THE COLONISATION OF NON-STERILE WHEAT STRAW BY FUNGI

BY

C O L I N M c S H A N E B.Sc. (Hons.)

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SUMMARY

A survey of the national production of surplus straw and the measures taken to reduce this surplus is given. This is followed by a review of the current knowledge of the genus Coprinus and the species Coprinus cinereus, a Basidiomycete. The temperature and pH relations of three strains of the fungus in pure culture are outlined and from this, values for temperature and pH are suggested to give optimal growth as measured by several methods. Also in pure culture, the preferences for nitrogen sources and carbon/nitrogen ratios are also studied.

Having studied the growth characteristics under sterile conditions, the suggested optima were incorporated into a non-sterile, wheat straw substrate. One of the strains was selected and its growth and colonisation on this straw substrate charted. Various aspects of such growth are then described.

ACKNOWLEDGEMENTS

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C H A P T E R 1

Introduction

Introduction

1.1 Surplus straw - The nature and size of the problem

Due to the present high saleable value of cereals throughout the world, there has been a substantial increase in cereal production. It is widely held that this trend must continue, at least within the next few decades, in an attempt to narrow the ever increasing gap between world food production and consumption. Ohlin (1967) and Lowry (1970) express this Malthusian concept in more contemporary terms.

On a more regional basis, in England and Wales there is at present a larger acreage of land under arable crops than at any other time since 1872, (N.F.U. Working Party on Straw Disposal, 1973). The Industrial Revolution was undoubtedly the cause of the fall in agricultural practice subsequent to 1872, and the industrialisation of many more countries throughout the world must have consequently lowered the potential world food production. Less people producing food naturally results in more people needing to purchase food which, in turn, makes the formation of an emergency stock-pile of food all the more difficult. Improved farming techniques and machinery are attempting to allieviate this fluctuating supply, and the consistently high demand situation for food, a situation which is directly responsible for the increased saleable value of cereals.

England and Wales has, at present, 14 million acres of arable land under cultivation, approximately 9 million acres of which is used to grow cereals; chiefly barley, wheat and oats. Although this results in a large amount of cereal grain production, the fact that only the distal portion of a cereal shoot is of real commercial value, means that there is consequently a large amount of straw left after harvesting.

Figure 1.1 shows the distribution of this straw production throughout England and Wales and it is obvious from this figure that there is a much greater production of straw in the Eastern part of the country (approx. 70%) than in the West. Livestock production is largely centred in the West of the country and is therefore spatially separated from a large supply of straw which has traditionally been used chiefly for animal bedding. The amount of straw used for bedding and food is only slightly more than the amount produced in the West. It is a point of conjecture as to whether more straw would be used if it were available but the point seems academic since high transport costs of a bulky, low cost commodity would make movement of straw uneconomical. Thus, the situation arises whereby England and Wales annually produce 3.57 million tons of straw which is defined as surplus to requirements (A.D.A.S. Review No. 11, 1973, N.F.U. Working Party on Straw Disposal, 1973).

Figure 1.1 Estimated yields of straw (England and Wales) in thousand tons based on 1972 acreages. (A.D.A.S. Review No. 11, 1973, N.F.U. Working Party on Straw Disposal, 1973).

	ACREAGES OF:-			TOTAL STRAW YIELD	% OF NATIONAL TOTAL
	WHEAT	BARLEY	OATS plus mixed corn		
Eastern region	1,415.7	1,235	112.5	2,763.2	29.7
South Eastern Region	452.8	683.7	98.4	1,235	13.3
East Midland Region	719.4	788.4	110.3	1,618.1	17.4
West Midland Region	280.6	432.2	125.3	838.1	9.0
South Western Region	324.9	636.3	168.8	1,130.1	12.1
Northern Region	101.8	459.1	59.3	620.2	6.7
Yorks & Lancs. Region	277.8	565.1	60.4	903.3	9.7
England	3,573.0	4,799.9	735.1	9,108.0	97.9
Wales	18.1	108.6	71.4	198.1	2.1
England and Wales	3,591.1	4,908.5	806.5	9,306.1	100

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1.2 Uses of Straw

The two major uses of straw have already been mentioned; as an animal bedding material, straw attains a further use when used as a traditional manure after becoming soaked in animal excrement. None of the cereal straws are of any particular nutritive value to farm animals if used alone (see later table for analyses) and therefore the straw is used largely as a fibrous filler, along with various concentrates. Because of this low nutritive value it would appear that there is little hope that the 15.0% of straw already being used in this way, will increase and thereby reduce the surplus. This aspect will be further discussed later.

The ploughing-in of straw is one way in which it is hoped that more surplus straw could be disposed of. This idea, however, has received mixed reactions from farmers. Although not necessary, chopping machines to reduce the straw to short lengths, do make the process much more practical, but the high initial cost of plant as well as increasing running costs make the scheme economically unattractive. Persuading farmers to employ this method is made even more difficult since many recent experiments have failed to show any substantial benefit to the soil of this practice.

Although straw is reasonably cheap, its uses outside agriculture are, at present, of only minor importance. Any increase in non-agricultural uses is hampered on seve-

ral counts; it is bulky, limited to one season (or requires carefully controlled storage), and is variable in quality. The same situation prevails throughout Europe where only two non-agricultural uses of straw are of any consequence.

Firstly straw has been used for many years as a packing material; but the chances of this area expanding, especially in view of the many other suitable, cheap and abudant, man-made materials, is low.

Secondly, straw is being used to produce low-grade building boards by a British firm. Although no figures are available the amount of straw used is increasing but it is highly unlikely that any massive increase in this area will occur.

Various experimental uses of straw outside agriculture are also being considered. The three most important of these are as a brick filler, as a tobacco substitute and as a wood substitute in paper making. Little definite hope can be extended to any of these since, as yet, they are still in the relatively early stages of development.

Thus it is generally considered that, at present, there seems to be no use for straw which could drastically reduce the 36.6% of total production which is being disposed of by burning. Indeed, until quite recently, little thought was even given to alternatives to burning. The practice has gone on for centuries all over the world, since it is a cheap, quick and relatively low-labour process. However,

concern has recently been directed towards this practice from two different areas.

Many environmentalists, ecologists and members of the general public have begun to question the right of farmers to destroy much of the soil and hedgerow flora and fauna. Although definite correlations between straw burning and the depletion of organisms has not yet been proved, farmers are generally not concerned unless there is a threat to their crops as a consequence of a species' mortality. Burning has even been shown to be beneficial in some areas since it helps to keep down crop diseases. Much public interest in burning was aroused in 1973 when a heavy pall of smoke fell on the M1 motorway causing several fatal accidents. This latter point raises the question of the position of straw burning in the law. There are four areas of legislation which may be invoked in suitable cases.

(i) The Clean Air Act 1956

The intention of this act is to provide powers to deal with persistent pollution in urban areas and has no provision for damage to trees, hedges or wildlife and is therefore of limited relevance to straw burning.

(ii) The Highways Act 1959

This act makes the lighting of a fire within fifty feet of the centre of a highway illegal. Again this is of limited relevance, especially since the penalty is a maximum of £20.00 for first offenders and £50.00 for subsequent offences.

(iii) Tree Preservation Orders

These are made by local planning authorities to prevent wilful damage to trees and hedgerows.

(iv) Local Government Act 1933

After 1st April 1974, County, Borough and District councils have the authority to make byelaws, "for the good rule and government of the whole part of the county, borough or district, as the case may be and for the prevention and supression of nuisance therein". This is sufficient provision to prevent straw burning by law, but some difficulty has been experienced in the submission of proposals which are deemed by the Home Office to be workable.

In addition to these laws which may be used to prevent straw burning, the National Union of Farmers have themselves drawn up a new code of practice for straw burning in an attempt to make the farmer more aware of the influence of the practice on the environment.

The objectors to burning have illustrated its shortcomings, but they have not produced any satisfactory alternatives to the problem. Interest in this area has been shown however and this will be discussed in section 1.4 but firstly it is proposed to evaluate straw in terms of its chemical composition.

1.3 Analyses of Straws

The term 'straw' as used in the previous text has been used in its broadest sense, but a more detailed investigation into the chemical composition of straw requires a discrimination into the three major types.

Figure 1.2

Crude Analyses of Various Straws

FRACTION	% COMPOSITION		
	WHEAT	BARLEY	OAT
Dry Matter	86.0	86.0	86.0
Crude Protein	2.1	3.3	1.9
Oil (as ether extract)	1.3	1.8	1.5
Crude fibre	36.6	33.9	34.6
Ash	5.3	4.6	4.9
Carbohydrate (as N-free extract)	40.7	42.4	43.1

(After Kent-Jones and Amos, 1967)

Barley straw is produced in slightly higher amounts than wheat straw which, in turn is produced in an amount approximately five times that of oats. The straw used experimentally for the following report was wheat straw which was used as a function of its availability.

The burning of straw by definition releases, and to all intents and purposes, wastes a large amount of energy. Recent trials in the use of straw as a source of energy have been given more than the usual amount of publicity due to this large energy release upon ignition. However, large amounts of ash, together with its bulkiness are large deterrents to the potential of straw as a fuel.

Much of the nitrogen in the straw may be returned in the ash-residue after burning, but it is felt by several workers that this nitrogen may be better utilized into animal feedstuffs where its conversion into organic material may be more efficient.

1.4 Current Work on Straw Utilization

As has already been stated, there are relatively large amounts of utilizable chemicals within straw which are effectively lost by burning each year. However, recent rapid rises in the cost of animal feeds has aroused much interest, in several circles, in the possibilities of using these previously wasted chemicals in straw, to supplement these high-cost feeds. There are, however, two major problems with straw as a feed; firstly it is itself relatively low in nitrogen as well as being poorly digested by ruminants. It has been with regard to improving the second of these two shortcomings that recent research has been directed.

Several authorities, including BOC Silcock (1975) as well as the Rowett Research Institute, Aberdeen, (1972) have been engaged in a study of the use of a dilute solution of sodium hydroxide to increase digestibility of straw and grasses to sheep. The process has been used traditionally and it does increase the acceptability to the animals. However, large volumes of sodium hydroxide and water are required to produce relatively small batches of product on a farmyard scale. Research is being continued to assess the feasibility of the process, both economically, technically and scientifically, on a large scale.

R.D. Hartley and N.J. King working (1974) at the Grassland Research Institute, Maidenhead and Princes Risborough Laboratories respectively, have been investigating physical,

chemical and mycological treatments of various sawdusts and barley grain, again to increase their digestibilities. The sodium hydroxide methods have been assessed and although work is still progressing along these lines, they suggest that one fungus in particular, Fomes lividus, shows promise in its ability to convert wood waste and straw to useful animal feed components.

As well as the previously mentioned work on sodium hydroxide treatment, the Rowett Research Institute is also investigating the use of fungi, particularly Aspergillus oryzae, to increase the protein content of barley grain. However, the suggested process is carried out under sterile conditions which inevitably requires expensive plant but nevertheless this research may provide some relevant information to help solve the straw problem.

1.5 The Need for a New Approach

The limitations of the sodium hydroxide treatment have already been discussed. The major disadvantage with any of the fungal treatments so far suggested have also been mentioned; i.e. sterile conditions are required thereby making a high initial cost for plant necessary, as well as increasing costs for energy inevitable. This would almost certainly result in a centralized industry to produce this product which would obviously require transport of straw from farm to plant and transport of product back. The economics of the situation are clearly undesirable. Yet another factor to take into consideration is that such is the process that there would essentially be a waste product at the end of it. Thus one is using a waste product but simultaneously creating a different one. The fact that the majority of the waste is liquid may make it easier to dispose of than bulky straw, but the fact remains that it is produced.

With these points in mind it was decided to investigate the possibilities of a low-cost, low-technology, non-sterile, farmyard process for the 'upgrading' of straw using a particular fungus. This upgrading may take the form of an increase in the nutritive value, or the digestibility or, as it was hoped, both factors of the straw.

Very preliminary experiments at the Biodeterioration Information Centre, Aston University, had indicated that a Basidiomycete, Coprinus cinereus was capable of growing

well on non-sterile wheat straw to which had been added a small amount of nitrogen and a dilute solution of various salts.

This was, of course, merely the germinal stage of any form of definitive process but it did provide a starting point for a wide variety of research. There were several possible pitfalls which could be forseen and it was into these areas to which the initial research was directed. Firstly a moist, non-sterile fermentation would seem at first sight to be insurmountably difficult to be kept free of infection. Secondly would the protein content and hence the food value of the straw be increased? The palatability and toxicity of the product also had to be tested and although the facilities for these tests were non-existent in the laboratories, the Ministry of Agriculture, Fisheries and Food were able to assess some products on these two counts. One other, extremely important, yet often omitted aspect of the research was the question of acceptability of the product and process to the farmer. Although any product was not intended for human consumption, the process did involve micro-organisms, which are still viewed with scepticism by the public, farmers and some scientists alike. It was felt, however, that one method of dispelling at least some of these doubts was by making it clear that a consistent product could be produced. This, in essence, involved one possessing the ability to control the organism throughout the process as one would with a chemical process. The brewing industry has reached such a sophisticated level of technology that the organism as well as the process can now

be controlled to within extremely narrow limits. Such is the consistency of the brewing industries products that the fact that micro-organisms are used is largely forgotten, or at least does not deter, the consumer.

To attain such a level of control over an organism it is obviously necessary to carry out extensive research into the organism itself and in particular, its physiological behaviour. To study and monitor as many of the physiological patterns in the behaviour of Coprinus cinereus, both in pure culture and in non-sterile conditions, this formed the basic philosophy behind the work described in this thesis.

1.6 The Organism - Coprinus cinereus

To say that there is much confusion over the correct nomenclature of the genus Coprinus would be an understatement, as a survey of Pinto-Lopes (1971) would show. Briefly it states that C. cinereus in particular has been given many synonyms, the four major ones being:- Coprinus fimetarius by Buller (1931), Coprinus lagopus by Anderson (1959), Casselton and Lewis (1967), Cowan and Lewis (1966), Day and Anderson (1961) and Day and Roberts (1968), Coprinus stercorarius by Kniep (1920) and Mounce (1921) and Coprinus macrorhizus by Buller (1924) and Dickson (1936). To avoid confusion, the species used for this work was Coprinus cinereus (Schaeff. ex Fr.) S.F. Gray sensu Konr. which is suggested as the unequivocal authority for the species.

Although the work to be carried out on C. cinereus within this study is essentially for application to a practical problem, such is the lack of information on the physiology of the organism that the results will be useful in their own right, especially with regard to possibly elucidating the ecological significance of the organism. Indeed, the majority of references to the genus Coprinus as a whole, can be found in ecological research works where the appearance of fruiting bodies seems to be the major point of interest. Even here the inclusion of most of the references to Coprinus seem to be as an afterthought. However, it is not surprising that this preoccupation with sporophore production pervades the study of most Agarics since these are of paramount importance in identification,

as well as being their only economically important product.

Webster (1970) in a presidential address to the British Mycological Society, presented a paper entitled, "Coprophilous Fungi", but, here again the genus Coprinus was only briefly mentioned. Most of Websters' comments however were taken from what must be regarded as the only standard reference works on the physiology of Coprinus; those published by Lisbeth Fries in 1955 and 1956. In all, twenty-six different strains, representing at least nineteen species of Coprinus were examined, but C. cinereus is not included. A precis of the current knowledge of the physiology of Coprinus and C. cinereus is given in the introductory sections of each chapter.

One aspect which is not dealt with later however, but which is of importance, is that C. cinereus, in common with most fungi, possesses strain variation. An illustration of this phenomenon is given by Fries (1955), where two strains of C. fimetarius produced dry weights of 52.9mg and 8.1mg respectively, under exactly the same physiological conditions using urea as a nitrogen source. Since such large discrepencies were possible, it was deemed necessary to use several different strains of C. cinereus to avoid obtaining exceptional results.

Initially, strain difference was based purely on the geographical position at which the strains were isolated. It was soon evident from the varying degrees of difference

in experimental results that this geographical separation was, in fact, reflecting a real strain variation on a physiological basis.

Three strains of C. cinereus were used, and each one is referred to in the text by the following names:-

1) Aston Strain

This was isolated from wheat straw in early 1973 at the Biodeterioration Information Centre. The cultures were kept on malt extract agar (Oxoid) and for one month prior to the commencement of experimental work they were kept on malt but under sterile paraffin oil.

2) Aberystwyth Strain

This was first isolated by Hedger from wheat straw compost at Cambridge in 1970 but is now being stored at University College Wales, Aberystwyth. The cultures were originally kept on yeast peptone - soluble starch agar (YPSS Oxoid) but were given the same treatment as the Aston strain for the final month in culture.

3) Java Strain

Again, this strain was isolated by Hedger, but from paddy straw compost in Java during September 1973. Its cultural history up to the commencement of experimental work was identical to the Aberystwyth strain.

It was hoped that by using these various cultural histories and strain variations, a reasonably wide spectrum of physiological activities would result from which significant average figures could be drawn.

1.7 Aims of Research

These can be summarized as follows:-

1) To study the growth of *Coprinus cinereus* under various physiological conditions in pure culture.
2) To complement the current knowledge on the physiology of the genus *Coprinus* as a whole and *C. cinereus* in particular.
3) To study the growth of *C. cinereus* under non-sterile conditions on a straw based substrate.
4) To study the feasibility of applying results from sterile culture work to non-sterile methods.
5) Provide some of the necessary knowledge required to control the growth of *C. cinereus*.
6) To provide some of the ground work for possible future production of a novel animal foodstuff.

C H A P T E R 2

The effect of temperature on the growth of *Coprinus cinereus*

2.1 Introduction

Of all the environmental conditions studied by ecologists with interests in any section of biology, the temperature is usually the easiest and hence the first to be recorded. Ingraham (1962) suggests that temperature is undoubtedly the most important environmental variable affecting the growth of bacteria.

Although the above statement may not be strictly true for bacteria, fungi or any other living organism, few biologists would doubt that temperature does play a cardinal role in the vital metabolic processes of all living things; this effect being usually mediated through the enzymal, hormonal and other chemical control systems within an organism. Fungi can be regarded as being physiologically, relatively simple organisms and as such are quite sensitive to any change in their environment. Since the temperature of most natural environments can fluctuate quite drastically both diurnally and seasonally, it follows that temperature can be regarded as extremely important in fungal growth studies.

The term "growth" also has a somewhat special meaning with respect to fungi since the unit of growth is entirely dependent upon the method used in its study (Cochrane, 1958). Probably the two most widely used methods of measuring growth are:

(i) By dry weight

The organism to be studied is grown either on the surface of, or forms submerged pellets in, a liquid nutrient medium. The form of vegetation depending

upon the treatment of the growth containers. At specified time intervals the mycelium or pellets are filtered off, washed, dried to constant weight, then weighed.

(ii) Linear expansion on semi-solid media

This is the least laborious method and because of this many replicates can be prepared giving more significant results. The movement of nutrients and metabolic products through the agar media used can cause drawbacks in using this method in nutritional studies as outlined by Chaudhuri (1923), Fries (1956) and Lilly and Barnett (1951). For temperature studies, however, this method is generally regarded as being satisfactory, (Chaudhuri, 1923; Domsch, 1955).

The method is particularly suited to the theme of this study as a whole, since surface spread of mycelium over agar should be quite closely correlated to the speed of colonisation of the materials to be studied since they are all relatively loosely packed, as well as having a continuous surface coating of nutrient salts solution. The physical conditions of growth may thus quite closely resemble those on agar.

The actual speed of colonisation, as opposed to gain in mycelial dry weight of the fungus becomes even more important when one considers non-sterile conditions which, it is hoped, will eventually be used. It is imperative that the fungus should establish itself throughout the

substrate quickly, to prevent contaminating organisms establishing themselves. Once this has been successfully achieved, the organism can then begin to degrade the substrate without interference.

With regard to this ability to colonise large areas of substrate quickly and thus resist competition for nutrients, Basidiomycetes in general and the genus Coprinus in particular seem to illustrate these abilities very well. The Basidiomycetacae in general appear to be able to produce an extensive exploratory mycelium by producing thick strands of hyphae along which nutrients are able to move, from an already colonised nutrient source (Buller, 1971; Milne, 1969). This therefore allows the parent colony to carry out an extensive "search" of the surrounding substratum for fresh nutrient supplies.

