124	.93150	.73361	.95126	.89192	.89747	11.12914	0.64168
0.5	.84722	.77721	.97172	.87401	.52602	.92746	.94878	.67461	.74875	.66015	.82184	.91500	.79210	.80719	.89453	12.18659	0.81244
0.2	.65371	.73510	.95082	.51000	.77755	.92848	.85804	.85860	.78520	.84934	.43193	.81685	.91690	.81843	.90338	11.79433	0.78629
0.1	.60811	.62287	.90213	.79067	.8701	.72670	.95459	.49802	.86795	.42662	.86232	.86197	.89892	.94172	.89177	11.54137	0.76942
0.066	.92955	.93721	.68054	.61489	.71751	.87823	.87384	.45457	.80715	.70602	.75645	.43668	.85292	.50731	.82117	10.97406	0.73160
0.050	1.03758	.75369	1.00614	.61166	.54514	.96747	.84237	.60746	.67121	.62891	.30445	.31284	.36851	.32909	.33306	9.31958	0.62130
Total for each Cu treatment				34.65223				35.09502								38.59883	
Mean				0.69304				0.70170								0.77198	

Least significant difference between means at 0.05 probability level for copper treatment = \pm 0.15627 grams.

Least significant difference between means at 0.05 probability level for ratio treatment = \pm 0.28479 grams.

TABLE 96. The dry weight yield, in grams, obtained from cultures of *Penicillium glaucum* in Experiment 12.

Copper: Zinc Ratio	Level of Copper Supply in p.p.m.															Total for each ratio treatment	Mean
	2.5					5.0					10.0						
500	.31852	.29519	.31223	.31532	.36974	.39635	.34331	.48486	.42547	.33831	.65206	.32003	.53112	.54355	.39033	6.03639	0.40243
50.0	.41226	.37876	.40351	.41160	.39120	.36498	.57372	.44969	.53083	.41142	.55268	.38632	.45443	.69396	.42644	6.84430	0.45632
5.0	.59787	.59971	.53625	.83809	.81745	.55425	.73693	.62481	.65893	.59623	.39643	.49288	.66023	.65036	.41593	9.27645	0.61843
2.5	.62503	.54915	.70476	.61210	.30181	.60176	.76185	.67367	.50486	.51006	.59091	.41828	.76451	.51509	.47713	9.12197	0.60813
1.0	.65804	.55525	.44070	.68386	.64927	.60575	.60473	.62131	.62070	.52454	.58760	.46376	.63746	.64391	.43011	8.73699	0.58247
0.5	.42742	.57898	.71345	.70133	.74745	.70015	.35933	.48178	.75054	.59919	.52052	.59054	.61322	.57336	.52206	8.83938	0.53929
0.2	.59935	.65214	.58083	.50732	.74027	.37727	.54078	.69323	.56298	.63684	.56599	.35638	.58129	.35614	.63221	8.38302	0.55830
0.1	.55856	.50029	.69961	.65860	.53914	.54441	.36438	.41369	.60554	.51832	.36160	.35101	.42219	.36153	.57946	7.57883	0.50526
0.066	.48721	.53552	.50702	.58351	.53832	.50027	.49285	.55306	.51626	.37943	.55051	.39201	.42100	.39206	.51434	7.46387	0.49759
0.050	.63401	.55486	.40480	.61732	.55732	.38629	.39298	.35414	.42260	.41093	.36415	.39615	.43416	.42569	.50674	6.86214	0.45748
Total for each Cu treatment	28.07881					26.18516					24.87937						
Mean	0.56158					0.52370					0.49760						

Least significant difference between means at 0.05 probability level for copper treatment = ± 0.03930 grams.

Least significant difference between means at 0.05 probability level for ratio treatment = ± 0.07175 grams.

TABLE 97. The results of an analysis of variance, performed on the dry weight yield data, obtained from cultures of Aspergillus niger in Experiment 12.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Ratio	9	1.31741	0.14638	1.38	Not significant
Copper	2	0.18744	0.09372	0.88	Not significant
Replication	4	0.09429	0.02332	0.22	Not significant
Error	135	14.29568	0.15890		

TABLE 98. The results of an analysis of variance performed on the dry weight yield data, obtained from cultures of Penicillium glaucum in Experiment 12.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Ratio	9	0.74377	0.08264	8.22	Significant at 0.1%
Copper	2	0.10350	0.05175	5.14	Significant at 1%
Replication	4	0.10058	0.02514	2.50	Significant at 5%
Error	135	1.35785	0.01005		

TABLE 99. The results of a regression analysis of the dry weight yield data obtained from cultures of Aspergillus niger in Experiment 12.

Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	10.1183	2.02237	7.79	Significant at 0.1%
Residual	24	6.22948	0.25956		

$$y = 4.375 + 0.069x_1 + 0.088x_2 - 0.057x_3 + 0.154x_4 - 0.163x_5 \text{ (Graph 23).}$$

TABLE 100. The results of a regression analysis of the dry weight yield data, obtained from cultures of Penicillium glaucum in Experiment 12.

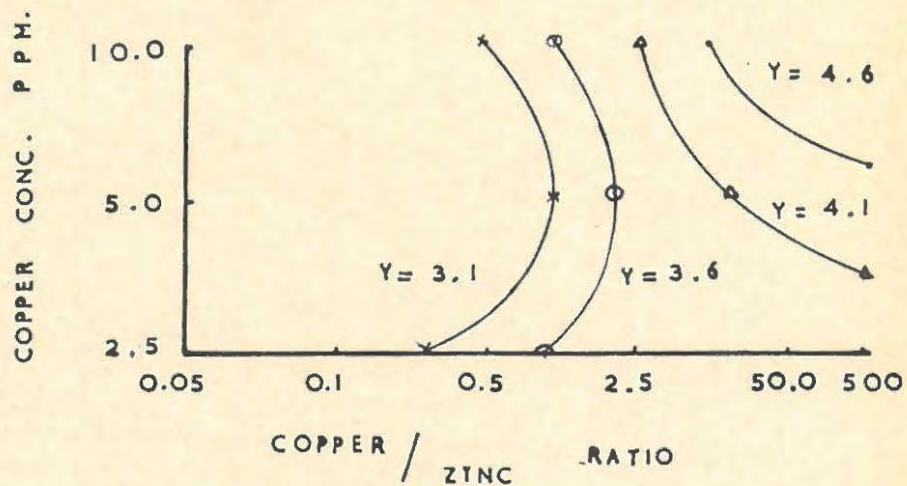
Factor	Degrees Freedom	Sum squares	Mean sum squares	F value	Significance
Regression	5	3.93224	0.78445	9.79	Significant at 0.1%
Residual	24	1.92268	0.08011		

$$y = 2.525 + 0.467x_1 + 0.180x_2 - 0.041x_3 + 0.029x_4 - 0.063x_5 \quad (\text{Graph 24}).$$

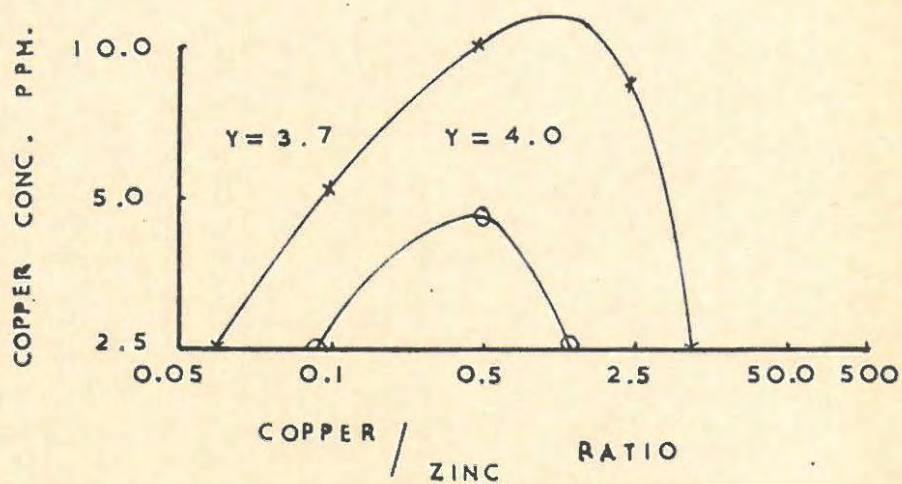
The results are considered under the following headings:

- (a) The effect of varying the level of copper supply, irrespective of the Copper:Zinc ratio in the medium.
- (b) The effect of varying the Copper:Zinc ratio, irrespective of the levels of copper and zinc supply in the medium.
- (a) The effect of varying the level of copper supply, irrespective of the Copper:Zinc ratio in the medium.