Webster (1970) provides the following table of maximum temperatures for growth of some Coprinus species:-

Coprinus ephemerus	30-35	Fries 1956
C. friesii	35-40	" "
C. hiascens	35-40	" "
C. curtus	44	" "
C. tardus	40	" "
C. fimetarius	44+	Fries 1956

and he also mentions that the optimum temperature for Coprinus cinereus is about 35^{o}C. No reference could be found as to the exact optimal temperature for this species, therefore it was considered useful to ascertain this on its own merits and for further practical usage. Cochrane (1963),

however, would doubt the validity of any one figure for any one species since the optimum temperature is affected by conditions of time, medium used, nutrients, physical conditions and methods of measurement. For this work however, the time scale is stated, the medium used is standard and easily available, the method of measurement is simple allowing many replications, and with regard to physical conditions, the H^+ ion concentration was varied to observe what effect this had. Bearing these points in mind it was decided that the concept of an optimum temperature for growth as measured by radial spread would be useful in providing one of the basic growth parameters for use in further practical work.

2.2 Materials and Methods

2.2.1 Organisms

All three strains of Coprinus cinereus, as described in chapter 1 were used. They were grown up on malt extract agar, pH 5.3, at 25°C for five days before being used as inocula.

2.2.2 Materials

Oxoid Malt Extract Agar, was used in all the following experiments. pH adjustment was by addition of 0.1N Sodium hydroxide and pH measurement carried out on a Pye temperature compensated pH meter.

2.2.3 Methods

The malt extract agar was prepared according to the manufacturers instructions, a range in pH being achieved by the addition of varying amounts of 0.1N NaOH. This was added prior to autoclaving, but it was found necessary to retain random samples of agar after autoclaving to retest the pH. As reported by Allsopp (1973) there was found to be a drop in pH during autoclaving of between 0.1 - 0.4 pH units. Figure 2.1 shows the range of pH both before and after autoclaving:-

Figure 2.1

To show the effects of adding various amounts of 0.1N NaOH to malt extract agar, prior to autoclaving.

Amount of NaOH added to agar (mls.)	pH of agar before autoclaving	pH of agar after autoclaving
0	5.7	5.3
1.0	6.0	5.8
2.3	6.5	6.4
5.0	7.2	7.0
9.1	8.1	7.7

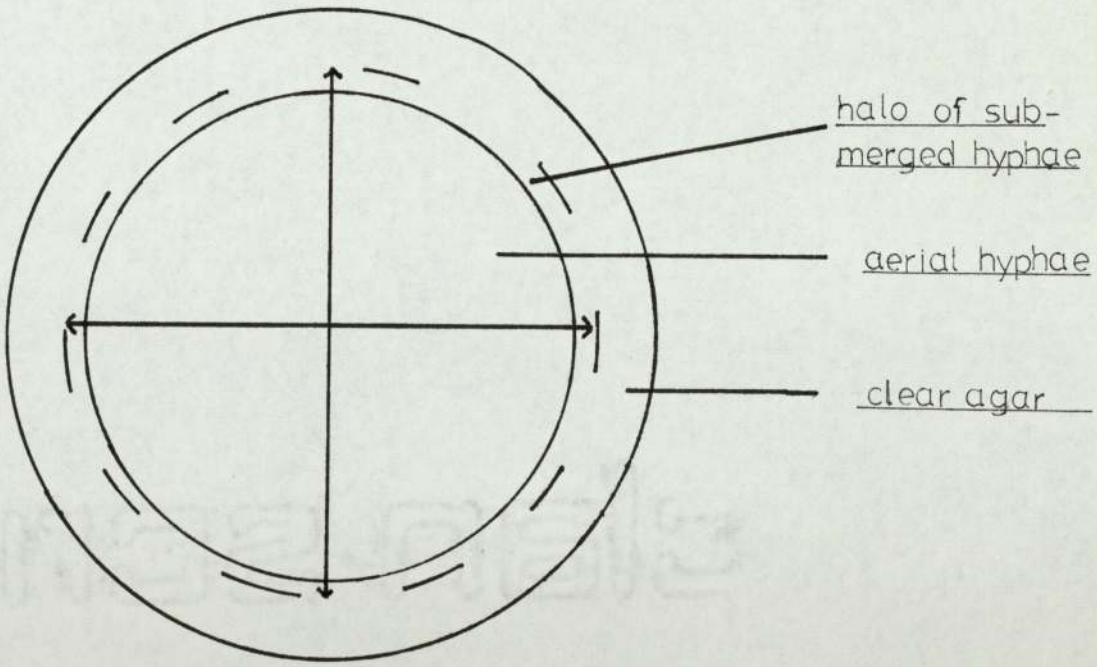
It was found impractical to use any higher pH since however much NaOH was added above an initial pH of about 8.4, this figure always dropped to pH 8.0 during autoclaving.

Cartwright and Findlay (1934) in their work with wood destroying Basidiomycetes provided figures showing that the depth of the agar used (also malt extract) did not significantly affect the growth of their species. Brancarto and Golding (1953), Brown (1923) and Chaudhuri (1923) suggest that the depth of medium is important, but chiefly where growth is measured by dry weight rather than radial expansion. It was therefore decided that no great detail would be paid to exacting the depth of agar in each plate and an amount [approximately to 20mls] was added to each one.

A 6mm cork borer was used to prepare inocula from the edges of the five day old stock cultures previously mentioned. A single plug of inoculum was transferred aseptically to the centre of each petri-plate, with the aerial mycelium resting on the surface of the sterile agar.

A range of six temperatures was used (see results) and for each temperature therewere five different pH's and three fungal strains. Each variable was provided with ten replicates.

At periods of 75, 100 and 125 hours the plates were examined and the colony diameters measured. This was done using a transparent rule placed on the underside of the petri-plate, thus enabling the "halo" of submerged hyphae around the edge of the aerial colony to be taken into account. All three strains investigated formed very regular, circular, smooth colonies on the agar and hence it was thought necessary to take measurement along only two diameters opposed at 90^{o} :-



The average figure for the two diameters was used in the results.

After the 125 hour period, the mycelium was scraped off the surface of the agar and for each group of variables the agar was "pooled" and gently re-melted in a water bath. The pH was then re-measured and in all cases was found to be 7.1 ± 0.4. Although this method is only useful as a broad guide, it has been used previously (Allsopp, 1973) and was found to be useful for future work.

2.3 Results

These are shown by figures 2.2 to 2.10. Cartwright and Findlay (1934) suggest that absolute growth figures are of only limited use but that comparison between strains or species are of much greater value. For this reason each figure depicts strain variation between one or more variables.

Figures2.2 to 2.6 show the strain variation in colony diameter over the time course of the experiment, each temperature being taken separately. There seems to be very little strain variation as shown by any of the histograms and although statistical analyses have not been carried out, it seems doubtful that the small differences would prove to be significant in practice. There are, however, two important trends appearing in all three strains:

a) The linear expansion for each time period increased with an increase in temperature up to 37^{o}C. Above this temperature the trend is reversed, less growth occurring for each time period. At 55^{o}C no growth was apparent in any of the plates. As an example of these variations with temperature figure 2.12 has been compiled from the results of the Java strain at pH 6.4 after 75 hours:

Figure 2.12

Colony diameters of the Java strain at pH 6.4 after 75 hours at various temperatures.

Temperature ^{o}C	Colony Diameter (mm)
21	25
27	33
33	72
37	79
41	51
55	0

b) An increase in the pH of the medium substantially increased the linear expansion of the colony in an almost linear fashion. To give an example of this, figure 2.13 has been compiled again from the Java strain at $27^{o}C$ after 75 hours growth:

Figure 2.13

Colony diameters of the Java strain at $27^{o}C$ after 75 hours at various pH levels.

pH of medium	Colony Diameter (mm)
5.3	30
5.8	31
6.4	34
7.0	39
7.7	44

Unfortunately for reasons mentioned this trend could not be followed to its conclusion.

Figures 2.7 to 2.9 express a condensed version of the histograms in graphical form to give a much more easily

Explanatory Notes for figures 2.2 to 2.6

The following histograms show the growth of the three strains of C. cinereus at different temperatures on malt agar of varying pH.

The pH of the media are shown on the horizontal axis, whilst the growth (diameter of colony in mm.) is shown on the vertical axis.

Within each pH group there are three bars, the first representing the ASTON strain, the second the JAVA and the third the ABERYSTWYTH .

The clear areas of the bars show the extent of growth at seventy-five hours, the black areas the extent of growth at 100 hours and the lightly shaded areas, the extent of growth at 125 hours.

Figure 2.2 Colony diameters of the three strains of C. cinereus at various pH levels at 21°C

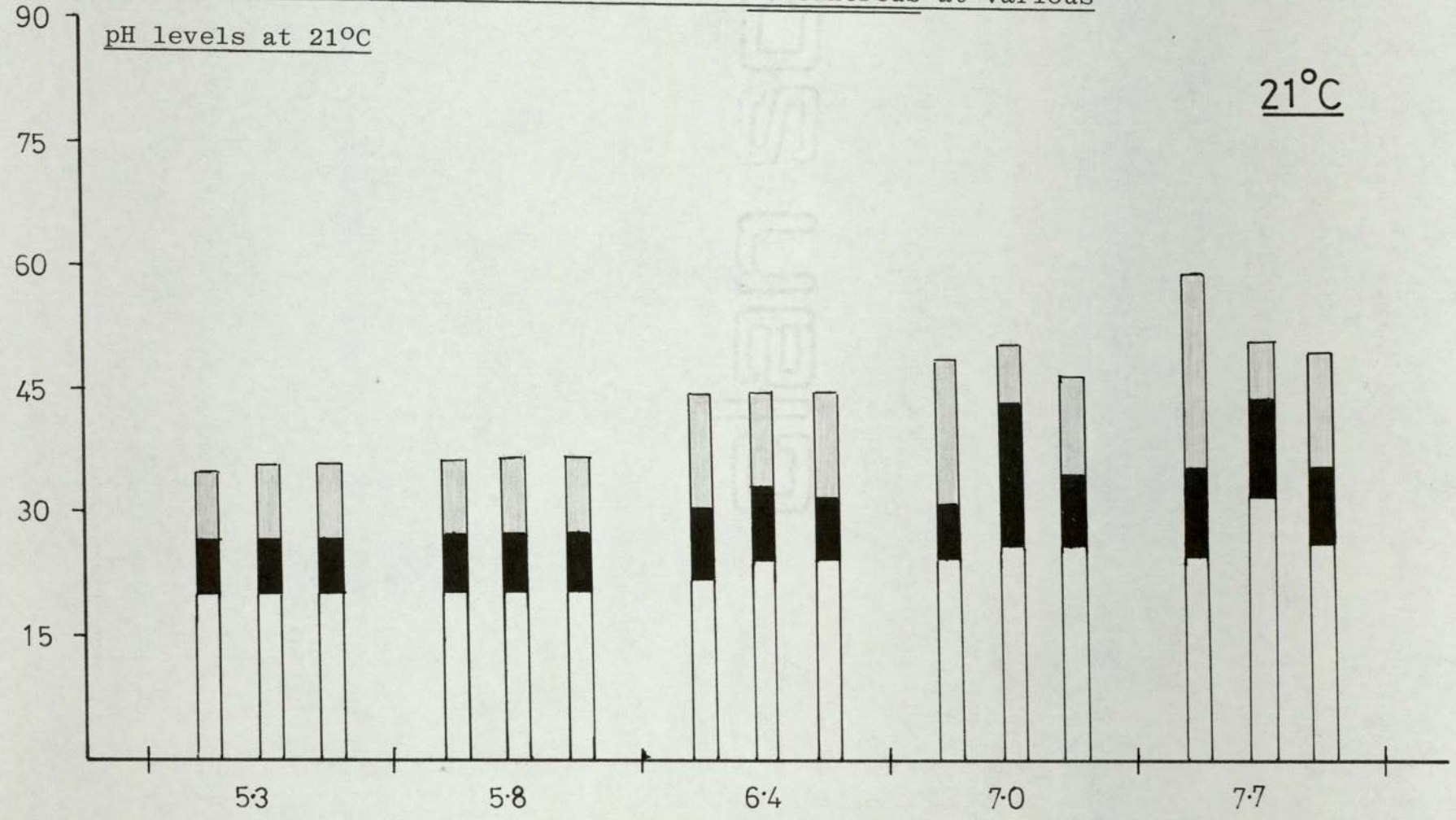


Figure 2.3 Colony diameters of the three strains of C. cinereus at various pH levels at 27°C

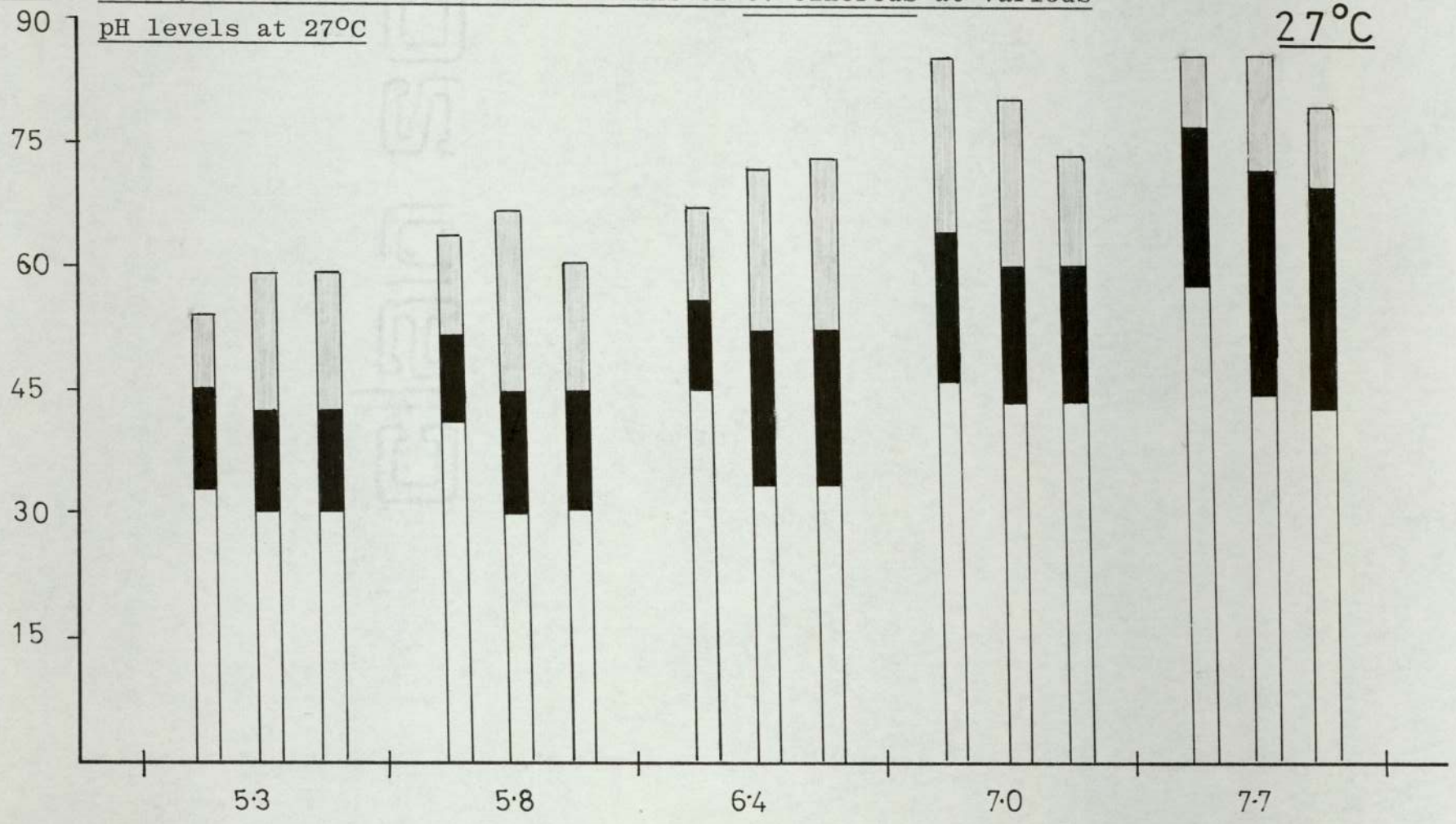


Figure 2.4 Colony diameters of the three strains of C. cinereus at various pH levels at 33°C

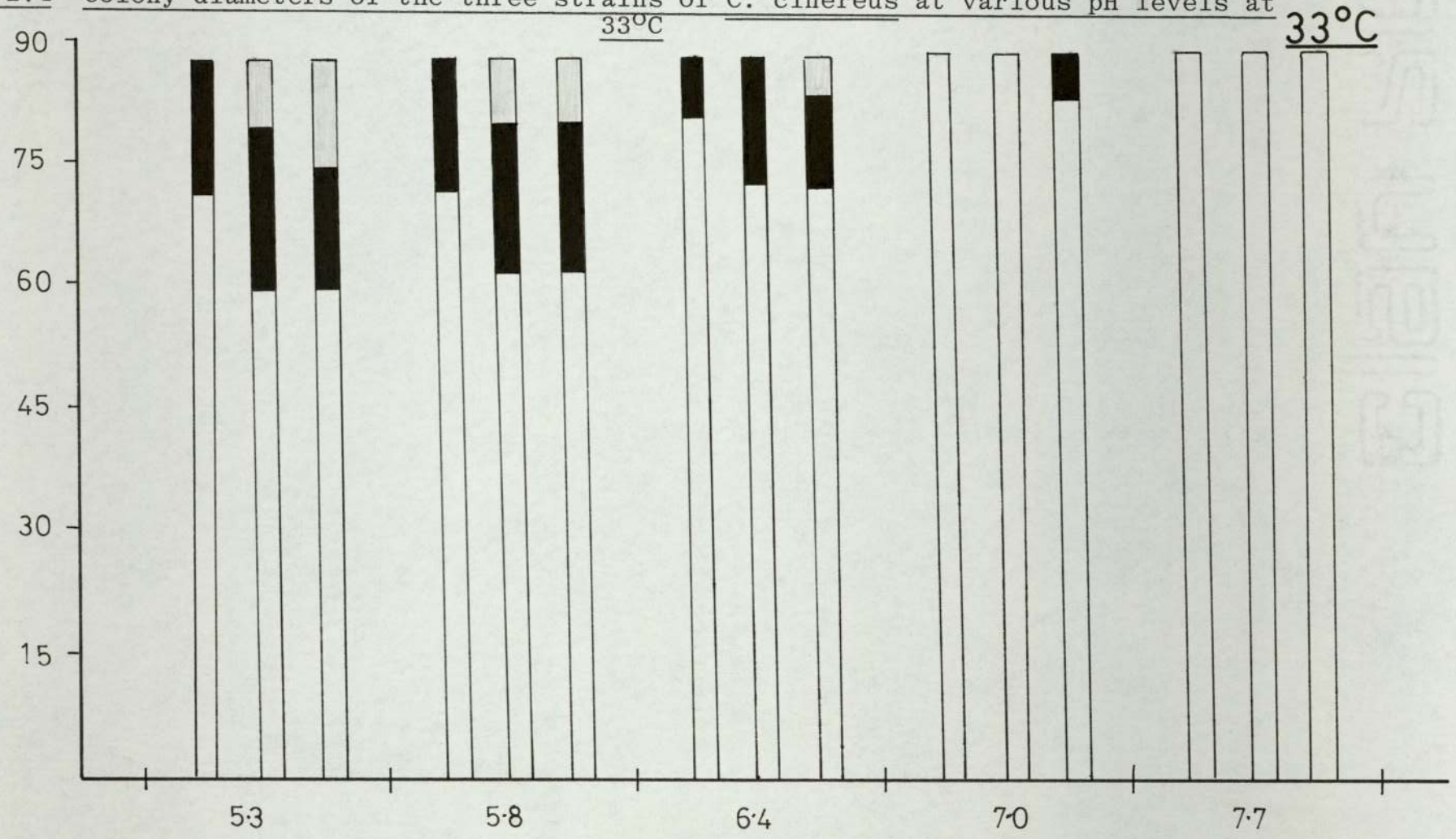


Figure 2.5 Colony diameters of the three strains of C. cinereus at various pH levels at 37°C

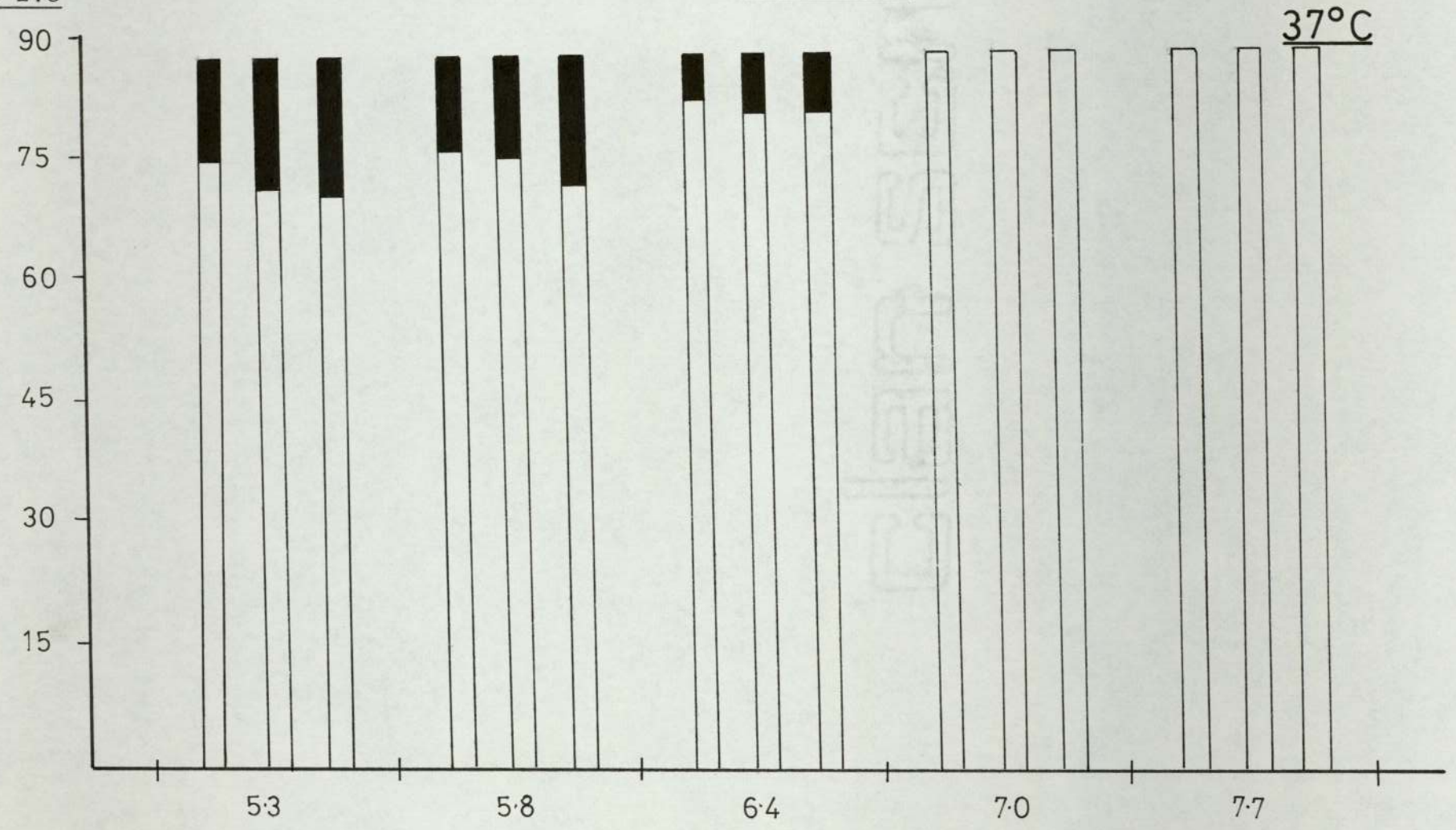


Figure 2.6 Colony diameters of the three strains of C. cinereus at various pH levels at 41°C

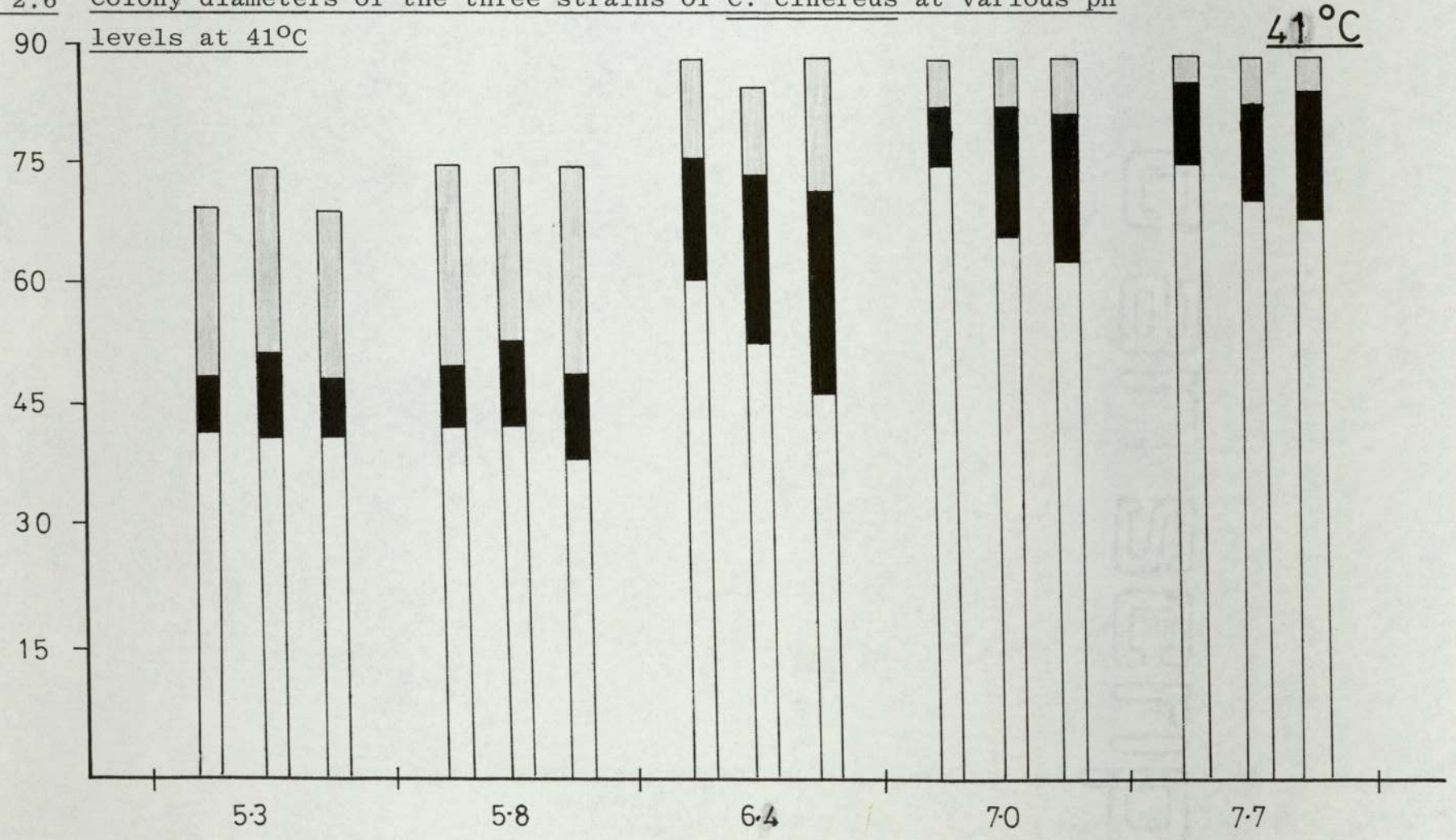


Figure 2.7 To show the extent of growth of the ABERYSTWYTH strain of C. cinereus at varying temperatures at three different pH's after 75 hours.

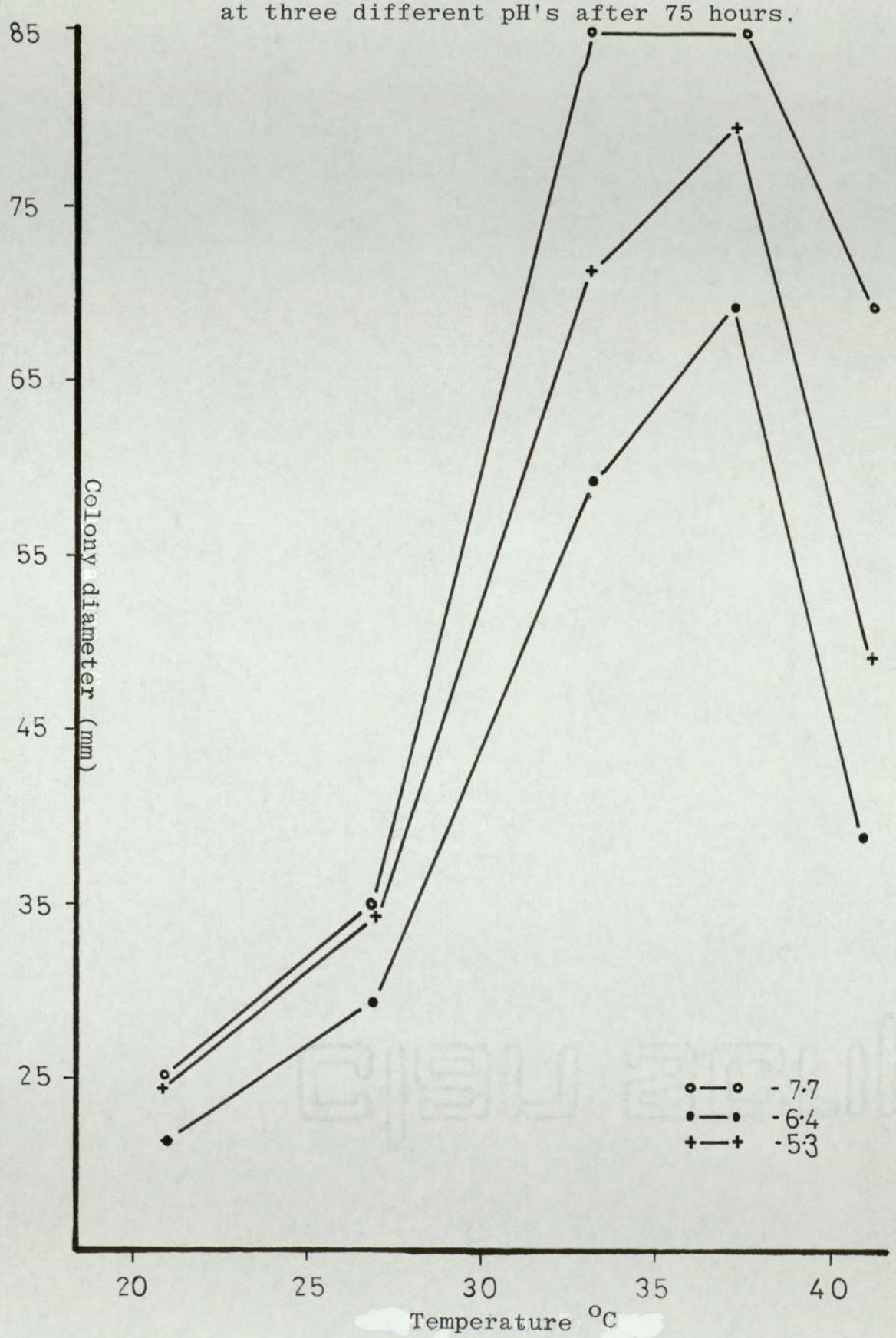


Figure 2.8 To show the extent of growth of the JAVA strain of C. cinereus at varying temperatures at three different pH's after 75 hours.

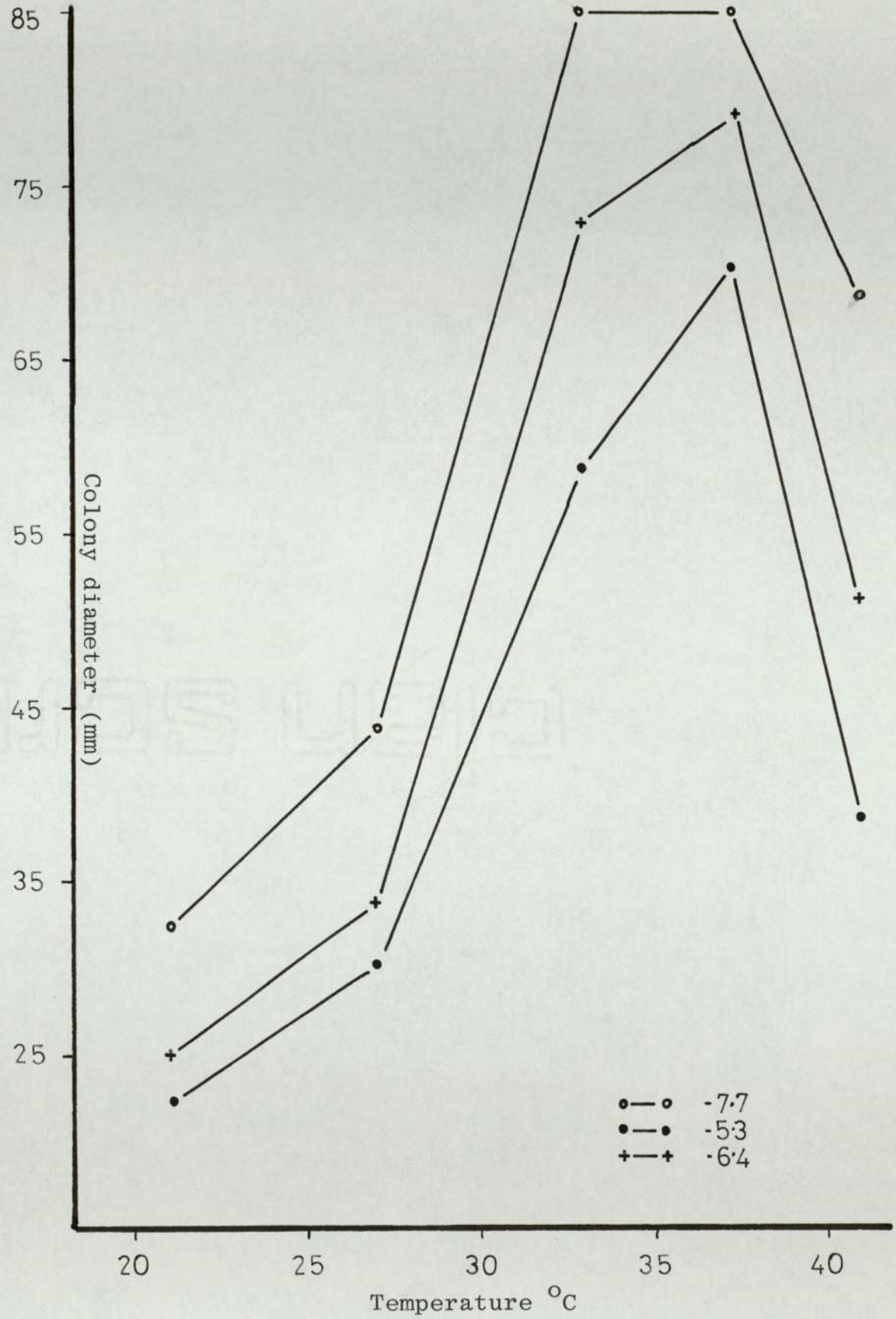


Figure 2.9 To show the extent of growth of the ASTON strain of C. cinereus at varying temperatures at three different pH's after 75 hours

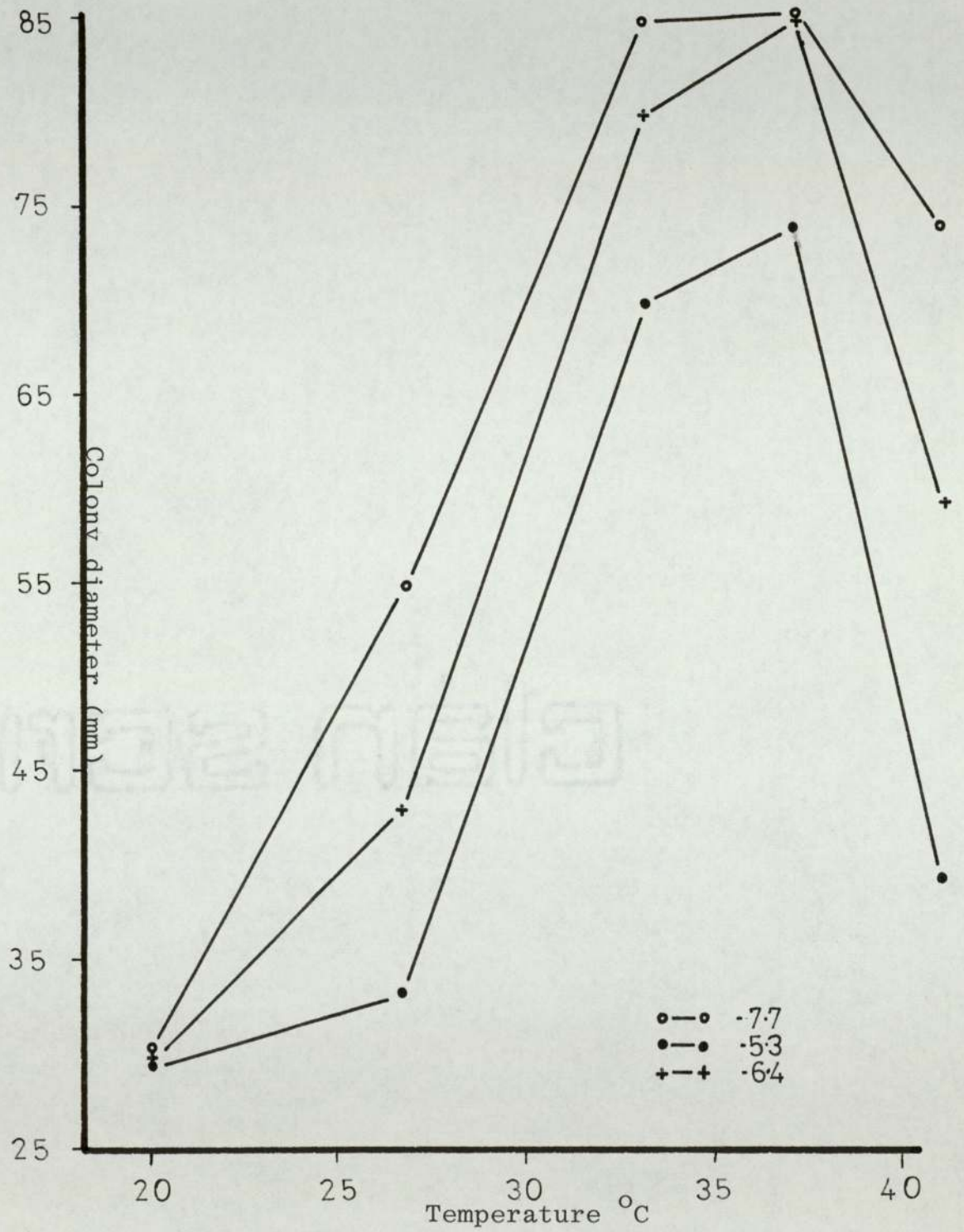
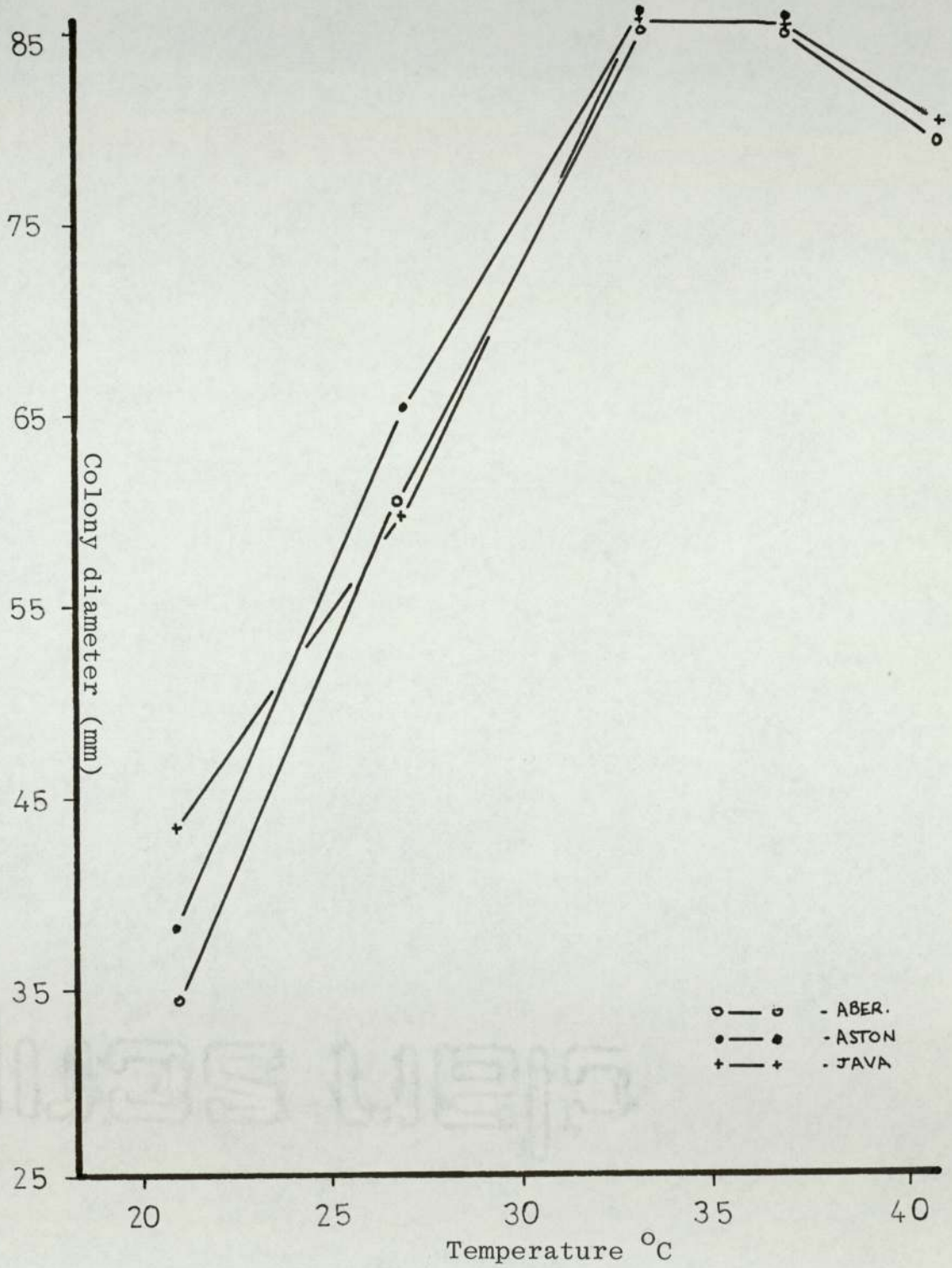