In cultures of Aspergillus niger, raising the level of copper supply from 2.5 to 10 p.p.m. had little effect on the dry weight yield (Table 95). The overall copper effect was shown by analysis of variance to be not significant (Table 97). In cultures of Penicillium glaucum, however, raising the level of copper supply from 2.5 to 10.0 p.p.m. had a significant effect on the dry weight yield (Table 98). It can be seen from the dry weight yield data (Table 96) that raising the copper concentration in the medium results in diminished yield. The amounts by which the yield is decreased, with each successive increase in copper concentration, are only slightly less than the value obtained for the least significant difference. Thus the copper effect is considered to



GRAPH 23. The effect of the level of copper supply and the copper:zinc ratio on the dry weight yield of Aspergillus niger.



GRAPH 24. The effect of the level of copper supply and the copper:zinc ratio on the dry weight yield of Penicillium glaucum.

be highly significant in the determination of the dry weight yield trends.

There is no evidence that the level of copper supply was important in determining the wrinkling, or sporulation of the felts, and the pH of the filtrate, in either cultures of Aspergillus or Penicillium (Tables 21 and 22 of Appendix).

- (b) The effect of varying the Copper:Zinc ratio irrespective of the levels of copper and zinc supply in the medium.

The effect of the Copper:Zinc ratio, in the determination of the dry weight yield trends, was shown to be highly significant in cultures of Penicillium glaucum (Table 98), but not significant in cultures of Aspergillus niger (Table 97). The yield trends, due to varying the Copper:Zinc ratio in the culture medium, are not noticeable in a study of the mean yield for each ratio treatment (Table 96). However, study of the regression analysis graph (graph 24) shows the effect of the Copper:Zinc ratio in the determination of the dry weight yield trends. At low levels of copper supply (2.50 p.p.m.) raising the Copper:Zinc ratio in the medium to 5.0 resulted in increased yield. Raising the ratio further to 500.0 resulted in diminished yields. At high levels of copper supply (10.0 p.p.m.), the optimum Copper:Zinc ratio was 1.0. Thus maximum dry weight yield was obtained when the Copper:Zinc ratio was in the range 0.5 to 1.0, or, in other words, when the Zinc:Copper ratio was between 1.0 and 2.0.

There was no evidence that the Copper:Zinc ratio in the medium was important in determining the wrinkling and sporulation of the felts, or the pH of the filtrate (Tables 21 and 22 of the Appendix).

Conclusion.

These results suggest that, in cultures of Aspergillus niger,

the level of copper supply, possibly due to the presence of a chelating agent, and the Copper:Zinc ratio in the culture medium do not play major roles in the determination of the dry weight yield trends. In cultures of Penicillium glaucum however, both the level of copper supply, and the Copper:Zinc ratio are important in determining yield trends. The optimum range of Zinc:Copper ratios 1.0 to 2.0 lies within the range shown in parts 1 and 2 of Experiment 11.

CHAPTER VI.Discussion and Conclusions.

It has been well established by numerous workers that the level of nutrient supply markedly influences the visual appearance and dry weight yield of fungal cultures. It has also been demonstrated that different fungi, and indeed different strains of the same fungus, may react differently under similar nutritional conditions.

In the Preliminary Investigations, where the level of supply of one heavy metal was varied, while all other nutrients were present in ample supply, the effect, on the visual appearance and yield, of varying the concentration, was demonstrated. Under these conditions, optimum levels of supply were demonstrated for manganese and copper in cultures of Aspergillus and Penicillium, and possibly for zinc in Penicillium. It was found that apart from the effect on the dry weight yield, the wrinkling of the felt of cultures of Aspergillus and Penicillium could, to a certain extent, be correlated with the level of heavy metal supply. Wrinkling became progressively more evident with increase in the heavy metal concentration. At low levels of supply this effect did not appear to be markedly influenced by the presence of a chelating agent. At high levels of supply, however, increasing the level of supply in the presence of a chelating agent had little effect on the wrinkling of the felt. Trends in sporulation were evident in the Preliminary Investigations, and tended to parallel the effect of the level of heavy metal supply on the dry weight yield. The presence of a chelating agent reduced the appearance of diminished sporulation at high levels of supply.

It is noteworthy that, when the levels of supply of two heavy metals were varied simultaneously, there was a marked tendency for the reduction of symptoms of toxicity. This was particularly evident in cultures of Aspergillus, when the levels of supply of

copper and zinc were varied simultaneously. These results suggest an interaction between the heavy metals, which controls either the uptake or precipitation of the metals in toxic quantities in the mycelium.

There is some evidence that the presence of a chelating agent, in particular Ethylene diaminetetra-acetic acid, reduces heavy metal toxicity. This was demonstrated for the four heavy metals studied, but in particular for iron and manganese in cultures of Aspergillus. Whether the E.D.T.A. functions to reduce metal uptake is not clear, but it seems more likely that it prevents the precipitation of heavy metals in the mycelium, and thereby reduces or prevents toxicity.

The evidence in favour of the Iron:Manganese ratio in the medium being important in the determination of growth and development of cultures of Aspergillus and Penicillium is not conclusive. There is some evidence which would suggest the ratio effect may be important, but less important than the individual effects of iron and manganese. It may also be that the use of a chelating agent, which is necessary at high levels of metal supply, serves to reduce the ratio effect, if such an effect exists, and thereby makes it difficult to distinguish the ratio effect from the individual effects of iron and manganese.

The Zinc:Copper ratio in the culture medium was shown to influence the dry weight yield obtained from cultures of Penicillium, but not in cultures of Aspergillus. It was found that the dry weight yield was related to the absolute concentration of copper and zinc, and to the Zinc:Copper ratio in the culture medium. Thus at low levels of supply the optimum Zinc:Copper ratio for yield was of the order 0.06, but as the absolute level of supply was raised, the Zinc:Copper ratio in the medium had to be increased to 16.0, for the maintenance of optimal nutritional conditions. There was no conclusive evidence that the Zinc:Copper ratio in the medium was

important in determining the sporulation and wrinkling of the felts or the pH of the filtrate.

The symptoms of too low a Zinc:Copper ratio in the medium were indistinguishable from those when the ratio was too high for optimal growth. It was therefore impossible to determine whether the effect of too low a ratio, or too high a ratio, could be remedied by the addition of zinc or copper respectively.

The precise relationship that exists between zinc and copper in determining the optimum ratio for growth is not understood, and it suffices, with the present information, to note its occurrence.

APPENDIX.

pH OF FILTRATE.

TABLE 1. The pH values of the filtrate obtained from cultures of Aspergillus niger in Experiment 3.

Level of supply in p.p.m.	Metal varied			
	Iron	Manganese	Copper	Zinc
0.0004	2.29	2.04	2.06	2.62
0.0016	2.36	2.01	2.01	2.52
0.0064	2.06	2.08	2.00	2.62
0.0256	2.09	2.02	1.99	2.41
0.1024	2.18	2.01	1.98	2.23
0.4096	2.14	1.96	1.95	1.99
1.6384	1.98	2.09	1.97	2.46
6.5536	1.99	1.97	2.12	2.06
26.2144	2.01	2.01	3.39	1.96

TABLE 2. The pH values of the filtrate obtained from cultures of Pencillium notatum in Experiment 3.