Figure 2.10 To show the extent of growth of all three strains of C. cinereus at varying temperatures at pH 7.0 after 100 hours.



interpreted account of the lack of strain variation, both in pH and temperature reactions. By extrapolating the graphs the optimum temperature for all three strains under the conditions of the experiment appears to be 35.5 $\pm$ 0.8^{o}C. This is an average figure over the range of pH since an increase in pH appears to lower the optimum temperature by a small amount in all cases which falls within the guidelines of temperature optima of Coprinus spp. provided by Webster.

Figure 2.10 again shows the close correlation between the strains since it depicts their colony diameters for varying temperatures measured after 100 hours at pH 7.0. The largest variations appear in the lower temperatures, but as the temperature becomes optimal the graphs coincide. This, however, may well be due to the fact that the diameter of the petri plate itself becomes a limiting factor under optimal conditions.

2.4 Discussion and Conclusions

Probably the most surprising result of this experiment was the extremely close correlation between the three strains, especially since one of the strains was isolated in a tropical country. It is not uncommon for tropical strains of a fungus to exhibit significantly higher optimal temperatures than those from temperate regions, (Cartwright and Findlay, 1934). The reason for this Coprinus species not showing variation may well be due to its ecological niche in that, being a litter and coprophilous fungus whose habitat is usually undergoing self heating during composting and is therefore independent to a certain degree of the ambient conditions. The figure for the optimum temperature viz 35°C approximately, is in very close agreement with the literature.

Yung Chang (1967) states that her strain of Coprinus cinereus also grew fastest at 35°C although the growth medium she used was Czapek-Dox Yeast agar with a pH of 6.5. In a previous publication of the same year, Yung Chang and Hudson provide evidence for the isolation of Coprinus species from wheat straw compost after about 22 days composting. By this time the average temperature of the compost heap had fallen to between 30°C and 40°C. These two papers seem to suggest that the temperature optima for this species is hardly affected by the substrate used providing enough nutrients are present, thus providing a much firmer basis for transposing laboratory results into more practical work using natural substrates.

The genus Coprinus is given a special section as 'doubtful thermophiles' in the standard text on thermophiles by Cooney and Emerson (1964). However, according to the authors' definition of thermophily, Coprinus cinereus does not qualify as a true thermophile due to its ability to grow below 20°C. Neither is it able to grow at or above 55°C, and one possible reason for this is given by Cochrane (1958). He cites Coprinus fimetarius growing poorly at 44°C due to the inability of the fungus to biosynthesise methionine at a sufficiently high rate. Apparently, if methionine is added to the medium , normal growth can be achieved at elevated temperatures. It was thought, that for C. cinereus in the context of this thesis that this particular line of investigation was not necessary.

All three strains also seem to react to an increase in pH in the same way, that is a steady increase in growth rate with an increase in pH, up to the highest pH it was possible to achieve. The mechanism of this reaction is beyond the range of the work in hand so the results are merely taken as being so.

In conclusion then, the following useful factors have emerged from this study which should prove extremely useful when endeavouring to propogate Coprinus cinereus on various materials under non-sterile conditions:-

(i) The growth of Coprinus cinereus as measured by linear expansion, possibly on any nutrient-rich substrate, is fastest at 35°C.

(ii) Few other fungi have their growth optima at this

temperature (Cochrane, 1958), therefore there is relatively less competition.

(iii) The linear expansion method of measuring growth seems particularly relevant to the theme of this work since it appears to give a guide to the ability of an organism to colonise large areas of substrate, quickly.

(iv) There does seem to be a correlation between this laboratory work and ecological studies on natural substrates as carried out by Yung Chang and Hudson (1967).

(v) Preliminary pH investigations suggest that Coprinus cinereus, as well as prefering fairly high temperatures, also prefers relatively high pH values for fungal growth, again lessening competition.

C H A P T E R 3

The effect of pH on the growth of *Coprinus cinereus*

3.1 Introduction

No study on the physiology of fungi can be considered complete without reference to the effects of hydrogen ion concentration (pH) on the organisms studied. The pH of any natural or synthetic medium is known to have a profound effect on the growth of fungi (Allsopp, 1973). Indeed it is often this effect which is responsible for the variation in fungal species found in different habitats. With respect to soil as one such habitat, Allsopp (1973) has made a comprehensive study of the effects of pH on the variation in fungal species isolated.

Many other workers have investigated the effects of pH on the growth of fungi in general. The areas of study have been diverse; for example Ling and Yang (1944) studied the effects of pH in spore germinations of several fungi, Cockwood (1937) studied the effects on perithecial production in several Ascomycetes, Allsopp (1973) observed the way in which pH affected the decay susceptibility of cellulosic materials by fungi. Fries (1956) along with many other workers have used the dry weight of mycelium produced as a criterion for measuring the effects of pH on the vegetative growth of fungi.

From the literature, probably the only general statement regarding the influence of pH on fungal physiology is that the relationship is a highly complex one, for several reasons:-

a) pH seems to affect the various stages in the life

histories of fungi in different ways.

b) pH is not a unitary factor since its action differs throughout its range. The different areas of a pH range can influence enzyme systems (Cochrane, 1958), metal solubilities (Fries, 1956), surface metabolic reactions, uptake of minerals and entry of organic acids into the cell (Overstreet, 1952) as well as entry of essential vitamins (Wyss et al., 1944).

c) The environmental conditions can moderate the effects of pH. Temperature (Pehrson, 1948), time of harvest (Neal, 1927) and nitrogen source (How, 1940) are just some of the environmental factors which can act in this way.

d) The pH of the growth medium is very often altered by the production of metabolites by the growing organism.

e) The toxicity of various chemicals can even be altered by differences in pH (Allsopp, 1973).

It appears then that there are many pitfalls in attempting to discover the effects of pH on the growth of a particular organism unless steps are taken to cover all the above contingencies. Probably the most widely studied of all these contingencies is the pH change due to growth. Allsopp (1973) gives a full account of this effect using Trichoderma viride, as well as describing the latest work being carried out at the B.I.C. in perfusion techniques which enable the pH of a growth medium to be kept constant throughout the growth of a fungal colony. However, by far the most widely used method of achieving this end is the incorporation of buffer solutions within growth media.

Amongst some recent work published in this field, Child and Knapp (1973) suggest that high concentrations of phosphate buffers can reduce the change in pH of various media. Knapp (1973) who suggests that high concentrations of phosphate buffers can reduce the change in pH of various media to 0.3 units after four days growth of Rhizopus sexualis. However, Child and Knapp themselves point out that phosphate itself may affect fungal growth directly since it is metabolizable. They also point out that the use of phosphate has a limited pH range. Two other commonly used buffers are mentioned, MES (Z-(N-morpholino) ethane sulphonic acid) and EMTA (3,6-endomethylene 1,2,3,6-tetrahydro-cis-pthalic acid), but even these are restricted to certain fungi since they are known to affect the growth of others directly, rather than through the pH of the medium. Due to this complex situation it was decided not to attempt to control the pH of the solution throughout the experiment. Instead, the initial and final pH of the media were monitored and these, together with the dry weights of mycelia produced were considered sufficient to produce a tolerance range and estimate an optimum initial pH for growth of the fungus to be used in further work.

A liquid malt extract medium was used throughout these experiments for the following reasons:-

a) Good growth of the Coprinus cinereus strains had been obtained on malt extract agar in previous studies.

b) The stock cultures of the fungus were kept on malt extract agar so there were no complications of conditioning of the fungus to an alien nutrient source.

c) Liquid media are preferable to agar media for dry weight determinations due to ease of harvest as explained in Chapter 2.

d) Liquid media are also preferable for pH work since ionic movements are less restricted than in agar. They are also easier to test the pH.

e) Malt extract broth was found to change pH relatively easily with small additions of sodium hydroxide or hydrochloric acid, (the adjustment of pH is explained fully in the method).

The pH of freshly autoclaved malt extract broth was 5.3 which was slightly higher than the lowest pH at which Coprinus cinereus will grow according to Hedger (1973). Only one lower pH, 4.5, was used since all the previous workers on Coprinus spp. suggest that their optima are in the neutral or alkaline regions. Fries (1956) investigated several habitats in which several Coprinus species were isolated, and found that in all cases the pH of the habitat was between 6.6 and 8.7. It was therefore decided that this range would constitute the major area of research. It was found necessary, however, to repeat the experiment using different methods of pH adjustment (see method) since it was considered that the results obtained using the first method could possibly have been masked by the chemical reactions within the media which would probably have taken place during autoclaving. The dilute caustic soda may well have drastically altered the chemical constitution of the malt broth, rather than merely altering the pH. A second series of experiments using the second method of pH adjust-

ment was performed in the hope that the possibility of these chemical reactions would be reduced. The two sets of results could then be regarded as complementary.

3.2 Materials and Methods

3.2.1 Organisms

All three strains of Coprinus cinereus, as described in chapter 1 were used. They were grown up on malt extract agar, pH 5.3 at 25°C for five days before being used as inocula.

3.2.2 Materials

The growth medium, (broth) was made up as shown below:-

Malt Extract, Oxoid No. L39	30g/1 H_2O
Mycological Peptone, Oxoid No. L40	5gm/1 H_2O

Adjustment of pH was by addition of either 0.1N HCl or 0.1N NaOH and measurement of pH carried out on a Pye temperature compensated pH meter with glass electrode. Non-wettable cotton wool bungs were used to stopper the 100ml conical flasks, within which the experiments were carried out.

3.2.3 Methods

As mentioned in the introduction to this chapter, the determination of the pH optima for the three strains of Coprinus were carried out in two similar ways; the point at which the pH of the broth was adjusted, being the main difference. There were, however, several practical implications in differing the methods which warrent a separate discussion of each:-

a) Adjustment of pH before autoclaving broth

The constituents of the broth were dissolved in distilled water and the solution divided into 400ml aliquots. To each was added either 0.1N HCl or 0.1N NaOH to give the desired pH range as shown in the centre panel of figure 3.1. 20mls of the broth was then introduced into each of the 100ml conical flasks by means of a 25ml syringe. The flasks were then plugged with cotton wool and autoclaved at 10p.si. for twenty minutes. This combination being used to lessen any possible effects on the constituents of the broth that a higher temperature and pressure would have had. When the medium had cooled to ambient, two flasks at each pH level were sacrificed and the pH determined to give the figures in the right-hand panel of figure 3.1.

Figure 3.1

To show the effects on the pH of malt extract broth, of adding various amounts of 0.1N HCl or 0.1N NaOH, prior to autoclaving

Mls 0.1N HCl/100mls broth	pH of broth initially	pH of broth after autoclaving
4	4.4	4.5
Mls 0.1N NaOH/100mls broth		
0	5.8	5.3
1	6.0	5.7
2	6.4	6.1
3.5	6.8	6.5
6	7.5	7.1
10.5	8.3	7.0
15	8.8	6.9
20	9.7	6.7

The sample variation of the pH after autoclaving was less

than 0.1 of a pH unit in all cases.

A 6mm cork borer was used to prepare inoculum plugs from the edges of the agar stock cultures. As explained in chapter 1, the size of the inoculum is considered to be important in liquid culture work, where growth is measured by dry weight determinations. For this reason, the stock cultures were grown up on exactly 20mls of malt extract agar per each petri plate so that that the thickness of the plug was virtually the same for each flask.

The plugs were then introduced into the flasks and incubated at 35^{o}C (the temperature optimum as determined in chapter 2) for seven days. After this time the mycelial mats were filtered off using a Buchner funnel and dried, on weighed filter papers. To remove any possible differences with regard to weight, between the original inoculum plugs, these were quite easily removed, immediately after the filtering process. The mycelia were washed with distilled water to remove any adhering salts, then dried at 105^{o}C for 24 hours and weighed.

Each variable was replicated six times and the remaining liquids after growth, of each variable were pooled and the final pH taken.

b) Adjustment of pH after autoclaving

Before the actual growth studies using this technique were carried out, several prelimimary studies were performed to determine exactly how much HCl or NaOH would be required

to obtain a similar range of pH as in section (a). 20ml aliquots of malt broth were measured into 100ml conical flasks using a 25ml pipette. The flasks were then plugged with non-wettable cotton wool, and autoclaved at 10p.s.i. for twenty minutes. Sterile 0.1N HCl and 0.1N NaOH were then added in the proportions shown in figure 3.2.

For the actual growth studies, the malt broth was made up in bulk, divided into 20ml aliquots, each being placed in 100ml conical flasks which were then plugged and autoclaved. When the solutions had cooled the pH range shown in figure 3.2 was achieved by the aseptical addition of 0.1N HCl or 0.1N NaOH.

Figure 3.2

To show the effects on the pH of malt extract broth, of adding various amounts of 0.1N HCl or 0.1N NaOH, after autoclaving

Mls 0.1N HCl/flask	pH of broth
1	4.5
Mls 0.1N NaOH/flask	
0	5.3
0.25	6.0
0.5	6.4
0.8	6.8
1.0	7.2
1.75	7.7
2.0	8.0
2.5	8.3
3.0	8.5

After this stage, the procedure was exactly similar to that in section (a) and again, six replicates of each major variable were used.

3.3 Results

These were expressed in tabular form, figures 3.3 and 3.4 as well as graphically, figures 3.5 to 3.16. However, since the experimental section was divided into two sections, the results have likewise been divided. Thus figures 3.3 and 3.5 to 3.10 relate to the section of pH adjustment prior to autoclaving whilst figures 3.5 and 3.11 to 3.16 represent the results of adjusting the pH of the medium after autoclaving.

Table 3.3 shows the initial pH, final pH, change in pH and dry weight of mycelium produced after seven days growth at 35°C. However, the last three sets of results, that is, those after an initial pH of 7.1 have been delimited and not used in the compilation of the graphs. This was thought necessary since these results appear to be examples of the complex chemical effects due to autoclaving caustic soda solution in the broth, as mentioned in the introduction. No matter how high the initial pH of the broth above 7.5 before autoclaving, it always dropped to between 7.0 and 6.5. This was probably due to the hydrolysis of the peptone in the broth, thus releasing amino-acids. It is worth noting here, the contrast to malt extract agar used in chapter 1. Here the maximum pH obtainable was 8.0 and this would suggest that the agar itself exertsabuffering capacity since the other components are exactly alike.

Graphs 3.5 to 3.7 show the variation in dry weight of mycelium produced at different initial pH's for the three

Figure 3.3

Initial pH, final pH, pH change and mycelial drywt. of the three strains after seven days growth at 35°C

(pH adjusted before autoclaving)

Figure 3.3

ASTON STRAIN

pH broth before growth	pH broth after growth	pH change	Dry weight of mycelium (mg)
4.5	7.1	2.6	128.9
5.3	8.1	2.8	174.9
5.7	8.4	2.7	256.4
6.1	7.9	1.8	210.8
6.5	8.3	1.8	204.0
7.1	8.4	1.3	217.2
7.0*	8.2	1.2	180.9
6.9*	8.2	1.3	168.3
6.7*	8.5	1.8	186.0

ABERYSTWYTH STRAIN

pH broth before growth	pH broth after growth	pH change	Dry weight of mycelium (mg)
4.5	4.5	0	0
5.3	7.0	1.7	150.2
5.7	7.4	1.7	287.2
6.1	7.7	1.6	233.4
6.5	7.9	1.4	233.1
7.1	8.1	1.0	215.5
7.0*	8.1	1.1	231.5
6.9*	8.1	1.2	201.7
6.7*	8.1	1.4	177.4

JAVA STRAIN

pH broth before growth	pH broth after growth	pH change	Dry weight of mycelium (mg)
4.5	4.5	0	0
5.3	7.4	2.1	210.4
5.7	7.9	2.2	253.5
6.1	7.8	1.7	243.4
6.5	7.8	1.3	219.4
7.1	8.1	1.0	222.4
7.0*	8.1	1.1	204.0
6.9*	8.1	1.2	172.4
6.7*	8.1	1.4	172.4

Figure 3.4

Initial pH, final pH, pH change and mycelial drywt. of the three strains after seven days growth at 35°C

(pH adjusted after autoclaving)

Figure 3.4

ASTON STRAIN

pH broth before growth	pH broth after growth	pH change	Dry weight of mycelium (mg)
4.5	7.6	3.1	181.6
5.3	7.9	2.6	218.7
6.0	8.0	2.0	189.7
6.4	8.2	1.8	219.9
6.8	8.2	1.4	218.4
7.2	8.2	1.0	232.7
7.7	8.2	0.5	219.8
8.0	8.2	0.2	202.2
8.3	8.3	0.0	201.2
8.5	8.3	-0.2	200.3

ABERYSTWYTH STRAIN

pH broth before growth	pH broth after growth	pH change	Dry weight of mycelium (mg)
4.5	4.5	0	0
5.3	7.7	2.4	231.7
6.0	7.2	1.2	232.1
6.4	7.6	1.2	210.3
6.8	7.5	0.8	253.5
7.2	7.5	0.3	252.8
7.7	7.9	0.2	261.1
8.0	7.9	-0.1	232.2
8.3	7.9	-0.4	223.3
8.5	8.0	-0.5	230.2

JAVA STRAIN

pH broth before growth	pH broth after growth	pH change	Dry weight of mycelium (mg)
4.5	4.5	0	0
5.3	7.2	1.9	219.7
6.0	7.8	1.8	237.1
6.4	7.7	1.3	257.2
6.8	8.0	1.2	280.9
7.2	7.7	0.5	292.9
7.7	7.8	0.3	266.6
8.0	7.9	-0.1	243.1
8.3	7.8	-0.5	273.9
8.5	8.0	-0.5	237.6

Figure 3.5

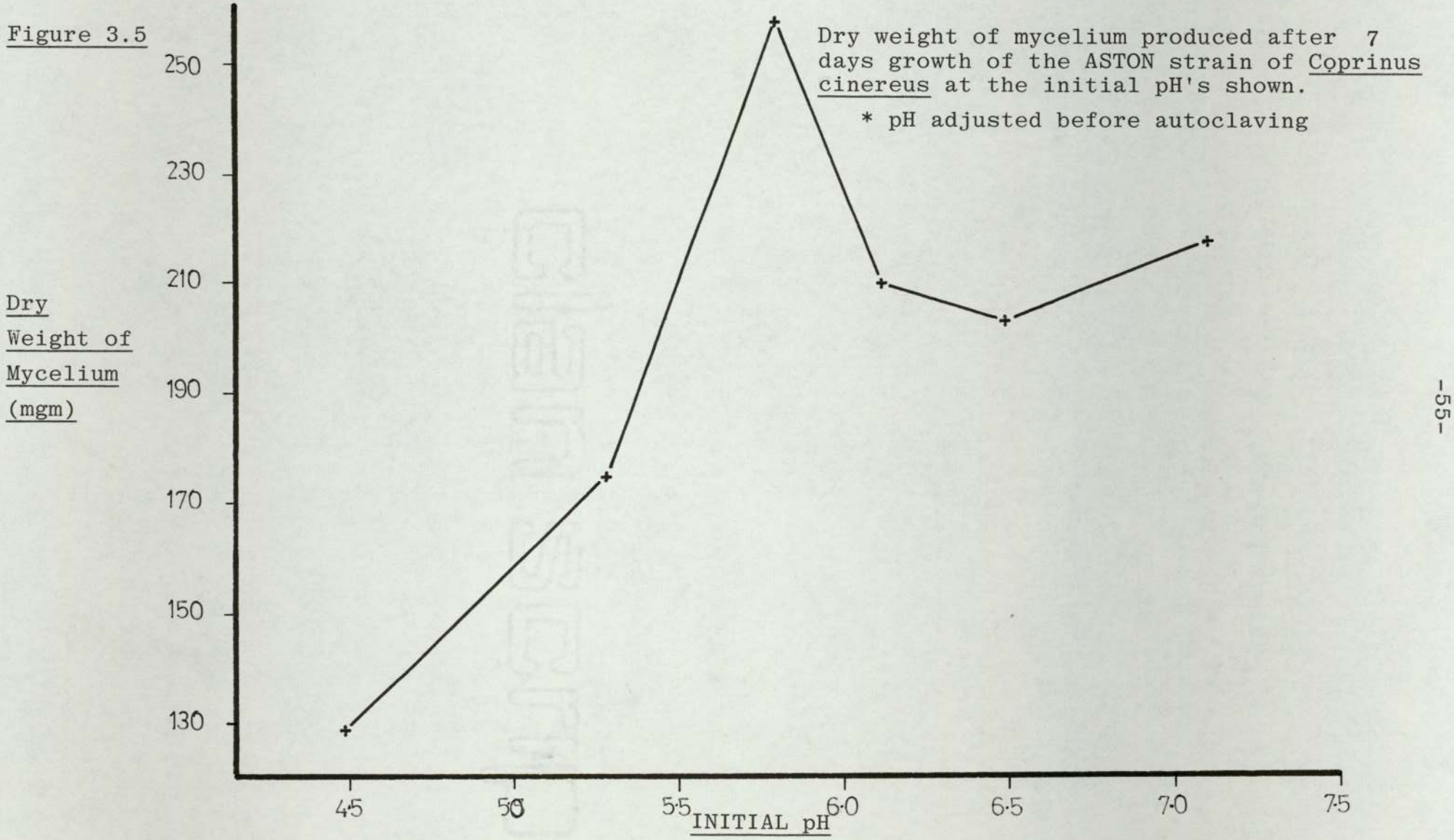
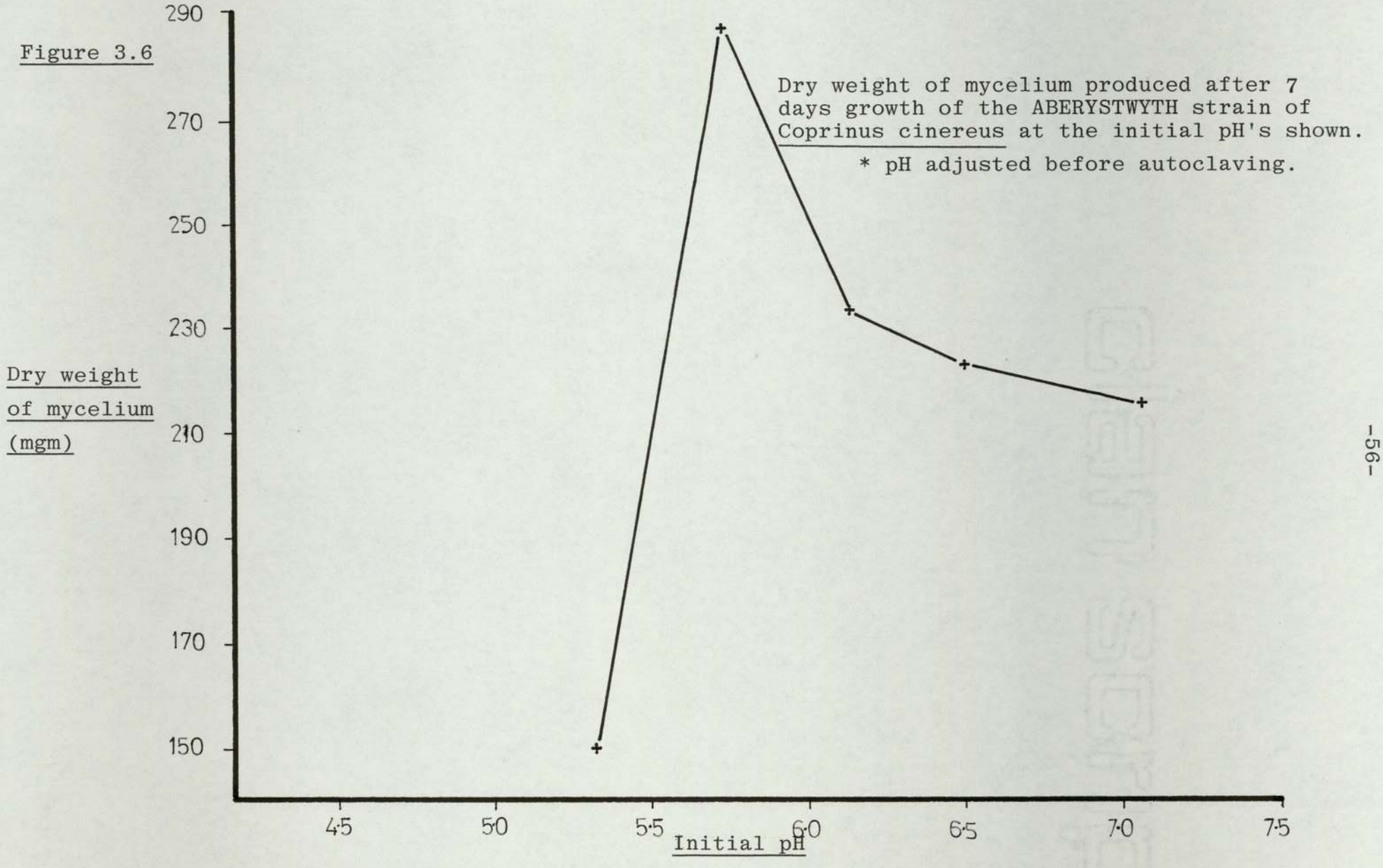

Dry weight of mycelium produced after 7 days growth of the ASTON strain of Coprinus cinereus at the initial pH's shown.
* pH adjusted before autoclaving
Dry Weight of Mycelium (mgm)
250
230
210
190
170
150
130
4·5
5·0
5·5
6·0
6·5
7·0
7·5
INITIAL pH

Figure 3.6



Dry weight of mycelium produced after 7 days growth of the ABERYSTWYTH strain of Coprinus cinereus at the initial pH's shown.

* pH adjusted before autoclaving.

Figure 3.7 Dry weight of mycelium produced after 7 days growth of the JAVA strain of Coprinus cinereus at the initial pH's shown.

* pH adjusted before autoclaving.

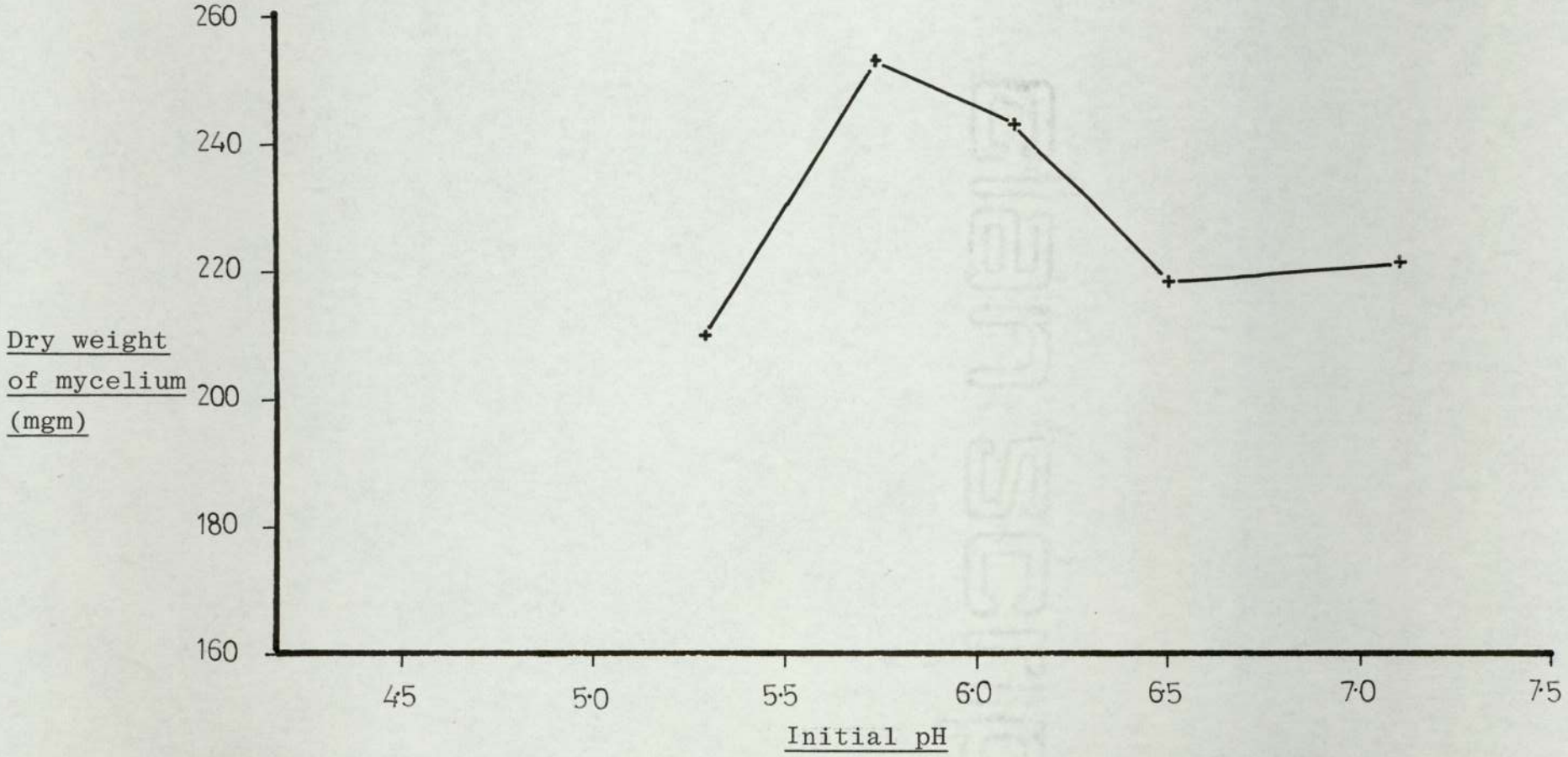
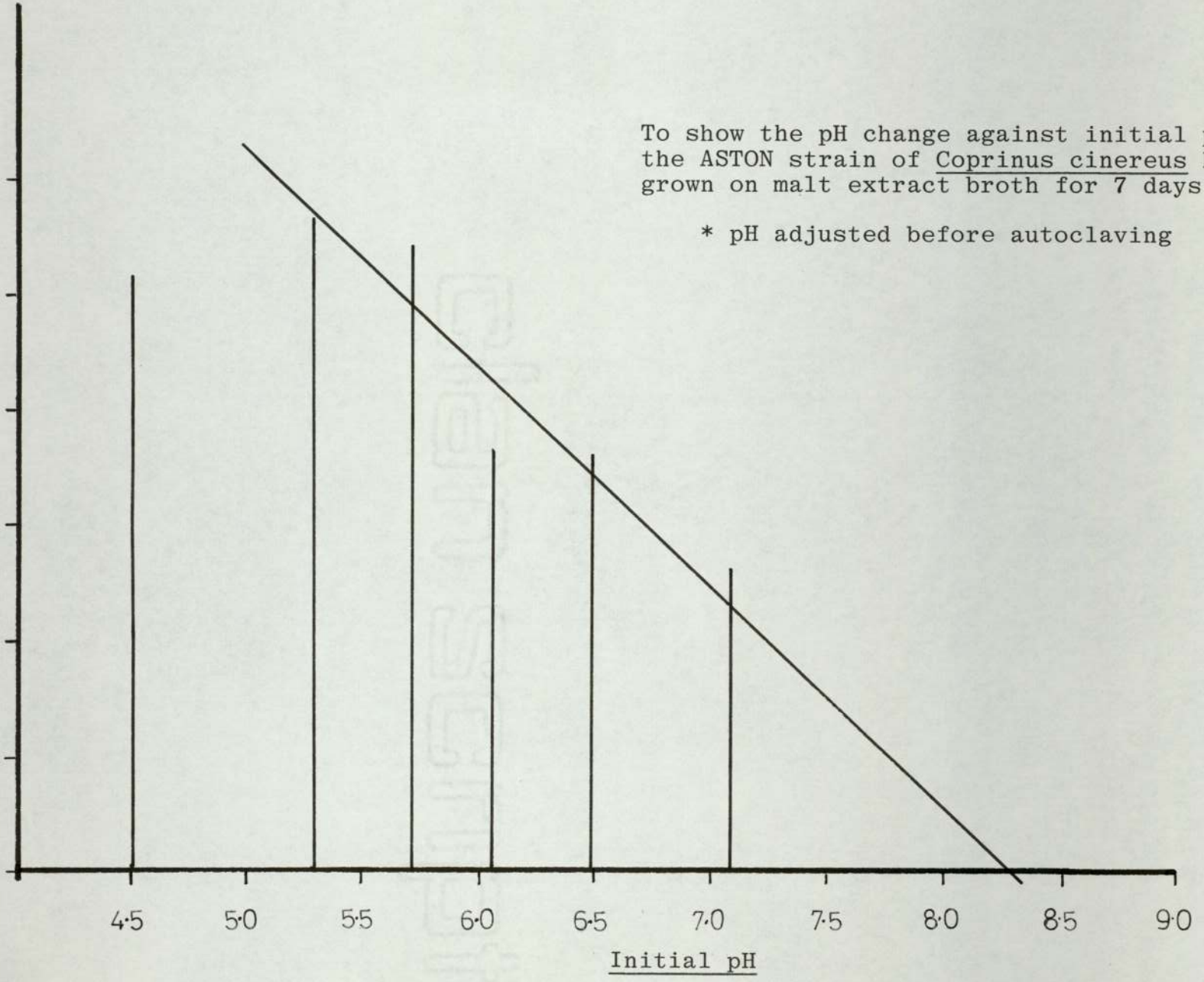


Figure 3.8



To show the pH change against initial pH after the ASTON strain of Coprinus cinereus had been grown on malt extract broth for 7 days.

* pH adjusted before autoclaving

Figure 3.9

To show the pH change against initial pH after the ABERYSTWYTH strain of Coprinus cinereus had been grown on malt extract broth for 7 days.

* pH adjusted before autoclaving.

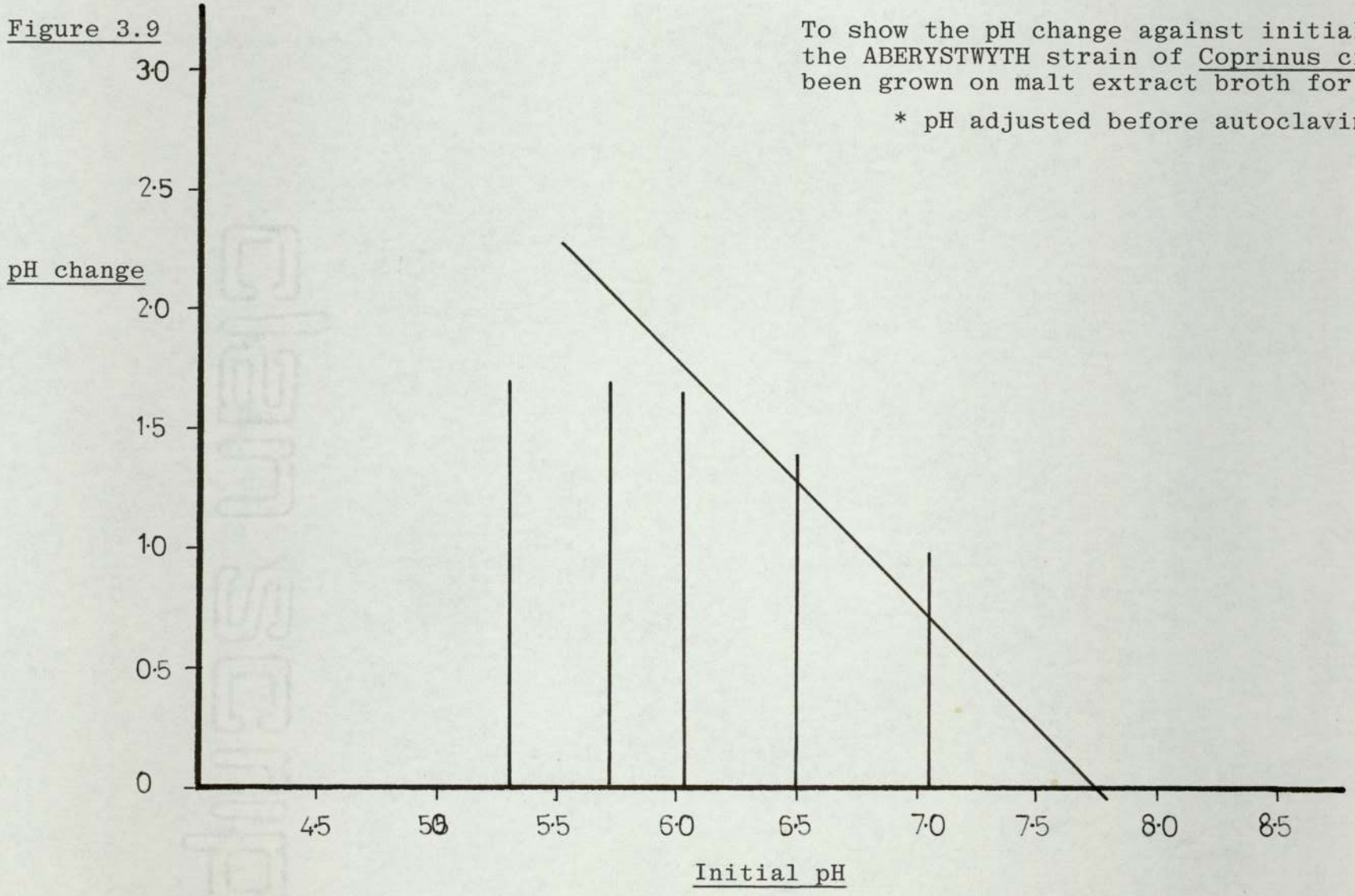


Figure 3.10

To show the pH change against initial pH after the JAVA strain of Coprinus cinereus had been grown on malt extract broth for 7 days.

* pH adjusted before autoclaving.