Level of supply in p.p.m.	Metal varied			
	Iron	Manganese	Copper	Zinc
0.0004	2.61	2.55	2.50	2.88
0.0016	2.72	2.58	2.59	2.71
0.0064	2.58	2.54	2.38	2.77
0.0256	2.41	2.51	2.35	2.65
0.1024	2.36	2.66	2.39	2.61
0.4096	2.38	2.46	2.45	2.48
1.6384	2.49	2.45	2.50	2.51
6.5536	3.32	2.42	2.45	2.62
26.2144	3.31	2.52	2.52	2.47

II

TABLE 3. The pH values of the filtrate obtained from cultures of Aspergillus niger in Experiment 4.

Level of supply in p.p.m.	Metals varied Ratio = 1.0					
	Iron and Manganese	Iron and Copper	Iron and Zinc	Manganese and Copper	Manganese and Zinc	Copper and Zinc
0.0004	2.21	2.37	2.28	2.07	2.28	2.29
0.0016	2.02	2.42	2.71	2.11	2.58	2.57
0.0064	1.99	2.21	2.48	2.03	2.63	2.31
0.0256	2.02	2.22	2.59	1.95	2.49	1.91
0.1024	1.99	2.15	2.41	1.92	2.22	1.98
0.4096	1.95	2.02	2.01	1.92	2.02	2.07
1.6384	2.00	2.08	1.99	1.95	2.38	2.00
6.5536	2.02	2.22	1.86	2.10	1.90	1.98
26.2144	2.20	2.36	2.06	2.68	1.88	2.00

TABLE 4. The pH values of the filtrate obtained from cultures of Penicillium notatum in Experiment 4.

Level of supply in p.p.m.	Metals varied Ratio = 1.0					
	Iron and Manganese	Iron and Copper	Iron and Zinc	Manganese and Copper	Manganese and Zinc	Copper and Zinc
0.0004	2.72	2.46	2.63	2.66	2.89	2.90
0.0016	2.56	2.39	2.64	2.98	2.92	2.90
0.0064	2.56	2.48	2.58	3.60	2.78	2.82
0.0256	2.48	2.32	2.55	2.45	2.60	2.68
0.1024	2.36	2.38	2.38	2.35	2.58	2.48
0.4096	2.41	2.41	2.48	2.41	2.41	2.32
1.6384	2.46	2.32	2.43	2.35	2.45	2.33
6.5536	2.51	2.56	2.59	2.29	2.37	2.31
26.2144	3.24	3.23	3.78	2.42	2.46	2.58

III

TABLE 5. The pH values of the filtrate obtained from cultures of Aspergillus niger in Experiment 5.

Level of supply in p.p.m.	Metal or metals varied		
	Iron	Manganese	Iron : Manganese Ratio = 1.0
0	3.12	3.17	3.18
	2.67	2.65	3.25
0.0004	2.83	2.49	2.83
	2.68	2.53	2.94
0.0016	2.50	2.96	2.88
	2.68	3.01	2.63
0.0064	2.45	2.99	2.82
	2.62	2.97	2.69
0.0256	2.41	2.98	2.58
	2.51	2.99	2.46
0.1024	2.35	3.04	2.29
	2.34	3.20	2.33
0.4096	2.31	3.30	2.25
	2.52	3.14	2.51
1.6384	2.43	2.98	2.61
	2.42	3.12	2.48
6.5536	2.47	3.08	2.52
	2.48	3.02	2.49
26.2144	2.65	3.12	2.50
	2.62	3.10	2.39
26.2144	2.63	3.05	2.32
	2.66	3.15	2.33
26.2144	2.61	3.22	2.36
	2.47	3.19	2.45
26.2144	2.49	3.21	2.49
	2.53	3.14	2.31
26.2144	2.43	3.22	2.52
	2.39	2.68	2.51

TABLE 6. The pH values of the filtrate obtained from cultures of Penicillium notatum in Experiment 5.

Level of supply in p.p.m.	Metal or metals varied		
	Iron	Manganese	Iron : Manganese Ratio = 1.0
0	2.62	2.63	2.53
	3.78	2.52	3.51
0.0004	3.40	2.78	3.43
	3.48	2.54	3.46
	3.34	2.46	3.22
0.0016	3.25	2.47	3.25
	3.30	2.56	3.26
	3.29	2.49	3.21
0.0064	3.58	2.42	3.21
	3.24	2.53	3.22
	2.81	2.49	3.21
0.0256	2.62	2.48	3.20
	2.79	2.56	3.21
	2.50	2.65	3.28
0.1024	2.47	2.60	3.25
	2.51	3.17	3.24
	2.44	2.56	2.39
0.4096	2.46	2.58	2.42
	2.47	2.51	2.49
	2.44	2.51	2.39
1.6384	2.58	2.56	2.48
	2.41	2.53	2.48
	2.53	2.45	2.32
6.5536	2.62	2.42	2.41
	2.61	2.48	2.49
	2.43	2.45	2.42
26.2144	2.39	2.48	2.46
	2.58	2.65	2.45

TABLE 7. The pH values of the filtrate obtained from cultures of Aspergillus niger (no chelate supplied) in Experiment 5.

Level of supply in p.p.m.	Metal or metals varied		
	Iron	Manganese	Iron : Manganese Ratio = 1.0
0	2.72	2.80	2.68
0.0004	2.65	2.70	2.60
0.0016	2.63	2.65	2.80
0.0064	2.59	2.70	2.75
0.0256	2.50	2.65	2.55
0.1024	2.45	2.67	2.60
0.4096	2.52	2.68	2.50
1.6384	2.61	2.68	2.60
6.5536	2.54	2.68	2.62
26.2144	2.50	2.65	2.65

TABLE 8. The pH values of the filtrate obtained with Penicillium notatum in Experiment 7. (No chelate supplied.)

Level of Manganese supply in p.p.m.	Level of Iron Supply in p.p.m.									
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144
0.0001	2.64	2.62	2.61	2.39	2.32	2.25	2.27	2.23	2.25	2.31
0.0004	2.74	2.62	2.55	2.51	2.34	2.25	2.22	2.25	2.29	2.29
0.0016	2.70	2.73	2.66	2.51	2.42	2.26	2.25	2.28	2.73	2.35
0.0064	2.69	2.88	2.51	2.48	2.46	2.30	2.23	2.31	2.28	2.39
0.0256	2.85	2.62	2.69	2.50	2.39	2.31	2.22	2.30	2.21	2.25
0.1024	2.76	2.75	2.76	2.47	2.55	2.32	2.21	2.28	2.33	2.51
0.4096	2.89	2.83	2.67	2.52	2.42	2.38	2.38	2.32	2.31	2.33
1.6384	3.02	2.87	2.75	2.55	2.50	2.40	2.50	2.48	2.30	2.32
6.5536	2.98	3.02	2.87	2.51	2.57	2.48	2.52	2.46	2.38	2.40
26.2144	3.06	2.88	2.71	2.63	2.47	2.51	2.47	2.48	2.42	2.47

TABLE 9. The pH values of the filtrate obtained with Penicillium notatum in Experiment 7. (E.D.T.A. as chelate.)

Level of Manganese supply in p.p.m.	Level of Iron Supply in p.p.m.									
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144
0.0001	3.16	3.18	3.02	2.91	2.60	2.75	2.82	2.71	2.90	3.60
0.0004	3.11	3.02	3.05	2.86	2.64	2.71	2.75	2.73	3.58	3.51
0.0016	2.88	3.02	3.00	2.82	2.65	2.68	2.60	2.70	2.92	3.58
0.0064	2.79	3.11	2.93	2.76	2.58	3.52	2.58	2.63	2.69	3.45
0.0256	2.82	2.66	2.82	2.69	2.49	2.55	3.51	3.45	3.28	3.34
0.1024	2.84	2.70	2.53	2.68	2.54	2.51	3.02	3.45	3.30	3.35
0.4096	2.66	3.02	2.78	2.72	2.51	2.50	2.85	3.48	3.28	3.41
1.6384	2.85	3.10	2.71	2.71	2.55	2.51	2.67	3.56	3.21	3.28
6.5536	2.58	2.98	2.70	2.63	2.58	2.53	2.56	3.23	3.42	3.32
26.2144	2.75	2.72	2.65	2.54	2.61	2.59	2.62	3.02	3.41	3.32

VII

TABLE 10. The pH values of the filtrate obtained with cultures of *Aspergillus niger* in Experiment 8 part 1.

Level of Mn supply in p.p.m.	Level of Iron Supply in p.p.m.																				
	0.064			0.128			0.256			0.512			1.024			2.048			4.096		
0.064	1.88	1.76	1.73	1.81	1.74	1.76	1.81	1.78	1.88	1.82	1.78	1.82	1.79	1.80	1.81	1.82	1.98	1.71	1.86	1.86	1.91
0.128	1.75	1.72	1.75	1.85	1.82	1.78	1.79	1.77	1.74	1.73	1.79	1.74	1.79	1.82	1.81	1.78	1.81	1.78	1.91	1.82	1.90
0.256	1.71	1.72	1.70	1.75	1.79	1.70	1.81	1.84	1.86	1.78	1.89	1.77	1.84	1.87	1.78	1.92	1.90	1.80	1.85	1.89	1.82
0.512	1.70	1.72	1.76	1.82	1.82	1.92	1.83	1.78	1.79	1.78	1.98	1.82	1.80	1.99	1.89	1.72	1.81	1.85	1.83	1.82	1.82
1.024	1.72	1.72	1.75	1.76	1.80	1.82	1.82	1.78	1.78	1.76	1.79	1.82	1.79	1.82	1.79	1.76	1.81	1.82	1.89	1.82	1.88
2.048	1.78	1.84	1.95	1.83	1.82	1.75	1.79	1.82	1.84	1.78	1.80	1.75	1.80	1.79	1.85	1.80	1.76	1.82	1.91	1.92	1.92
4.096	1.78	1.77	1.85	1.74	1.75	1.83	1.76	1.78	1.79	1.73	1.89	1.85	2.02	1.79	1.81	1.89	1.81	1.92	1.96	1.95	1.89

TABLE 11. The pH values of the filtrate obtained with cultures of *Penicillium glaucum* in Experiment 8 part 1.

Level of Mn supply in p.p.m.	Level of Iron Supply in p.p.m.																				
	0.064			0.128			0.256			0.512			1.024			2.048			4.096		
0.064	2.30	2.32	2.70	2.60	2.66	3.16	3.62	3.25	3.39	3.48	3.62	3.19	3.40	3.82	3.74	3.52	3.64	3.78	3.35	3.60	3.90
0.128	2.70	2.80	2.15	2.54	2.64	2.39	3.51	3.52	3.42	3.20	3.14	3.38	3.53	3.32	3.20	3.60	3.75	3.89	3.60	3.59	3.35
0.256	2.48	2.54	2.65	2.43	2.63	2.85	3.30	3.65	3.13	3.58	3.75	3.48	3.12	3.10	3.32	3.55	3.82	3.83	3.75	3.90	3.65
0.512	2.40	2.41	2.37	2.60	3.10	2.42	3.16	3.12	3.31	3.40	3.15	3.48	3.16	3.12	3.22	4.21	3.69	3.78	3.51	3.30	3.75
1.024	2.32	2.45	2.38	2.48	2.40	2.70	3.28	3.20	3.41	3.40	3.55	3.31	3.23	3.28	3.12	3.70	3.80	3.35	3.40	3.61	4.01
2.048	2.18	2.40	2.52	2.55	2.60	2.64	3.39	3.14	3.32	3.08	3.08	3.18	3.18	3.42	3.45	3.35	3.15	3.40	3.30	3.35	3.60
4.096	2.30	2.24	2.19	2.40	2.59	2.30	2.80	2.88	3.28	3.30	3.15	3.19	3.29	3.31	3.11	3.40	3.38	3.15	3.40	3.10	3.30

VIII

TABLE 12. The pH values of the filtrate obtained with cultures of *Aspergillus niger* in Experiment 8 part 2. (No chelate supplied.)

Level of Mn supply in p.p.m.	Level of Iron Supply in p.p.m.																				
	2.0			4.0			8.0			16.0			32.0			64.0			128.0		
2.0	1.85	1.75	1.99	2.05	1.95	1.82	1.80	1.95	1.80	1.85	2.00	1.85	1.80	1.75	1.75	1.75	2.15	2.00	1.91	1.96	2.00
4.0	1.80	2.00	1.80	1.78	1.78	1.75	1.90	1.90	1.85	1.90	1.94	1.95	1.70	1.75	1.75	1.80	1.85	1.80	1.99	1.93	1.93
8.0	1.80	1.75	1.80	1.70	1.80	1.75	1.83	1.75	1.80	1.90	1.90	1.95	1.70	1.70	1.75	1.70	1.75	1.75	1.80	1.82	1.80
16.0	1.95	1.85	1.79	1.79	1.79	1.75	1.75	1.70	1.70	2.04	2.05	2.10	2.05	1.90	1.80	1.80	1.85	1.75	1.91	1.99	1.89
32.0	2.05	1.79	1.80	1.90	2.50	1.90	1.75	1.98	1.80	1.99	2.10	2.05	1.70	1.75	1.70	2.30	2.00	2.00	2.00	2.00	1.9
64.0	2.10	1.90	1.95	1.95	2.09	2.15	1.90	1.90	1.35	1.93	2.00	2.10	1.85	1.95	2.00	2.10	2.18	2.08	2.1	2.1	2.00
128.0	2.05	2.15	2.07	2.18	2.30	2.25	2.15	2.15	1.99	2.25	2.10	2.05	1.90	2.25	2.01	2.00	2.40	2.12	2.10	2.01	2.01

TABLE 13. The pH values of the filtrate obtained with cultures of *Aspergillus niger* in Experiment 8 part 2, (Citric acid supplied as chelate)

Level of Mn supply in p.p.m.	Level of Iron Supply in p.p.m.																				
	2.0			4.0			8.0			16.0			32.0			64.0			128.0		
2.0	2.01	1.98	1.95	2.03	1.92	1.94	2.00	1.95	1.96	2.02	2.01	2.05	2.15	2.10	2.05	2.04	2.04	2.09	2.15	2.15	2.08
4.0	2.03	2.00	1.98	2.00	1.97	1.91	2.01	2.0	1.98	2.12	2.10	2.08	2.00	2.06	2.00	2.10	2.09	2.05	2.09	2.17	2.00
8.0	2.10	2.00	2.02	2.00	2.05	1.95	1.99	2.09	1.98	2.11	2.01	2.12	2.10	2.02	2.05	2.08	2.10	2.09	2.19	2.17	2.02
16.0	1.95	1.95	2.05	2.00	2.05	1.94	1.95	2.02	2.01	2.12	2.02	2.09	2.01	2.02	2.12	2.12	2.08	2.09	2.12	2.12	2.04
32.0	1.95	2.06	1.97	2.01	2.05	1.90	1.95	2.08	2.00	2.02	2.13	1.98	2.03	2.00	2.11	2.10	2.05	2.12	2.17	2.15	2.03
64.0	2.00	2.03	2.06	1.98	2.03	1.92	1.99	2.12	1.99	2.01	2.05	2.06	2.10	2.02	2.12	2.17	2.03	2.02	2.19	2.17	2.02
128.0	2.01	1.98	1.99	2.10	2.10	1.89	2.00	2.02	2.01	2.05	2.02	2.10	2.00	2.01	2.03	2.01	2.03	2.22	2.21	2.17	2.29

TABLE 14. The pH values of the filtrate obtained with cultures of *Penicillium glaucum* in Experiment 8 part 2.