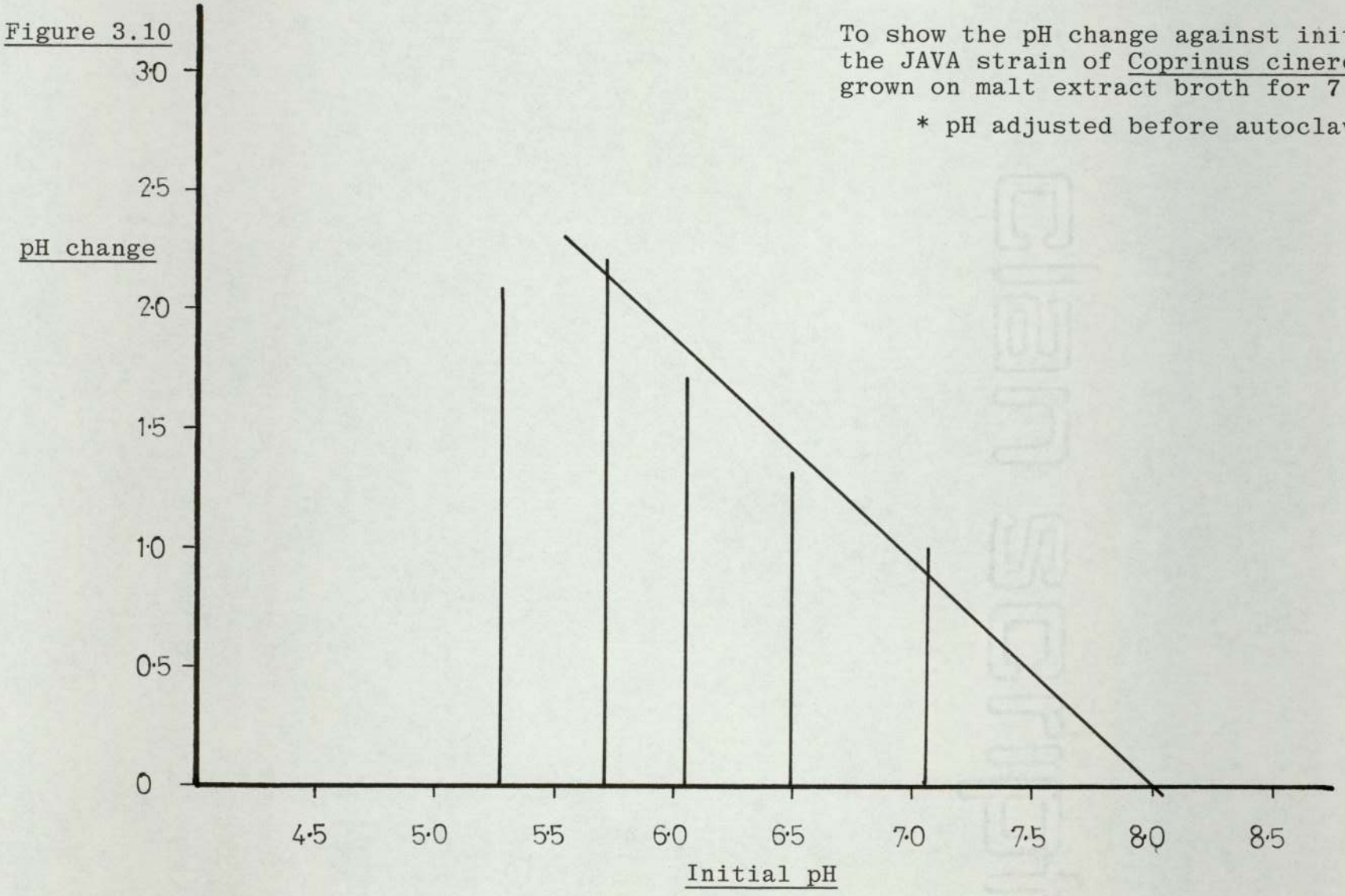


Figure 3.11

Dry weight of mycelium produced after 7 days growth of the ASTON strain of Coprinus cinereus at the initial pH's shown.

* pH adjusted after autoclaving.

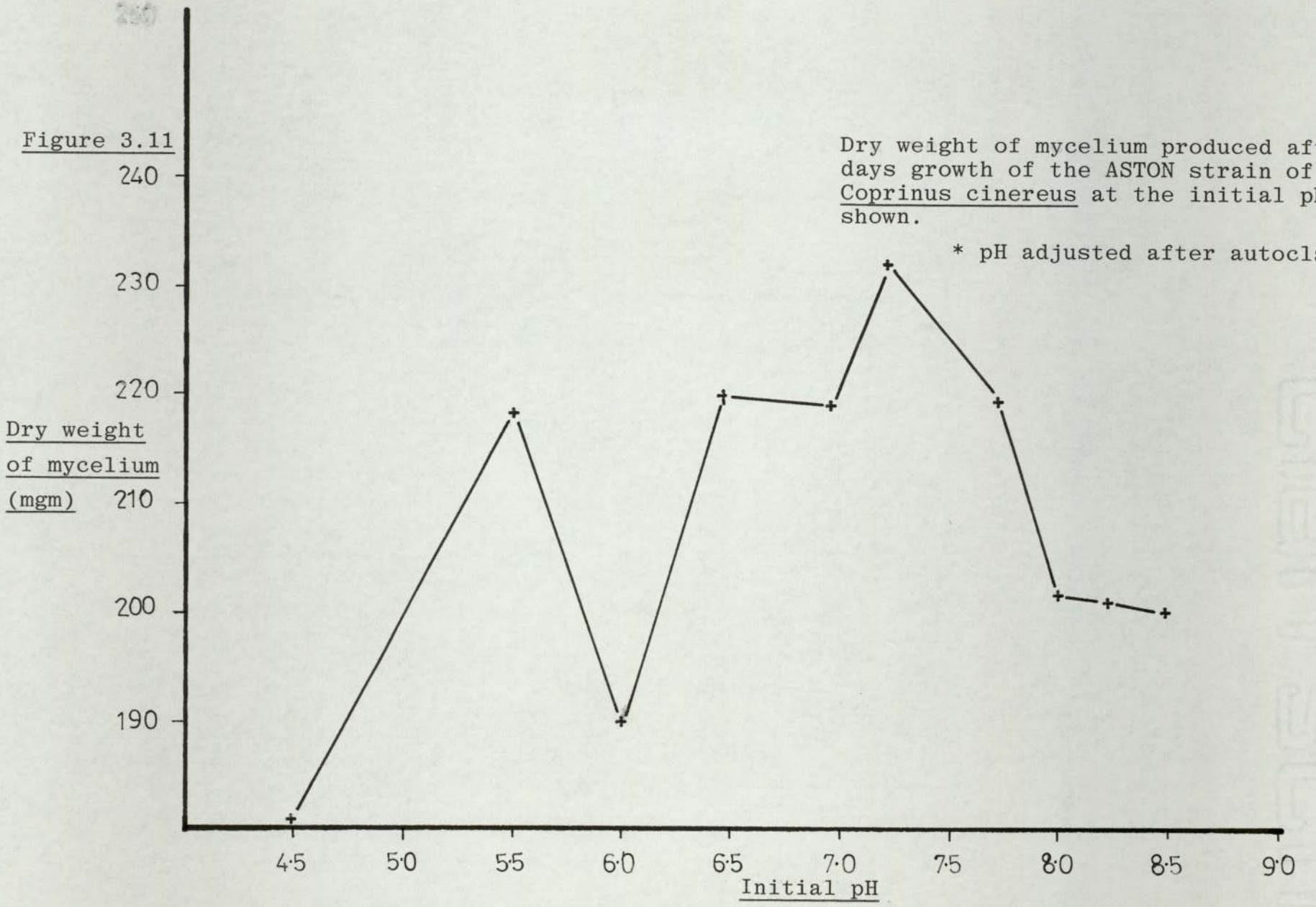


Figure 3.12

Dry weight of mycelium produced after 7 days growth of the ABERYSTWYTH strain of Coprinus cinereus at the initial pH's shown.

* pH adjusted after autoclaving.

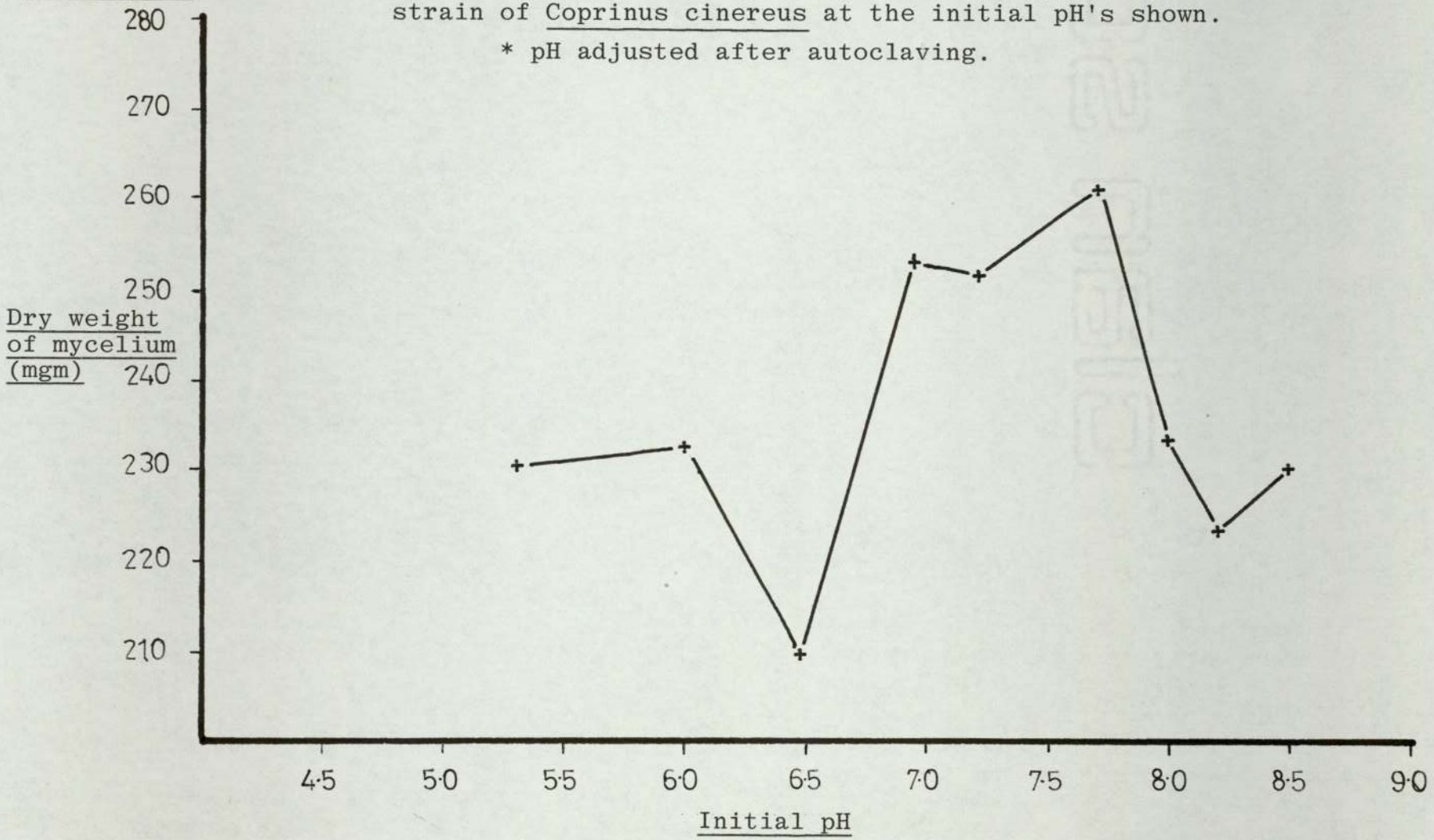


Figure 3.13

Dry weight of mycelium produced after 7 days growth of the JAVA strain of Coprinus cinereus at the initial pH's shown.

* pH adjusted after autoclaving.

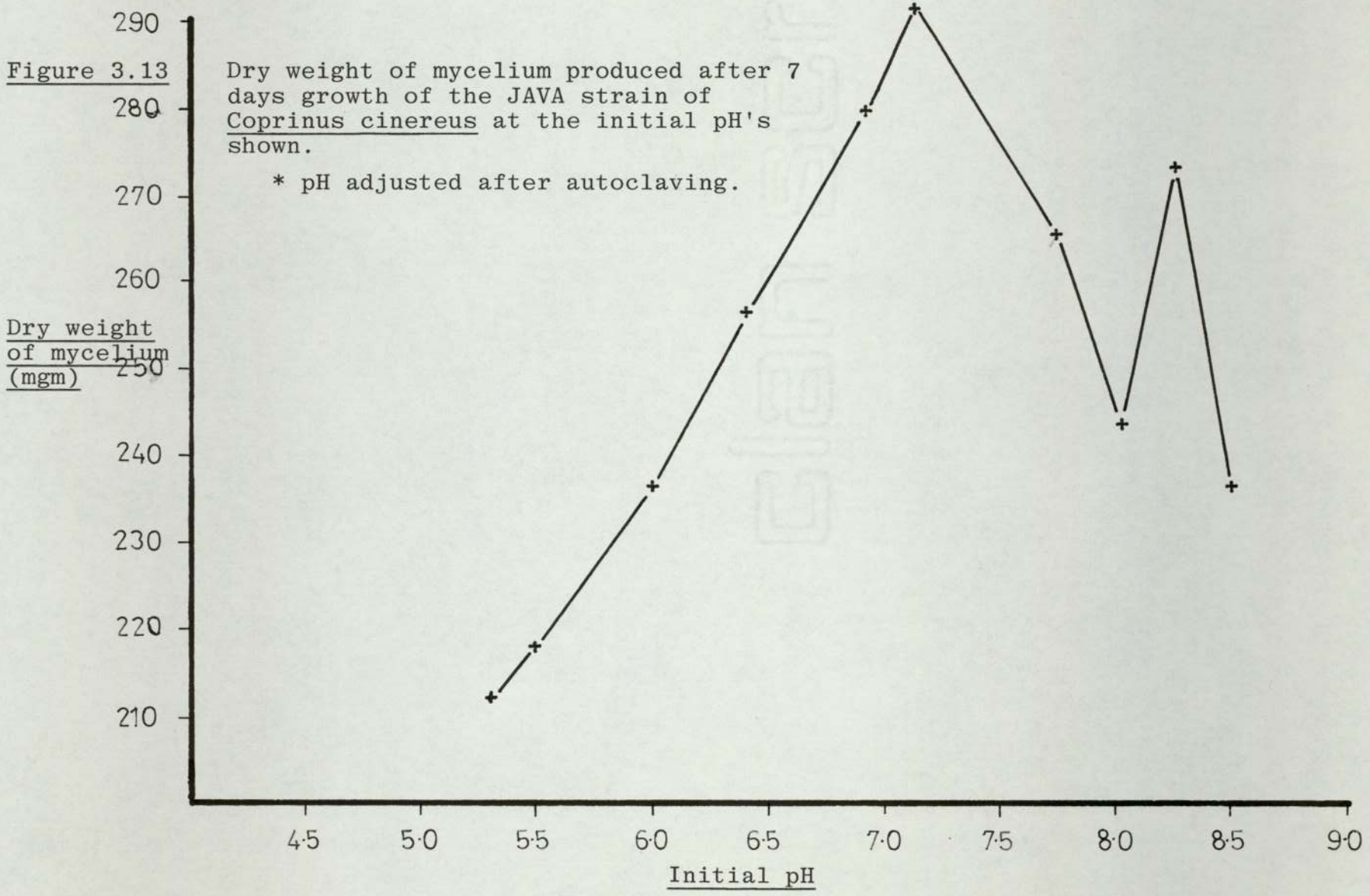
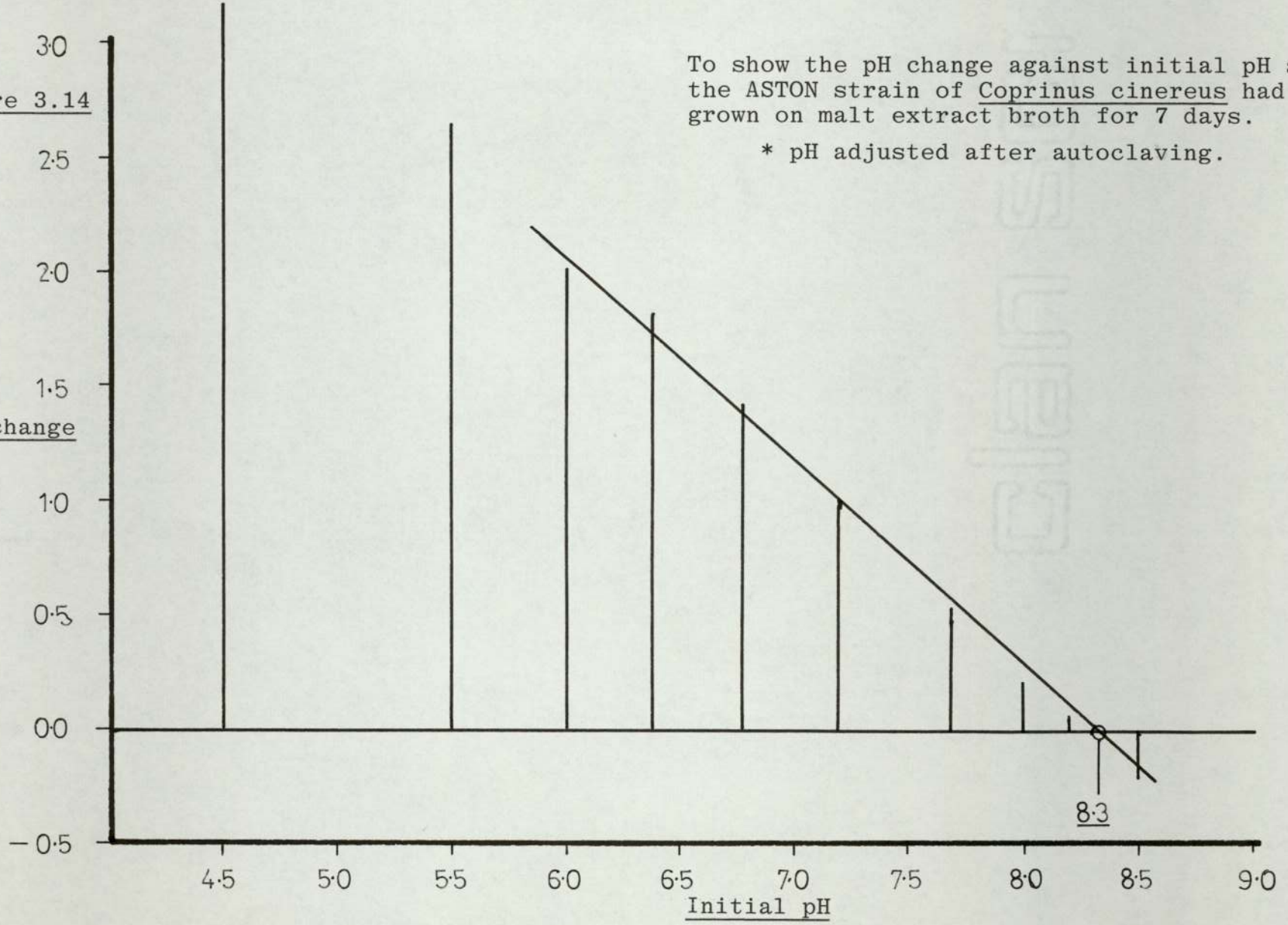


Figure 3.14



To show the pH change against initial pH after the ASTON strain of Coprinus cinereus had been grown on malt extract broth for 7 days.

* pH adjusted after autoclaving.

Figure 3.15

To show the pH change against initial pH after the ABERYSTWYTH strain of Coprinus cinereus had been grown on malt extract broth for 7 days.

* pH adjusted after autoclaving.