Level of Mn supply in p.p.m.	Level of Iron Supply in p.p.m.																				
	2.0			4.0			8.0			16.0			32.0			64.0			128.0		
2.0	3.63	3.60	3.51	3.79	3.91	3.76	3.76	3.82	3.72	3.69	3.64	3.73	4.26	3.70	4.01	3.11	3.52	3.01	3.60	3.22	3.52
4.0	3.60	3.42	3.72	3.61	3.52	3.46	3.79	3.83	3.91	3.72	3.89	3.84	3.71	3.83	4.01	3.56	3.32	3.56	3.69	3.61	3.53
8.0	3.62	3.41	3.72	3.76	3.78	3.72	3.80	3.81	3.82	3.75	3.17	3.91	3.60	3.91	3.76	3.55	3.58	3.46	3.75	3.60	3.49
16.0	3.62	3.58	3.11	3.68	3.71	3.76	3.39	3.62	3.72	3.88	3.40	3.71	3.53	3.59	3.49	3.42	3.68	3.49	3.51	3.64	3.68
32.0	3.37	3.71	3.31	3.72	3.62	3.69	3.82	3.81	3.91	3.63	3.79	3.63	3.72	3.53	3.80	3.65	3.03	3.62	3.67	3.53	3.52
64.0	3.81	3.38	2.96	3.12	2.88	3.53	4.07	3.64	3.30	2.82	3.73	3.59	3.46	3.98	3.79	3.72	3.65	3.76	3.55	3.72	3.65
128.0	3.28	2.68	2.72	3.48	3.77	3.79	3.72	3.62	3.56	3.86	3.68	3.78	3.65	3.51	3.42	3.60	3.57	3.68	3.61	2.82	3.92

TABLE 15. The pH values of the filtrate obtained with *Aspergillus niger* in Experiment 9.

Iron : Manganese Ratio	Level of Iron Supply in p.p.m.														
	2.5					5.0					10.0				
500	1.68	1.60	1.72	1.71	1.64	1.89	1.72	1.71	1.75	1.91	1.58	1.71	1.77	1.75	1.73
50.0	1.77	1.76	1.68	1.61	1.75	1.89	1.76	1.82	1.73	1.72	1.76	1.71	1.70	1.75	1.77
5.0	1.67	1.76	1.72	1.91	1.86	1.84	1.69	1.81	1.73	1.72	1.71	1.71	1.81	1.80	1.75
2.5	1.77	1.68	1.78	1.75	1.72	1.82	1.81	1.78	1.88	1.86	1.69	1.72	1.83	1.69	1.72
1.0	1.76	1.77	1.77	1.72	1.76	1.76	1.78	1.85	1.81	1.77	1.95	2.09	1.95	1.81	1.77
0.5	1.68	1.77	1.69	1.71	1.76	1.81	1.81	1.78	1.91	1.96	1.80	1.85	1.82	1.78	1.78
0.2	1.72	1.72	1.68	1.71	1.70	1.72	1.77	1.72	1.81	1.72	1.76	1.91	1.81	1.87	1.92
0.1	1.81	1.71	1.78	1.73	1.72	1.75	1.78	1.72	1.78	1.75	1.90	2.04	1.82	1.82	1.98
0.066	1.79	1.75	1.82	1.82	1.77	1.77	1.73	1.80	1.68	1.68	1.80	2.08	1.98	1.82	1.82
0.050	1.72	1.70	1.72	1.88	1.76	1.88	1.74	1.85	1.78	1.75	1.78	1.79	1.92	1.78	1.85

TABLE 16. The pH values of the filtrate obtained with Penicillium glaucum in Experiment 9.

Iron : Manganese Ratio	Level of Iron Supply in p.p.m.														
	2.5					5.0					10.0				
500.	3.99	3.84	2.50	2.56	3.59	2.52	3.82	3.81	3.53	2.98	3.02	2.51	2.37	2.32	3.43
50.0	2.21	2.62	2.22	2.21	2.20	3.04	2.72	2.29	2.34	2.67	3.42	2.47	3.97	2.68	2.59
5.0	2.30	2.16	2.22	2.21	2.15	3.04	2.58	2.91	2.29	2.42	2.52	2.78	3.61	2.48	2.35
2.5	2.21	2.20	2.16	2.12	2.12	2.51	3.12	2.99	2.42	2.41	2.37	2.22	2.25	2.28	2.28
1.0	2.15	2.18	2.16	2.18	2.11	2.12	2.12	2.29	2.42	2.21	2.32	2.19	2.21	2.26	2.34
0.5	2.17	2.14	2.21	2.12	2.14	2.12	2.12	2.15	2.11	2.11	2.30	2.48	2.42	3.31	2.31
0.2	2.18	2.11	2.15	2.26	2.12	2.16	2.19	2.24	2.18	2.18	2.17	2.24	2.17	2.22	2.15
0.1	2.11	2.06	2.13	2.12	2.11	2.12	2.09	2.13	2.03	2.04	2.22	2.13	2.17	2.18	2.19
0.066	2.13	2.08	2.07	2.09	2.08	2.10	2.12	2.12	2.12	2.06	2.12	2.03	2.11	2.22	2.21
0.050	2.22	2.18	2.13	2.14	2.14	2.02	2.10	2.12	2.15	2.15	2.29	2.29	2.29	2.37	2.48

TABLE 17. The pH values of the filtrate obtained with cultures of Penicillium glaucum in Experiment 10.

Level of Copper supply in p.p.m.	Level of Zinc Supply in p.p.m.									
	0.0001	0.0004	0.0016	0.0064	0.0256	0.1024	0.4096	1.6384	6.5536	26.2144
0.0001	2.29	2.64	2.70	2.76	2.62	3.17	3.02	2.45	2.52	2.70
0.0004	2.49	2.63	2.52	2.57	2.57	2.48	2.55	3.17	3.01	2.45
0.0016	2.53	2.60	2.52	2.38	3.02	2.41	2.52	2.69	2.45	2.43
0.0064	2.57	2.49	2.36	2.32	2.33	2.59	2.53	3.16	2.44	2.86
0.0256	2.53	2.48	2.32	2.33	2.21	2.40	5.88	3.79	3.15	2.49
0.1024	2.59	3.02	2.87	2.45	2.31	2.32	4.25	4.38	3.75	4.20
0.4096	2.78	2.52	2.42	2.35	2.40	3.40	4.21	4.62	4.59	3.98
1.6384	2.52	2.35	2.39	2.32	2.29	2.39	3.54	4.65	3.72	4.90
6.5536	2.49	2.34	2.56	2.39	2.38	2.68	3.18	3.93	3.80	3.51
26.2144	2.72	2.62	2.82	2.98	2.45	2.65	2.73	2.68	2.65	3.00

XII

TABLE 18. The pH values obtained with cultures of *Aspergillus niger* in Experiment 11, part 1.

Level of Copper supply in p.p.m.	Level of Zinc Supply in p.p.m.																				
	0.064			0.128			0.256			0.512			1.024			2.048			4.096		
0.064	1.81	1.73	1.76	1.91	1.89	1.82	1.84	1.77	1.78	1.79	1.73	1.72	1.78	1.85	1.78	1.95	1.79	1.86	1.94	1.98	1.82
0.128	1.81	1.80	1.82	1.81	1.79	1.80	1.80	1.82	1.82	1.86	1.88	1.83	1.89	1.83	1.82	1.96	1.85	1.84	1.86	1.84	1.88
0.256	1.98	2.00	2.02	1.98	2.01	2.01	1.98	1.96	1.89	2.00	1.94	2.00	1.90	1.95	2.00	1.99	2.02	2.01	2.00	1.98	2.01
0.512	1.81	2.03	2.11	2.11	2.10	2.06	2.12	2.01	1.99	2.04	2.02	2.12	2.09	2.11	2.09	2.06	2.09	2.01	2.09	2.06	2.05
1.024	2.05	2.01	2.03	2.00	2.01	2.01	2.00	2.02	2.03	2.00	2.04	2.02	2.01	2.03	2.06	2.02	2.06	2.01	2.02	2.02	1.80
2.048	1.89	2.12	2.13	2.12	2.01	2.18	2.12	1.85	2.00	2.08	2.09	2.07	2.02	2.02	2.01	2.03	2.05	2.08	2.06	2.07	2.06
4.096	2.02	2.04	2.09	2.10	1.90	2.12	2.10	2.11	2.12	2.16	2.13	2.15	2.16	2.18	2.21	2.20	2.16	2.18	2.21	2.06	2.25

TABLE 19. The pH values obtained with cultures of *Penicillium glaucum* in Experiment 11, part 1.

Level of Copper supply in p.p.m.	Level of Zinc Supply in p.p.m.																				
	0.064			0.128			0.256			0.512			1.024			2.048			4.096		
0.064	2.48	2.39	2.27	2.29	2.41	2.32	3.40	3.65	3.32	3.88	3.32	3.68	2.36	3.31	2.25	2.72	3.08	2.52	2.33	2.39	2.34
0.128	2.32	2.35	2.28	2.51	2.52	2.42	2.95	3.78	3.79	3.51	2.64	2.59	2.41	2.61	2.39	2.97	3.52	3.15	2.36	2.61	2.35
0.256	2.32	2.31	2.41	2.69	2.36	2.34	4.11	3.76	3.31	3.90	4.13	2.55	3.40	3.68	4.08	2.62	3.17	3.60	2.40	2.38	2.35
0.512	2.31	2.25	2.24	2.22	2.26	2.32	3.21	3.09	2.50	3.43	2.74	2.72	2.71	3.21	3.14	2.16	2.48	2.46	2.41	3.34	2.21
1.024	2.28	2.31	2.22	2.23	2.42	2.56	3.69	3.89	3.72	3.41	2.49	3.10	3.72	2.31	3.52	2.44	2.85	2.72	2.48	2.50	2.29
2.048	2.22	2.27	2.31	2.32	2.29	2.33	3.78	3.01	3.71	2.62	3.11	3.72	2.31	2.43	3.67	2.72	2.55	2.62	3.08	2.75	2.52
4.096	2.27	2.25	2.22	2.51	2.53	2.32	3.66	3.28	4.12	2.52	3.71	3.96	2.78	4.21	2.66	2.40	3.51	4.13	2.92	2.49	2.90

TABLE 20. The pH values of the filtrate obtained with cultures of *Penicillium glaucum* in Experiment 11, part 2.

Level of Copper supply in p.p.m.	Level of Zinc Supply in p.p.m.																				
	2.0			4.0			8.0			16.0			32.0			64.0			128.0		
2.0	2.22	2.22	2.19	2.19	2.64	2.69	2.47	2.17	2.09	3.51	2.92	2.20	2.11	2.20	2.22	2.16	2.16	2.14	2.16	2.18	2.12
4.0	2.12	3.32	2.22	2.21	2.90	2.48	2.13	2.22	2.16	2.11	2.08	2.14	2.14	2.18	2.22	2.12	2.11	2.17	2.17	2.14	2.16
8.0	2.86	2.12	3.19	2.49	2.40	2.16	2.36	2.11	3.43	3.12	2.31	2.11	2.82	2.08	2.09	2.12	2.10	2.12	2.11	2.21	2.12
16.0	3.35	2.26	2.23	2.12	2.11	2.49	2.93	2.18	2.45	2.79	2.13	2.27	2.10	2.12	2.31	2.18	2.08	2.15	2.46	2.13	2.18
32.0	2.11	2.09	2.21	2.08	2.09	2.04	2.10	2.08	2.03	2.15	2.12	2.10	2.12	2.10	2.11	2.14	2.19	2.21	2.20	2.16	2.21
64.0	2.09	2.10	2.12	2.12	2.09	2.10	2.09	2.11	2.25	3.51	3.17	2.22	2.16	2.15	2.15	2.18	2.14	2.15	2.16	2.15	2.19
128.0	2.11	2.07	2.09	2.04	2.09	2.09	2.06	2.12	2.12	2.13	2.15	2.11	2.15	2.15	2.15	2.18	2.14	2.15	2.19	2.26	2.22

TABLE 21. The pH values of the filtrate obtained with cultures of *Aspergillus niger* in Experiment 12.

Copper : Zinc Ratio.	Level of Supply of Copper in p.p.m.														
	2.5					5.0					10.0				
500	2.01	1.99	1.82	1.89	1.78	1.72	1.60	1.69	1.72	1.62	1.81	1.57	1.51	1.53	1.57
50.0	1.82	1.50	1.51	1.51	1.57	1.42	1.48	1.39	1.32	1.49	1.82	1.96	1.72	1.89	1.84
5.0	1.67	2.00	1.91	1.72	1.59	1.79	1.69	1.61	1.72	1.65	1.78	1.71	1.88	2.18	1.71
2.5	1.86	1.81	1.90	1.87	1.92	1.76	1.78	1.89	1.92	1.67	2.11	1.96	1.88	2.11	1.86
1.0	1.86	1.98	2.01	2.05	1.78	1.82	1.90	1.93	1.92	1.82	1.82	1.98	1.72	1.92	1.97
0.5	1.82	2.01	1.76	1.96	1.96	1.92	1.92	1.86	1.82	1.58	1.96	1.96	2.05	1.81	2.11
0.2	1.81	1.69	1.67	1.89	1.86	1.78	1.88	1.72	1.86	1.68	1.82	1.72	1.92	1.99	1.86
0.1	1.79	1.88	1.85	1.82	1.81	1.79	1.88	1.73	1.89	2.01	1.83	1.85	2.10	2.03	2.02
0.066	1.78	1.70	1.74	1.59	1.68	1.93	1.79	1.72	1.77	1.72	2.02	1.89	1.39	1.89	2.22
0.050	1.59	1.69	1.45	1.69	1.42	1.84	1.78	1.69	1.52	1.48	2.28	2.28	2.22	2.51	2.48

TABLE 22. The pH values of the filtrate obtained with cultures of Penicillium glaucum in Experiment 12.

Copper : Zinc Ratio.	Level of Supply of Copper in p.p.m.														
	2.5					5.0					10.0				
500	2.48	2.51	2.41	2.45	2.44	2.77	2.75	3.36	2.84	2.79	3.82	3.88	3.42	2.64	3.49
50.0	2.58	2.56	2.50	2.45	2.42	2.71	3.53	2.97	3.43	2.58	3.56	3.92	3.62	2.92	3.79
5.0	4.12	4.26	3.98	3.87	3.39	3.82	4.40	4.16	4.22	4.16	3.98	4.08	4.29	3.79	3.20
2.5	4.19	3.91	4.09	3.33	3.38	4.35	4.50	4.22	3.72	3.70	4.02	3.28	4.20	3.01	3.81
1.0	3.92	4.14	2.89	3.62	4.00	3.60	4.23	4.08	4.11	4.06	2.99	3.98	4.20	3.42	3.78
0.5	4.01	4.10	4.16	2.90	2.58	4.05	4.12	2.85	2.53	4.20	3.43	3.88	4.18	3.92	3.91
0.2	4.12	3.02	3.67	3.97	3.55	4.02	3.53	4.03	3.67	2.70	3.60	2.65	3.48	2.57	2.63
0.1	3.38	3.74	4.01	3.40	3.49	3.23	3.37	2.58	2.76	3.00	2.72	2.69	2.66	2.71	2.64
0.066	3.76	3.71	3.39	3.70	3.21	2.48	2.39	3.01	2.86	2.64	2.58	2.81	2.72	2.68	2.52
0.050	2.89	3.72	2.88	3.41	3.71	2.45	2.41	2.42	2.39	2.89	2.67	2.60	2.57	2.61	2.59

STATISTICAL METHODS.1. ANALYSIS OF VARIANCE.