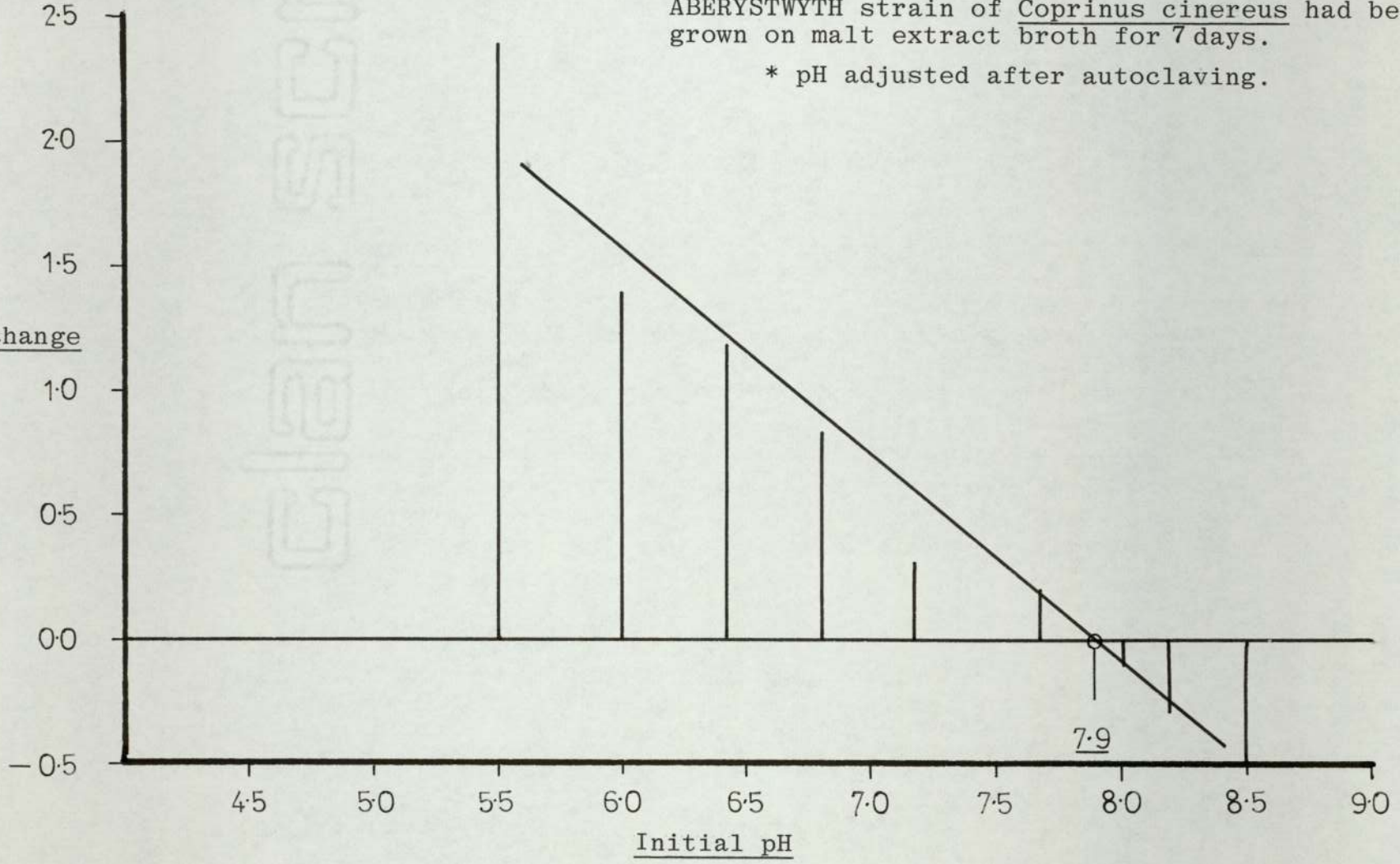
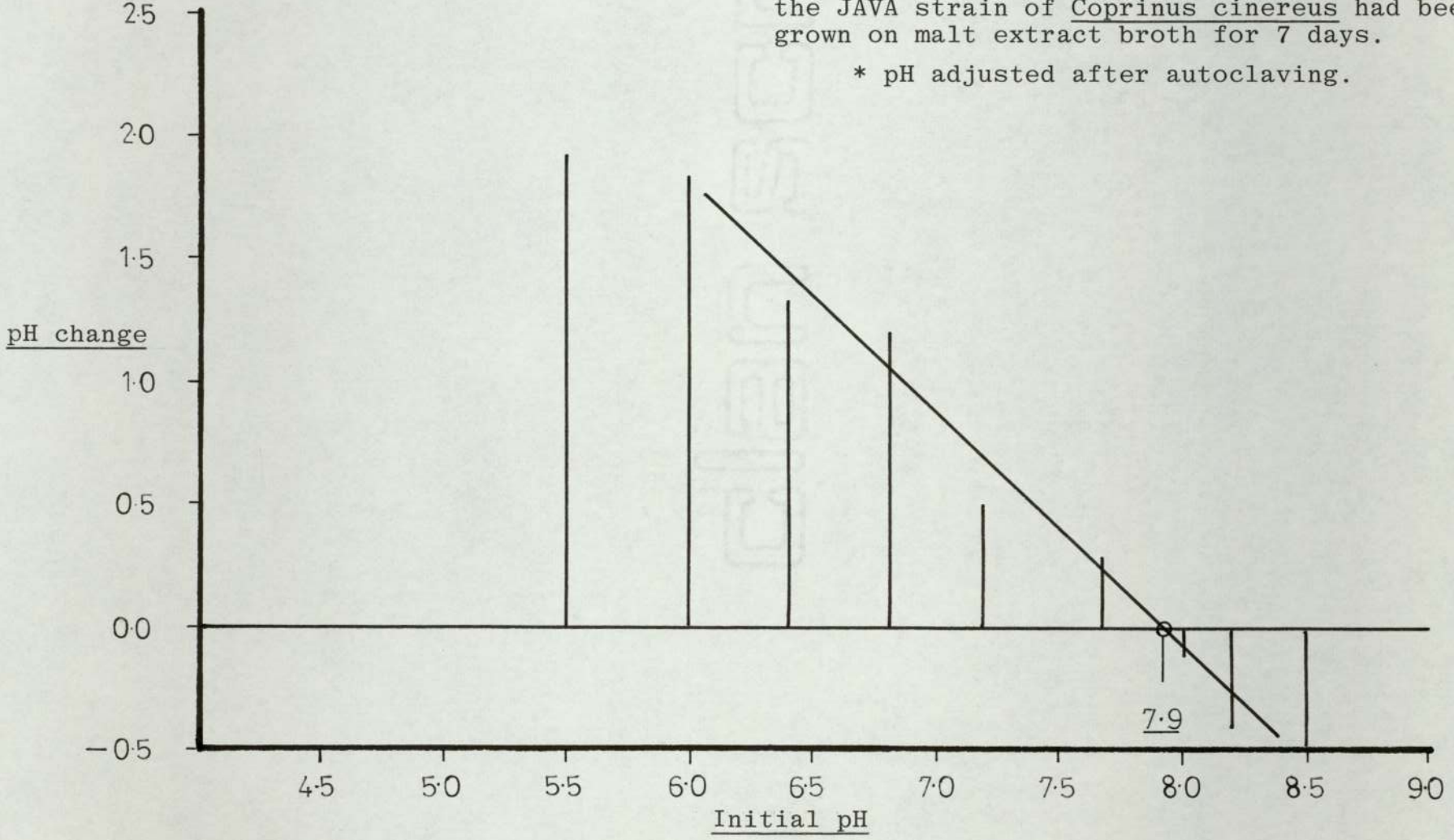


Figure 3.16

To show the pH change against initial pH after the JAVA strain of Coprinus cinereus had been grown on malt extract broth for 7 days.

* pH adjusted after autoclaving.



strains. Perhaps the most significant difference between the strains is that the Aston strain, unlike the others, was able to grow and produce a significant amount of mycelium at pH 4.5. All three strains were quite able to grow at the upper pH attained, 7.1 and all three showed a similar peak in mycelium production at initial pH between 5.5 and 6.0. Graphs 3.8 to 3.10 show the change in pH of the broth due to growth, compared to the initial pH. Only general trends can be seen from these graphs and an empirical extrapolation of the graphs suggests that at an initial pH of about 8.0 would be self buffering for all strains.

Figure 3.4 is of both singular and complementary value to figure 3.3 since using the second method of pH adjustment a much wider range of pH was obtained. All figures in table 3.4 were therefore used in compiling the graphs.

Graphs 3.11 to 3.13 show the dry weight of mycelium produced from various initial pH for the three strains. Again the Aston strain was the only one able to grow at pH 4.5. No upper pH limit was determined though growth at pH 8.5 was still quite heavy at about 200mg, for all strains. There was also correlation in the fact that two peaks of mycelial production were produced by the three strains although there were slight differences as to the initial pH's of these peaks as shown in figure 3.17:-

Figure 3.17

STRAIN	PEAK A (pH)	DRY WEIGHT OF MYCELIUM (mg)	PEAK B (pH)	DRY WEIGHT OF MYCELIUM (mg)
ASTON	5.5	220	7.5	235
JAVA	7.0	290	8.2	275
ABER.	6.0	230	7.5	260

Between the two peaks of mycelial production there was a significant drop in dry weight in all cases.

Graphs 3.14 to 3.16, by virtue of their linear fashion and close inter-strain correlation, were taken to be highly significant. From these graphs it would appear that an initial pH of approximately 8.0 would produce a high mycelial yield and be self buffering.

3.4 Discussion

The object of this exercise was to determine the pH tolerance of the three Coprinus cinereus strains and predict an optimum initial pH for further work. The outcome however, is that much more has resulted. Firstly, from both the literature survey and from practical experience, the difficulties of studying pH as a growth parameter have become much clearer. As previously explained, these difficulties are usually time-consuming to eradicate and even then the usefulness of the results obtained may still be questionable. From this has arisen another important consequence of this work. That is, a novel method of illustrating the results of pH work as shown in graphs 3.8 to 3.10 and 3.14 to 3.16. These graphs are based on the assumption that the initial pH of a medium which causes a fungus to change that pH least, yet still producing good growth, is likely to be the optimum initial pH for the growth of that fungus. The assumption seems to be correct in the case of Coprinus spp., if C. cinereus is at all typical, since the figures thus obtained fit in well with the literature and with practical experience. The figure obtained from these methods may not be medium dependent since the same initial pH was used on a different medium (see chapter 4) resulting in only a slight movement of the pH due to growth. Calam (1969) does indeed mention that monitoring the pH change of the medium can be useful in growth determinations, but he is concerned chiefly with fermentation process control.

With regard to the primary aim of this exercise, a more precise, though still incomplete range of pH tolerance for C. cinereus was compiled, to complement results already published. The range did agree well with the literature even to the point of a double peak in the graph of mycelial dry weight production against pH as described by Fries (1955 and 1956). The cause of these double peaks is a little obscure, but they could be due to some ion effects as suggested by Fries (1956). There could also be several emzyme systems in operation which are affected at different pH's although the need to elucidate the exact cause was beyond the scope of this study.

3.5 Conclusions

A new method using no buffers and monitoring the change in the pH of a medium due to growth is proposed which is less laborious than conventional methods and gives results of at least equal value which do appear to be applicable in practice. For this method to be used, liquid media must be used and initial adjustment of pH must be carried out after autoclaving.

Although a complete range of pH tolerance was not studied the Aston strain seemed to have the widest range since it was the only one able to grow at pH 4.5. All three strains were able to grow well at the highest pH studied, 8.5.

Using the novel method of study it appears that the optimum initial pH for the strains studied varied between 7.9 and 8.3.

C H A P T E R 4

The effect of various nitrogen sources and C:N ratios on the growth of *Coprinus cinereus*

4.1 Introduction

Nitrogen is an essential element in the nutrition of any living organism since it is a primary component of all proteinaceous material. Thus this nitrogen is bound up in many chemical forms within living things, due to the great diversity in protein structures. Although there is a known number of amino-acids, the building blocks of proteins, organisms must however develop specialised enzyme systems whereby these acids may be released from the protein structure before the nitrogen in them can be utilised per se or released from the amino-acid structure.

Broadly, all organisms can be placed into three groups with respect to their nitrogen requirements:-

a) Those organisms which can utilise atmospheric nitrogen.
b) Those organisms which can utilise inorganic nitrogen.
c) Those organisms which only utilise organic nitrogen.

As with many biological groupings however, these are by no means clearly defined since many organisms can adapt to two or, as in the case of the fungus Pullularia pullulans, all three sources of nitrogen (Schanderl, 1942). Although N^{15} studies have not yet proved its ability to utilise atmospheric nitrogen beyond doubt.

When dealing with fungi alone, however, one is usually concerned with a study of their utilisation of inorganic and organic nitrogen sources. Apart from the above example and certain yeasts, there are no definite records of

actual nitrogen fixation by filamentous fungi. It has never been shown in wood destroying fungi although Klingström and Oxbjerg (1964) have worked on this topic with seemingly little success. However, despite the lack of data, Merrill and Cowling (1966) and Cowling and Merrill (1966) suggest that the fixation of atmospheric nitrogen by decay fungi, possibly during their sporulating stage, could provide the necessary boost in the nitrogen content of decaying wood to maintain the rate of decay.

Nason and Takahaski (1958) suggest that inorganic nitrogen is the ultimate source of nitrogen for all living things since plants, which form the base of most food chains utilise nitrogen in this form. However, although the fungi are considered as members of the plant kingdom, they are either saprophytes or parasites and therefore have generally adapted their physiology to utilise organic nitrogen sources. Any ability on the part of fungi in general to employ inorganic nitrogen sources must therefore be considered a secondary adaptation.

Although Cochrane (1958) suggests that nitrogen metabolism in fungi has been neglected, he does quote over six hundred references, many of which show that this secondary adaptation to inorganic nitrogen utilisation has been made by many, but by no means all groups of fungi.

The majority of work on inorganic nitrogen sources is confined to nitrates, nitrites and ammonium salts, since it appears that other nitrogen sources can only be utilised

if they can easily be changed into one of these forms (Steinberg, 1939).

Fungi seem to vary considerably in their efficacy towards nitrates, many varieties of fungi being able to produce excellent growth (Kaarik, 1960; Brewer, 1959; Malca, 1966; Hasckaylo et al., 1954; Lilly and Barnett, 1951; Morton and MacMillan, 1954), others, especially the higher Basidiomycetes produce only poor growth, (Fries, 1956; Hacskaylo, 1954; Norkrans, 1950; Jennison et al., 1955; Henningsson, 1967; Lindeberg, 1944; Treschow, 1944), whilst others such as the Saprolegniacae (Reischer, 1951), the Blastocladiales (Cantino, 1955) and many other groups (Dulaney, 1948; Erikson, 1952; Thornberry and Anderson, 1948) including parasitic species definitely could not grow on media where nitrate was the only source of nitrogen.

Much less work has been carried out on nitrite as a nitrogen source to fungi, probably since it appears to be toxic under the acid conditions which favour many fungal species, (Steinber, 1944; Cochrane, 1958; Steinberg and Thorn, 1940 and 1942). However, nitrite does appear to be able to support the growth of several fungi which have been studied (Tally and Blank, 1942; Morton and MacMillan, 1954; Steinberg, 1939) including several Coprinus species (Fries, 1955).

The ammonium ion is generally regarded as being a suitable nitrogen source for many fungal types (Morton and MacMillan, 1954), with only a few members, especially in

the lower Phycomycetes being unable to utilise it: (Golueke, 1957; Barner and Cantino, 1952; Schade and Thimann, 1940). However, problems can arise using ammonium compounds from two areas. Firstly ammonia in high concentrations can be toxic to fungi (Gunter, 1956; Neal et al., 1933) and secondly, removal of the NH_4^{++} ion by the growing fungus can result in a severe drop in pH which often results in producing conditions unsuitable for fungal growth (Isaac, 1949; Dulaney et al., 1956). This drop in pH has been well charted by many workers using many different fungi and it appears quite conclusively that the cation is preferentially utilised forming disociated mineral acids in the medium (Barnett and Lilly, 1956; Crasemann, 1954; Shirahawa, 1955; Curtis et al., 1951; Treschow, 1944). Morton and MacMillan (1954) and Kinsley (1961) showed that when ammonium nitrate was incorporated into a medium, the ammonium ion was used and a feedback mechanism actually prevented nitrate utilisation until the level of ammonia within the medium had dropped.

Several workers have also shown another interesting aspect of ammonium utilisation in some fungi. During the phase of ammonia assimilation, various organic nitrogen compounds are formed in the growth medium which cannot be accounted for by autolysis. Morton and Broadbent (1955) showed that these compounds were chiefly peptides which could not be reutilised by the fungus that produced them. Dulaney et al. (1956) using Ustilago maydis showed that with ammonium hydrogen orthophosphate as a nitrogen source, 83% of the nitrogen added remained in the medium; 40% of this

had been converted into organic nitrogen compounds of which 53% was in the form of a wide variety of amino-acids.

Previously it was stated that the ability to utilise inorganic nitrogen was probably a secondary adaptation by fungi, yet it appears that practically all fungi can utilise the ammonium ion. An explanation of this can be found upon examination of the basic chemistry of nitrogen assimilation by fungi which can be split into two major areas: Firstly, the 'normal' method is for fungi to produce enzymes which can de-polymerise proteins and peptides of plant and animal material, thereby releasing amino-acids which can be absorbed as such, and utilised by the fungus. The second method is where the fungus has the ability to produce enzymes to transform inorganic nitrogen sources into ammonia which can then be absorbed to produce glutamic acid. Other amino-acids are then produced by transamination. This work by Nason (1956) therefore suggests that most fungi are able to produce protein by assimilation of ammonia whilst when faced with other inorganic nitrogen sources protein production cannot take place unless ammonia can be produced from the particular nitrogen source.

Organic nitrogen compounds are composed chiefly of amino-acids, amines, nucleic acids, various alkaloids and urea. With the exception of the latter, all are bound up in plant or animal material and can be considered to be the 'natural' nitrogen sources for most fungi. Due to the many naturally occurring amino acids, selecting a representative is difficult. L-asparagine was chosen due to its availabil-

ity and previous proven results (see later). This is a relatively expensive source of nitrogen so urea, which is cheaper, was also tested.

Whilst L-asparagine does not produce the greatest growth of Venturia inaequalis (Pelletier and Keitt, 1954) it is generally regarded as being acceptable to a wide range of fungi (Cochrane, 1958). Similarly, urea has been shown to be suitable for many fungi including various Mucorales (Bach, 1927), Aspergillus spp. (Nicholson et al., 1931), Penicillium spp. (Dox, 1909), fleshy Basidiomycetes (Garren, 1938) and particularly Coprinus spp. (Fries, 1955). The most complete work on the nitrogen requirements of Coprinus species was by Fries (1955), but Coprinus cinereus is not included in that study and therefore a study on the growth of this fungus on various nitrogen sources was of particular interest as well as providing more information required in connection with the main theme of this study.

No study of the nitrogen nutrition of fungi should be considered complete without reference to the carbon to nitrogen ratio of the nutrient solution. This ratio is often referred to simply as the C:N ratio, and it is extremely important, especially in natural situations where, in the presence of abundant carbohydrates, the amount of nitrogen can be limiting; (Stotsky and Norman, 1961a; Finstein and Alexander, 1962). The C:N ratios of commonly used nutrient solutions is given by Levi and Cowling (1969), an extract of that paper shows:-

Medium	C:N ratio
Difco agar	290:1
Malt extract agar	115:1
Potato dextrose agar	180:1
Malt extract broth	32:1
Nutrient broth	10:1

There is obviously a wide variation between these media, and therefore the C:N ratio of any medium should be borne in mind when viewing results, especially if these results are to be compared with those obtained by other workers. Although not stated as such, Fries (1955) used a C:N ratio of 40:1 whilst Hacskaylo (1954) used 25:1 and Hedger (1972) used 50:1, when carrying out nitrogen nutrition studies on Coprinus and other Basidiomycete species.

Previous experiments within this study have used differing C:N ratios within different media and therefore it was decided to study the behaviour of Coprinus cinereus under different C:N ratios using the same medium. However, cellulose was used as the carbon source for this experiment since this, rather than glucose (which has been used in the majority of these types of test), forms a major part of the carbon source of the materials to be used in later work. Although hemicelluloses are also a very important constituent of natural plant materials, these chemicals are so complex and often species specific that an attempt at producing the correct chemical makeup of natural materials within a liquid broth was considered unjustified for a study of this type.

4.2 Materials and Methods

A stock solution was made up, to which various amounts of the nitrogenous compounds were added to give 0.1gm of nitrogen per litre of nutrient solution. The chemical constituents of the stock solution was as follows:-

Glucose	10gm
NaCl	0.2 gm
$MgSO_4$ $7H_2O$	0.2 gm
$CaCl_2$	0.1 gm
K_2H PO_4	0.87gm
KH_2 PO_4	0.14gm
$ZNSO_4$ $7H_2O$	4.05mgm
$MnSO_4$ $4H_2O$	4.43mgm
Thiamine	100 mgm
H_2O	1000 mls

The following nitrogenous compounds were tested:- Potassium nitrate, Sodium nitrite, Ammonium nitrate, Ammonium phosphate, Ammonium tartrate, Urea and L-asparagine. All chemicals were of analytical reagent quality.