Treat- ment.	IRON						<u>TOTAL:</u>	
	A	B	C	D	E	F		
1	a	0.45505	0.60996	0.49590	0.47070	0.48998	0.32495	8.83900
	b	0.37340	0.40505	0.62895	0.52140	0.61284	0.49946	
	c	0.31495	0.47464	0.38256	0.54895	0.61721	0.61305	
2	a	0.41846	0.54600	0.33755	0.60443	0.60000	0.40446	9.00764
	b	0.33021	0.42330	0.59621	0.42506	0.73760	0.45020	
	c	0.56141	0.69015	0.33785	0.54745	0.66590	0.47140	
3	a	0.62195	0.60730	0.42448	0.53760	0.61430	0.48770	9.58967
	b	0.55716	0.71435	0.43882	0.59761	0.60975	0.45005	
	c	0.42860	0.31400	0.41511	0.70749	0.59485	0.48855	
4	a	0.55250	0.38965	0.30806	0.53085	0.50511	0.52704	8.98347
	b	0.38800	0.51620	0.97034	0.32725	0.51290	0.44012	
	c	0.50320	0.50955	0.31055	0.54560	0.68750	0.45905	
5	a	0.51245	0.69397	0.58816	0.65220	0.65425	0.40910	10.79132
	b	0.32445	0.73031	0.71257	0.74140	0.65609	0.40600	
	c	0.65735	0.55215	0.69536	0.77807	0.50935	0.46809	
6	a	0.28920	0.66251	0.36945	0.63410	0.73212	0.53150	9.93041
	b	0.42096	0.68149	0.66010	0.65125	0.59446	0.43770	
	c	0.41600	0.38635	0.64775	0.72497	0.59170	0.44830	
<u>TOTAL:</u>		8.10530	9.75693	9.32027	10.65638	10.98591	8.31722	

MANGANESE.

Correction factor.

$$\frac{\text{Total yield}^2}{108} = \frac{57.14201^2}{108} = \underline{30.23341}$$

Sum of squares for all analysis.

$$\begin{aligned} &\text{Total sum of squares - Correction factor} \\ &= 32.03709 - 30.23341 = \underline{1.80448} \end{aligned}$$

Sum of squares for iron effect.

$$\frac{(8.10530)^2 + (9.75693)^2 + (9.32027)^2 + (10.65638)^2 + (10.98591)^2 + (8.31722)^2}{18}$$

- Correction factor.

$$30.62143 - 30.23341 = \underline{0.38802}$$

Sum of squares for manganese effect

$$\frac{(8.83900)^2 + (9.00764)^2 + (9.58967)^2 + (8.98347)^2 + (10.79182)^2 + (9.93041)^2}{18}$$

- Correction factor.

$$30.38942 - 30.23341 = \underline{0.15583}$$

Sum of squares for replication effect.

$$\begin{aligned} &\frac{(\text{Sum a})^2 + (\text{Sum b})^2 + (\text{Sum c})^2}{36} - \text{C.F.} \\ &= \frac{(18.64299)^2 + (19.63301)^2 + (18.86601)^2}{36} - 30.23341 \\ &= 30.24837 - 30.23341 = \underline{0.01496} \end{aligned}$$

Sum of squares for Interaction effect.

		IRON					
		A	B	C	D	E	F
MANGANESE	1	1.14340	1.48965	1.50741	1.54105	1.72003	1.43746
	2	1.31008	1.45945	1.27161	1.63694	2.00350	1.32606
	3	1.58771	1.63565	1.27841	1.84270	1.81890	1.42630
	4	1.44370	1.41485	1.58895	1.40370	1.70551	1.42621
	5	1.49425	2.02643	1.99659	2.17167	1.81969	1.28319
	6	1.12616	1.73035	1.67730	2.06032	1.91828	1.41800

XVII

Sum of squares = 93.21717

Sum of squares for interaction effect = $\frac{93.21717}{3}$

C.F. - S.S. Fe. effect - S.S. Mn. effect

= 31.07239 - 30.23341 - 0.15583 - 0.38302

= 0.29514

Sum of squares for error.

Total sum of squares - C.F. - S.S. Fe. effect - S.S. Mn. effect

- S.S. Rep. effect.

= 32.03789 - 30.23341 - 0.15583 - 0.38302 - 0.29514

= 0.96549

Analysis of Variance.

Factor	Degrees Freedom	Sum of squares	Mean sum of squares	F Value	Significance
Iron	5	0.38802	0.07760	5.63	Sig. at 0.1%
Manganese	5	0.15583	0.03117	2.26	Not sig.
Interaction	25	0.29514	0.01181	0.86	Not sig.
Replication	2	0.01496	0.00748	0.005	Not sig.
Error	70	0.96549	0.01379	-	-

2. REGRESSION ANALYSIS.

Postulate Quadratic Surface.

- Code: X_1 = Balance effect
 X_2 = Copper effect
 X_3 = X_1^2 quadratic balance effect
 X_4 = X_2^2 quadratic copper effect
 X_5 = $X_1 X_2$ quadratic balance and copper effects

Code	X_1 X_2	+1 -1	0 0	+1 +1	Yield Totals	
Concentration Copper p.p.m.		2.5 p.p.m.	5.00 p.p.m.	10.00 p.p.m.		
Code	Ratio					
X_3	X_1					
1	1	500	1.64414	2.15085	3.63305	7.42804
4	2	50.0	2.49992	3.18434	4.56160	10.24586
9	3	5.0	4.03988	3.51446	4.14009	11.69440
16	4	2.5	3.81852	3.99719	4.21100	12.02671
25	5	1.0	3.58172	3.87660	4.45576	11.12514
36	6	0.5	3.99618	3.95975	4.23066	12.18659
49	7	0.2	3.62718	4.27966	3.88749	11.79433
64	8	0.1	3.61079	3.47388	4.45670	11.54157
81	9	0.066	3.87972	3.71981	3.37453	10.97406
100	10	0.05	3.95421	3.71742	1.64795	9.31958
Yield Totals		34.65223	35.08502	38.59883		

Total Yield : 108.33608

Sum of Squares of Yield : 407.59296

Sums:

- $\sum X_1 = 165$
 $\sum X_2 = 0$
 $\sum X_3 = 1155$
 $\sum X_4 = 20$
 $\sum X_5 = 0$

Crude sums of squares:

$$\begin{aligned} \sum x_1^2 &= (1)^2 + (2)^2 \dots\dots + (10)^2 \quad \times 3 = 1155 \\ \sum x_2^2 &= (1)^2 + (0)^2 + (1)^2 \quad \times 10 = 20 \\ \sum x_3^2 &= (1)^2 + (4)^2 \dots\dots + (100)^2 \quad \times 3 = 75999 \\ \sum x_4^2 &= (1)^2 + (0)^2 + (1)^2 \quad \times 10 = 20 \\ \sum x_5^2 &= (-1)^2 + (-2)^2 \dots\dots + (-10)^2 + (1)^2 + (2)^2 \dots\dots + (10)^2 \\ &= 770 \end{aligned}$$

Correction factor:

$$\frac{\text{Sum } x \text{ Total yield}}{30}$$

Corrected sums of squares:

$$\begin{aligned} \text{Balance: } X_1 &= 1155 - 907.5 = 247.5 \\ X_2 &= 20 - 0 = 20 \\ X_3 &= 75999 - 44467.5 = 31531.5 \\ X_4 &= 20 - 13.3333 = 6.6667 \\ X_5 &= 770 - 0 = 770 \end{aligned}$$

Sums of products:

$$\begin{aligned} \sum x_1 x_2 &= 0. & \sum x_2 x_3 &= 0. & \sum x_3 x_4 &= 770 \\ \sum x_1 x_3 &= 9075 & \sum x_2 x_4 &= 0. & \sum x_3 x_5 &= 0 \\ \sum x_1 x_4 &= 110 & \sum x_2 x_5 &= 110. & \sum x_4 x_5 &= 0 \\ \sum x_1 x_5 &= 0. & & & & \end{aligned}$$

Correction factor sums of products:

$$\frac{\text{Sum of 1st factor } \times \text{ Sum of 2nd factor}}{30}$$

Corrected sums of products:

$$\begin{aligned} \text{Balance: } X_1 X_2 &= 0 \\ X_1 X_3 &= 9075 - 6352.5 = 2722.5 \\ X_1 X_4 &= 110 - 110 = 0 \\ X_1 X_5 &= 0 \\ X_2 X_3 &= 0 \end{aligned}$$

XX

$$\begin{aligned}
 X_2 X_4 &= 0 \\
 X_2 X_5 &= 110 - 0 = 110 \\
 X_3 X_4 &= 770 - 770 = 0 \\
 X_3 X_5 &= 0 \\
 X_4 X_5 &= 0
 \end{aligned}$$

Matrix of corrected sums of squares and products.

	X_1	X_2	X_3	X_4	X_5
X_1	247.5	0	2722.5	0	0
X_2	0	20	0	0	110
X_3	2722.5	0	31531.5	0	0
X_4	0	0	0	6.6667	0
X_5	0	110	0	0	770

Coded matrix.

a_{11}	a_{12}	a_{13}	a_{14}	a_{15}
a_{21}	a_{22}	a_{23}	a_{24}	a_{25}
a_{31}	a_{32}	a_{33}	a_{34}	a_{35}
a_{41}	a_{42}	a_{43}	a_{44}	a_{45}
a_{51}	a_{52}	a_{53}	a_{54}	a_{55}

COMPUTATION OF INVERSE MATRIX.

Result of Forward Solution.

	<u>1.</u>	<u>2.</u>	<u>3.</u>	<u>4.</u>	<u>5.</u>
A_{1j}	247.5	0	2722.5	0	0
B_{1j}	1	0	11.000	0	0
A_{2j}		20	0	0	110
B_{2j}		1	0	0	5.5
A_{3j}			1584.0	0	0
B_{3j}			1	0	0
A_{4j}				6.6667	0
B_{4j}				1	0
A_{5j}					165
B_{5j}					1

Computation of forward solution.

j = indicates position in matrix 1 - 5

A_{1j} write down first row of matrix

B_{1j} divide each of these figures by a_{11}

A_{2j} is computed from the formula:

$$A_{2j} = a_{2j} - (a_{12} B_{1j}) \quad j = 2, \dots, 5.$$

$$B_{2j} = \frac{A_{2j}}{A_{22}}$$

A_{3j} is computed from the formula:

$$A_{3j} = a_{3j} - (A_{13} B_{1j} + A_{23} B_{2j}) \quad j = 3, \dots, 5.$$

$$B_{3j} = \frac{A_{3j}}{A_{33}}$$

A_{4j} is computed from the formula:

$$A_{4j} = a_{4j} - (A_{14} B_{1j} + A_{24} B_{2j} + A_{34} B_{3j}) \quad j = 4, 5.$$

$$B_{4j} = \frac{A_{4j}}{A_{44}}$$

A_{5j} is computed from the formula:

$$A_{5j} = a_{5j} - (A_{15} B_{1j} + A_{25} B_{2j} + A_{35} B_{3j} + A_{45} B_{4j}) \quad j = 5.$$

$$B_{5j} = \frac{A_{5j}}{A_{55}}$$

Result of backward solution (inverse matrix)

	C_1	C_2	C_3	C_4	C_5
C_1	0.08042891	0	-0.00694441	0	0
C_2	0	0.23333348	0	0	-0.03333336
C_3	-0.00694441	0	0.00063131	0	0
C_4	0	0	0	0.14999925	0
C_5	0	-0.03333336	0	0	0.0060601

Inverse matrix (Code)

$$\begin{pmatrix} C_{11} & C_{12} & C_{13} & C_{14} & C_{15} \\ C_{21} & C_{22} & C_{23} & C_{24} & C_{25} \\ C_{31} & C_{32} & C_{33} & C_{34} & C_{35} \\ C_{41} & C_{42} & C_{43} & C_{44} & C_{45} \\ C_{51} & C_{52} & C_{53} & C_{54} & C_{55} \end{pmatrix}$$

COMPUTATION OF BACKWARD SOLUTION.

$$C_{55} = \frac{1}{A_{55}}$$

$$C_{45} = -C_{55} B_{45}$$

$$C_{35} = -C_{45} B_{34} - C_{55} B_{35}$$

$$C_{25} = -C_{35} B_{23} - C_{45} B_{24} - C_{55} B_{25}$$

$$C_{15} = -C_{25} B_{12} - C_{35} B_{13} - C_{45} B_{14} - C_{55} B_{15}$$

$$C_{54} = C_{45}$$

$$C_{44} = \frac{1}{A_{44}} - C_{45} B_{45}$$

$$C_{34} = -C_{44} B_{34} - C_{45} B_{35}$$

$$C_{24} = -C_{34} B_{23} - C_{44} B_{24} - C_{45} B_{25}$$

$$C_{14} = -C_{24} B_{12} - C_{34} B_{13} - C_{44} B_{14} - C_{45} B_{15}$$

$$C_{53} = C_{35}$$

$$C_{43} = C_{34}$$

$$C_{33} = \frac{1}{A_{33}} - C_{34} B_{34} - C_{35} B_{35}$$

$$C_{23} = -C_{33} B_{23} - C_{34} B_{24} - C_{35} B_{25}$$

$$C_{13} = -C_{23} B_{12} - C_{33} B_{13} - C_{34} B_{14} - C_{35} B_{15}$$

$$C_{52} = C_{25}$$

$$C_{42} = C_{24}$$

$$C_{32} = C_{23}$$

$$C_{22} = \frac{1}{A_{22}} - C_{23} B_{23} - C_{24} B_{24} - C_{25} B_{25}$$

$$C_{12} = -C_{22} B_{12} - C_{23} B_{13} - C_{24} B_{14} - C_{25} B_{15}$$

$$\begin{aligned}
 C_{51} &= C_{15} \\
 C_{41} &= C_{14} \\
 C_{31} &= C_{13} \\
 C_{21} &= C_{12} \\
 C_{11} &= \frac{1}{A_{11}} - C_{12} B_{12} - C_{13} B_{13} - C_{14} B_{14} - C_{15} B_{15}
 \end{aligned}$$

COMPUTATION OF REGRESSION COEFFICIENTS.

$$\begin{aligned}
 \sum X_1 Y &= 606.70865 \\
 \sum X_2 Y &= 3.94660 \\
 \sum X_3 Y &= 4200.36089 \\
 \sum X_4 Y &= 73.25106 \\
 \sum X_5 Y &= -5.25996
 \end{aligned}$$

Correction factor for yield.

$$\frac{\text{Sum of factor } \times \text{ Total yield}}{30}$$

Corrected sums of products (P_i)

$$\begin{aligned}
 \text{Yield } \times \text{ Balance: } P_1 &= 606.70865 - 595.84844 = + 10.86021 \\
 P_2 &= 3.94660 - 0 = + 3.94660 \\
 P_3 &= 4200.36089 - 4170.93908 = + 29.42181 \\
 P_4 &= 73.25106 - 72.22405 = + 1.02701 \\
 P_5 &= -5.25996 - 0 = - 5.25996
 \end{aligned}$$

Regression coefficients ($B_i = \sum_j P_j C_{ij}$)

$$\begin{aligned}
 B_1 &= P_1 C_{11} + P_3 C_{13} = + 0.66916 \\
 B_2 &= P_2 C_{22} + P_5 C_{25} = + 0.88664 \\
 B_3 &= P_1 C_{31} + P_3 C_{33} = - 0.05684 \\
 B_4 &= P_4 C_{44} = + 0.15405 \\
 B_5 &= P_2 C_{52} + P_5 C_{55} = - 0.16343
 \end{aligned}$$

$$P_i \begin{pmatrix} + 10.86021 \\ + 3.94660 \\ + 29.42181 \\ + 1.02701 \\ - 5.25996 \end{pmatrix} \qquad B_i \begin{pmatrix} + 0.66916 \\ + 0.88664 \\ - 0.05684 \\ + 0.15405 \\ - 0.16343 \end{pmatrix}$$

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Using different values of Y, substitute in equations (i), (ii) and (iii) and then solve for X according to:

$$X = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

This gives values for X_1 .

Plot values for X_1 (balance effect) against values for X_2 (concentration effect).

These results are given in Graph 23.

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