20ml portions of the nutrient broth were placed in 100ml conical flasks and autoclaved, in all cases except where urea was used as the nitrogen source. Urea dissociates during autoclaving so this was filter sterilised in solution. The pH of the nutrient broth was then adjusted to 7.8 by the addition of 0.5mls of sterile 0.1N sodium hydroxide to each flask. Each flask was then inoculated with a 6mm plug of Coprinus cinereus mycelium which had been grown up on plates containing 20mls of malt extract

agar, pH 5.3. Incubation was at 35°C for seven days after which time the mycelial mats were filtered, washed, dried and weighed as described in chapter 3. The residual solutions were tested for pH and due to some interesting colour changes, were photographed (see plates 4A, 4B, and 4C). All three strains of Coprinus cinereus were again used and there were four replicates of each strain for each nitrogen source.

Although the results of the nitrogen study showed that L-asparagine was in general a marginally better nitrogen source for Coprinus cinereus than urea (see later), the latter was used in the C:N ratio experiments due to its low cost which was an important consideration regarding further practical applications of the work. The same stock solution constituents were used as for the nitrogen work except that cellulose was used as the sole carbon source instead of glucose as explained in the introduction to this chapter.

100gms of Whatman cellulose powder per litre of distilled water was ball-milled for seventy-two hours and to the resulting suspension were added the constituents of the stock solution. After gentle warming of the whole to dissolve the various salts, the suspension was divided into several batches to which urea was to be added to give a range of known C:N ratios. 20ml portions were poured into 100ml conical flasks which were then plugged with cotton wool and autoclaved. After autoclaving, a filter sterilised solution was aseptically pipetted into each flask to give

the required range of C:N ratios. The pH of each flask was then adjusted to 7.8. Inoculation and incubation was carried out as described previously; the only change being a twelve day incubation period rather than seven days.

Some difficulty was encountered upon harvesting due to the fungus forming a semi-solid mass throughout, as well as on the surface of the growth medium. A method was devised whereby the mycelium was harvested by flotation, since it seemed to be less dense than the cellulose when the contents of the flasks were poured into a beaker containing about 500mls of water. The mycelium, along with small amounts of adhering cellulose could then be removed with a spatula for washing, drying and weighing. Although this method was rather crude it was easy to perform and gave consistent results judging by the small deviations from the mean in the dry weight results for each major variable.

4.3 Results

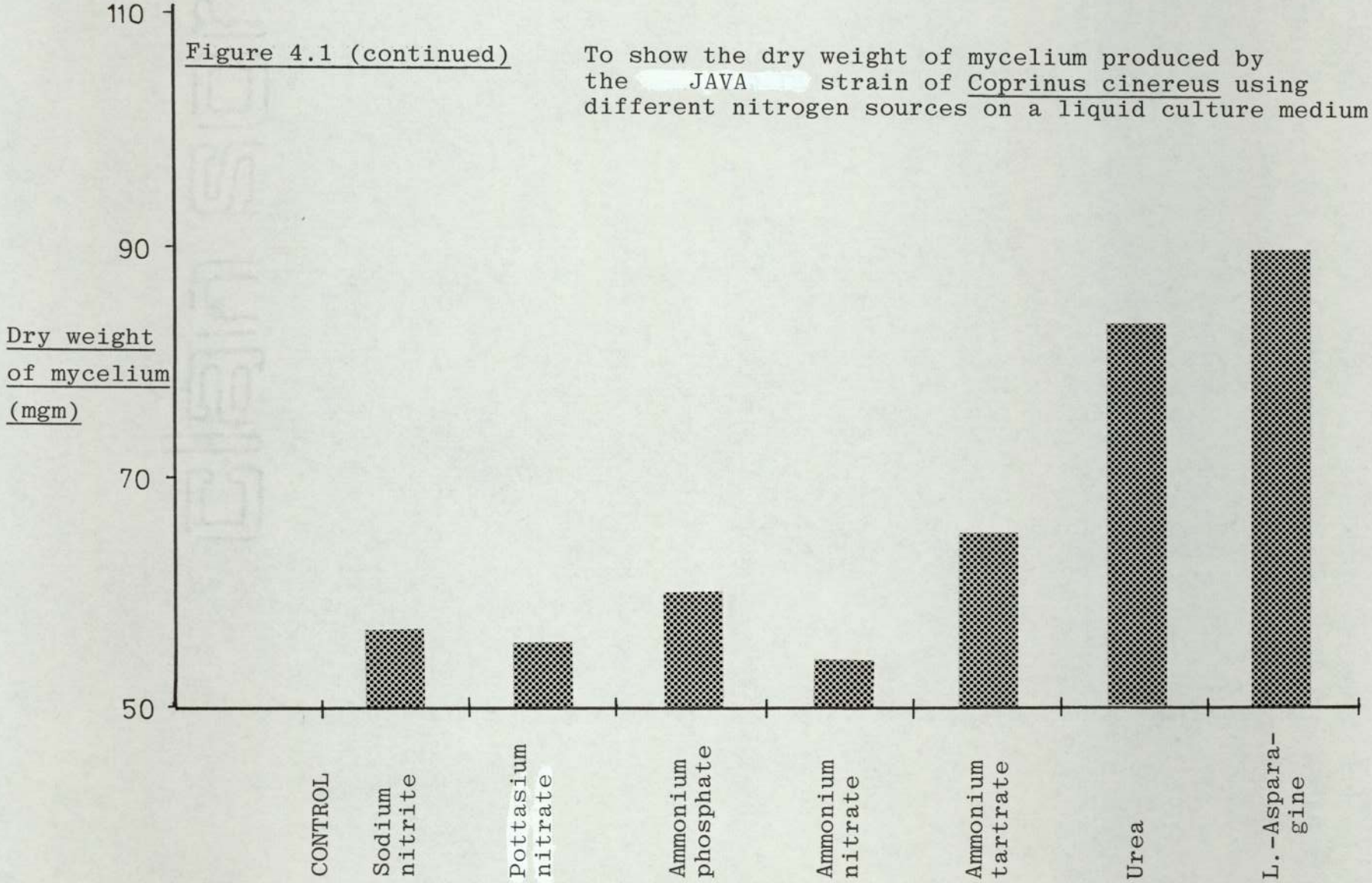
The results of the nitrogen source experiments are expressed in figures 4.1 to 4.3 and those of the C:N ratio experiments in figures 4.4 to 4.6.

It is probable that most of these results will be of greatest value if viewed with regard to general trends rather than specific instances. With this in mind, figures 4.1 and 4.2 are designed to enable any trends to be distinguished rapidly. Figure 4.1 shows that in the case of the Java and Aston strains of Coprinus cinereus, there is a tendency towards preference to organic nitrogen compounds although ammonium nitrate did produce good growth in the Aston strain. This trend does not appear in the Aberystwyth strain since there is a slight, though definite preference for inorganic nitrogen sources; ammonium nitrate giving the best growth in this case. The actual weight of this growth was, however, considerably smaller than the mycelial weights of the Aston and Java strains when grown on their most suitable nitrogen source.

Figure 4.2 shows the strain variation between the strains for each different nitrogen source, together with the actual average dry weight of mycelium produced per flask. The general trends from figure 4.2 are firstly that on all nitrogen sources except urea, the Aston strain produced heavier growth than the other two strains; and secondly that the Java strain produced more growth than the Aberystwyth strain on organic nitrogen whilst the reverse

Figure 4.1 To show the dry weight of mycelium produced by the ASTON strain of Coprinus cinereus using different nitrogen sources on a liquid culture medium

Figure 4.1 (continued) To show the dry weight of mycelium produced by the JAVA strain of Coprinus cinereus using different nitrogen sources on a liquid culture medium



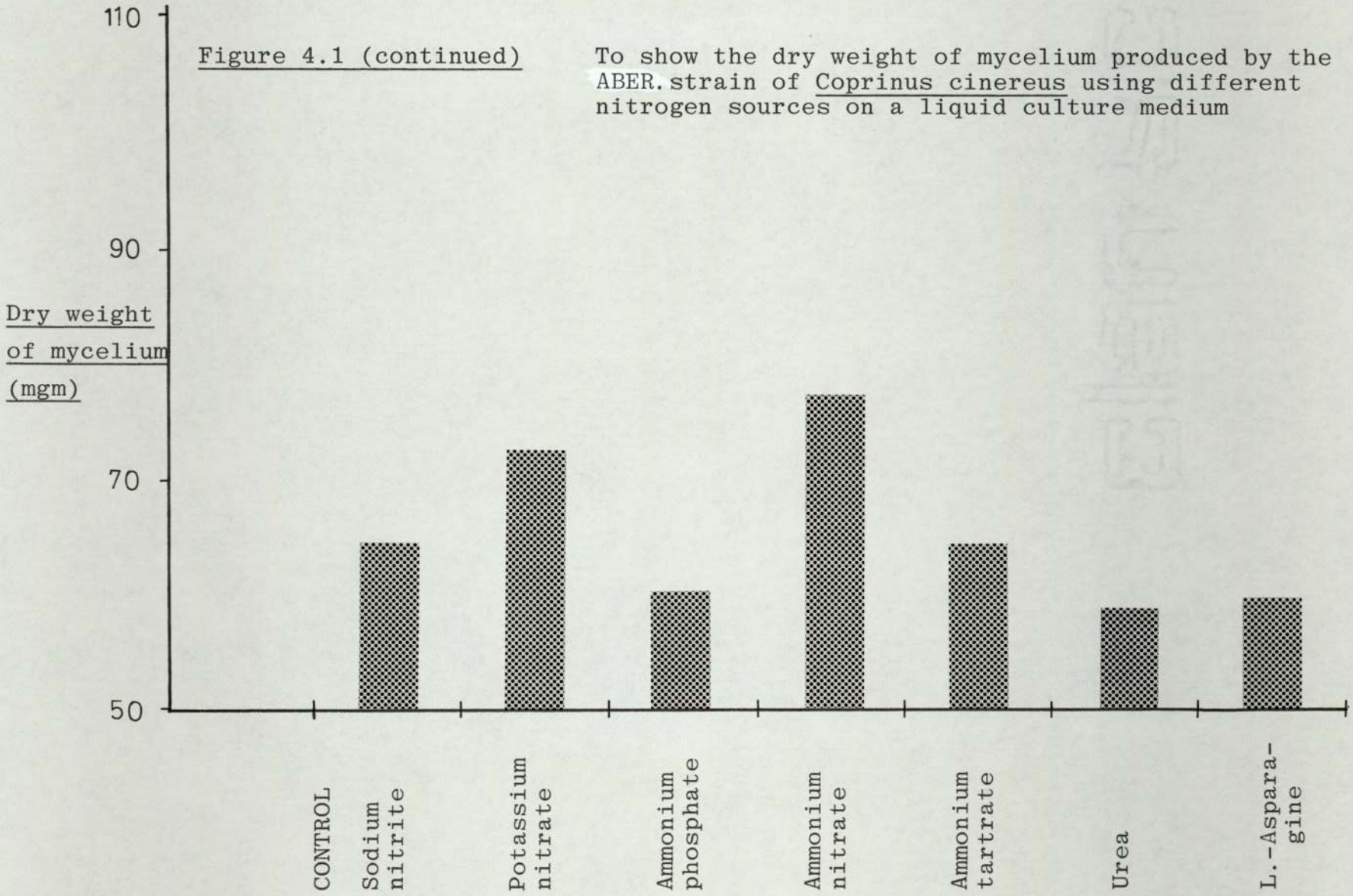


Figure 4.1 (continued) To show the dry weight of mycelium produced by the ABER. strain of Coprinus cinereus using different nitrogen sources on a liquid culture medium

Figure 4.2 Comparative mycelial dry weight production in grams by the three strains of C. cinereus on various nitrogen sources.

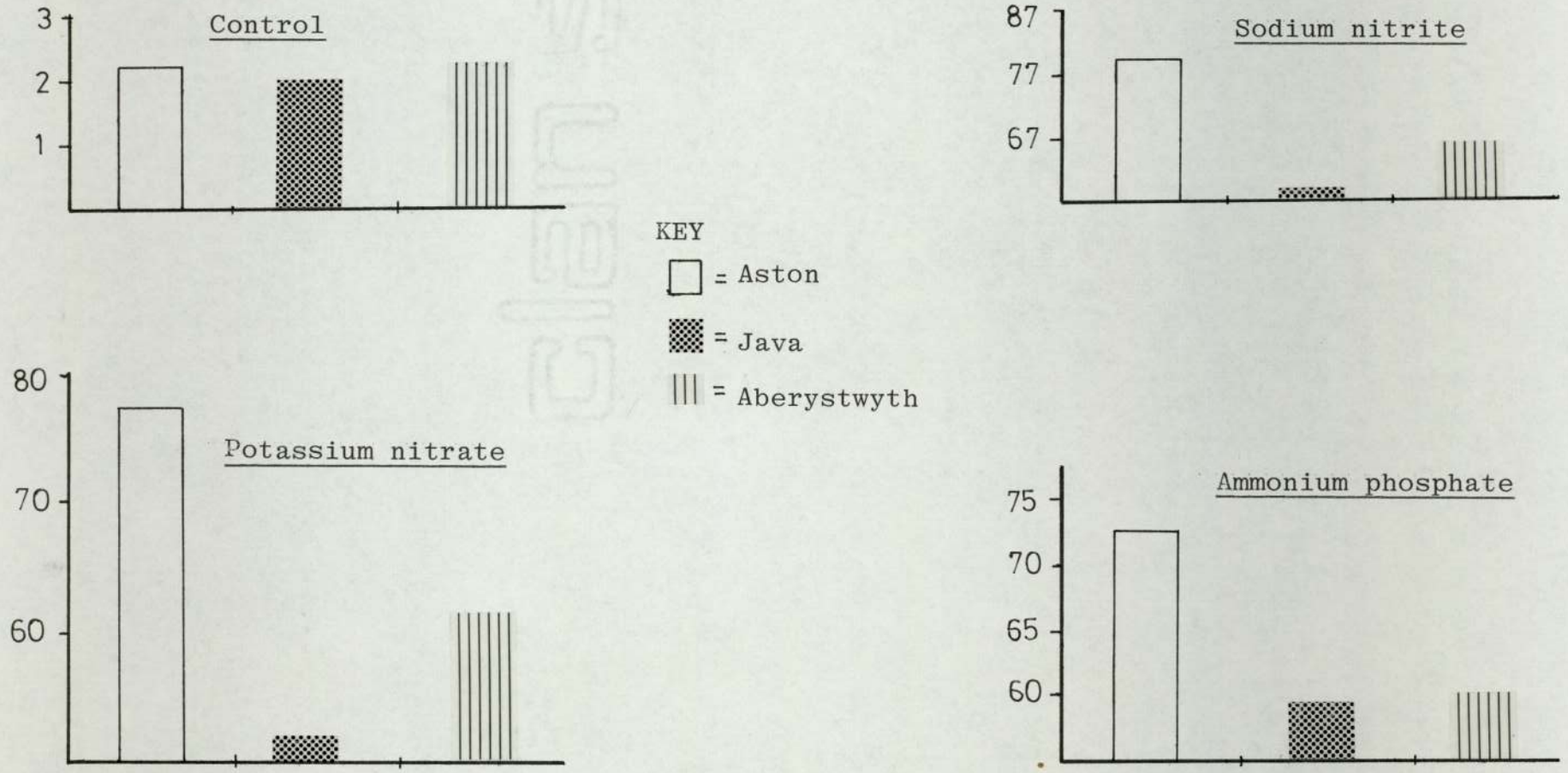
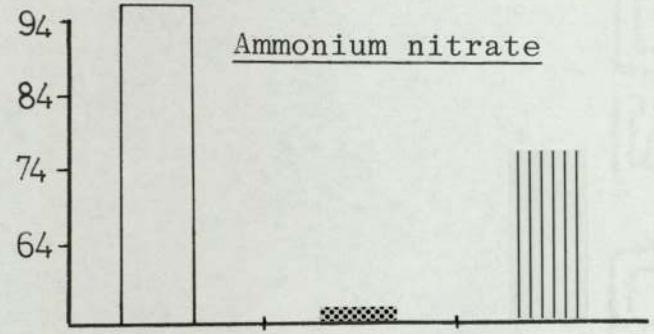
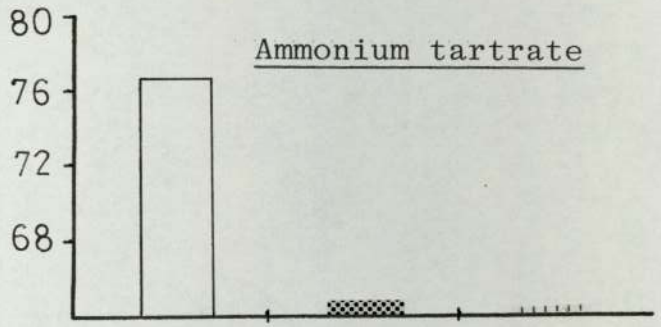
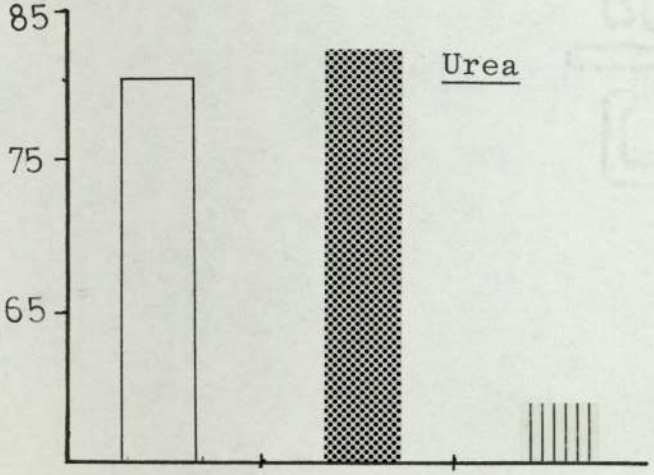


Figure 4.2 (continued) Comparative mycelial dry weight production in grams by the three strains of C. cinereus on various nitrogen sources







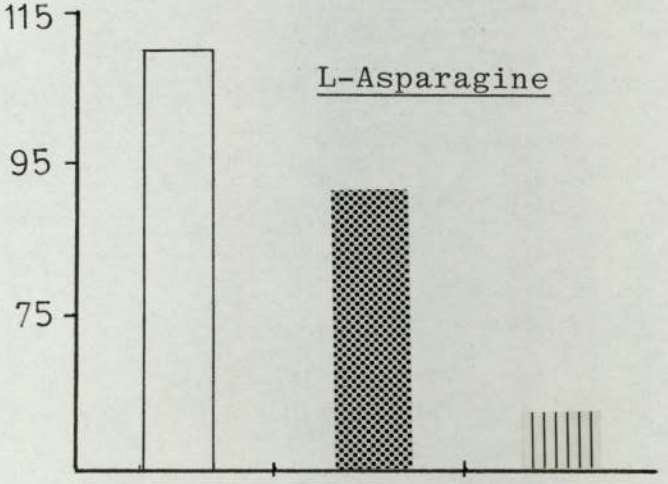


Figure 4.3

To show the mycelial dry weight production and final pH when the three strains of C. cinereus were grown on different nitrogen sources

JAVA	ABER.	ASTON	Average dry weight of mycelium per flask (mg)
			Nitrogen source
88.4	58.9	107.0	L. Asparagine
7.4	7.3	7.2	Final pH
82.7	58.6	80.1	Urea
7.3	7.4	7.4	Final pH
64.8	64.0	77.9	Ammonia tartrate
4.9	7.3	4.4	Final pH
54.5	77.8	96.2	Ammonium nitrate
7.1	7.3	6.7	Final pH
59.9	60.0	72.3	Ammonium phosphate
4.8	6.4	5.8	Final pH
54.6	72.4	78.7	Potassium nitrate
7.9	7.8	7.8	Final pH
57.3	63.9	78.3	Sodium nitrite
7.4	7.4	8.0	Final pH
2.0	2.0	3.4	CONTROL
7.8	7.8	7.8	Final pH

Figure 4.4 Variation of mycelial dry weight production by the three strains of C. cinereus at different C:N ratios

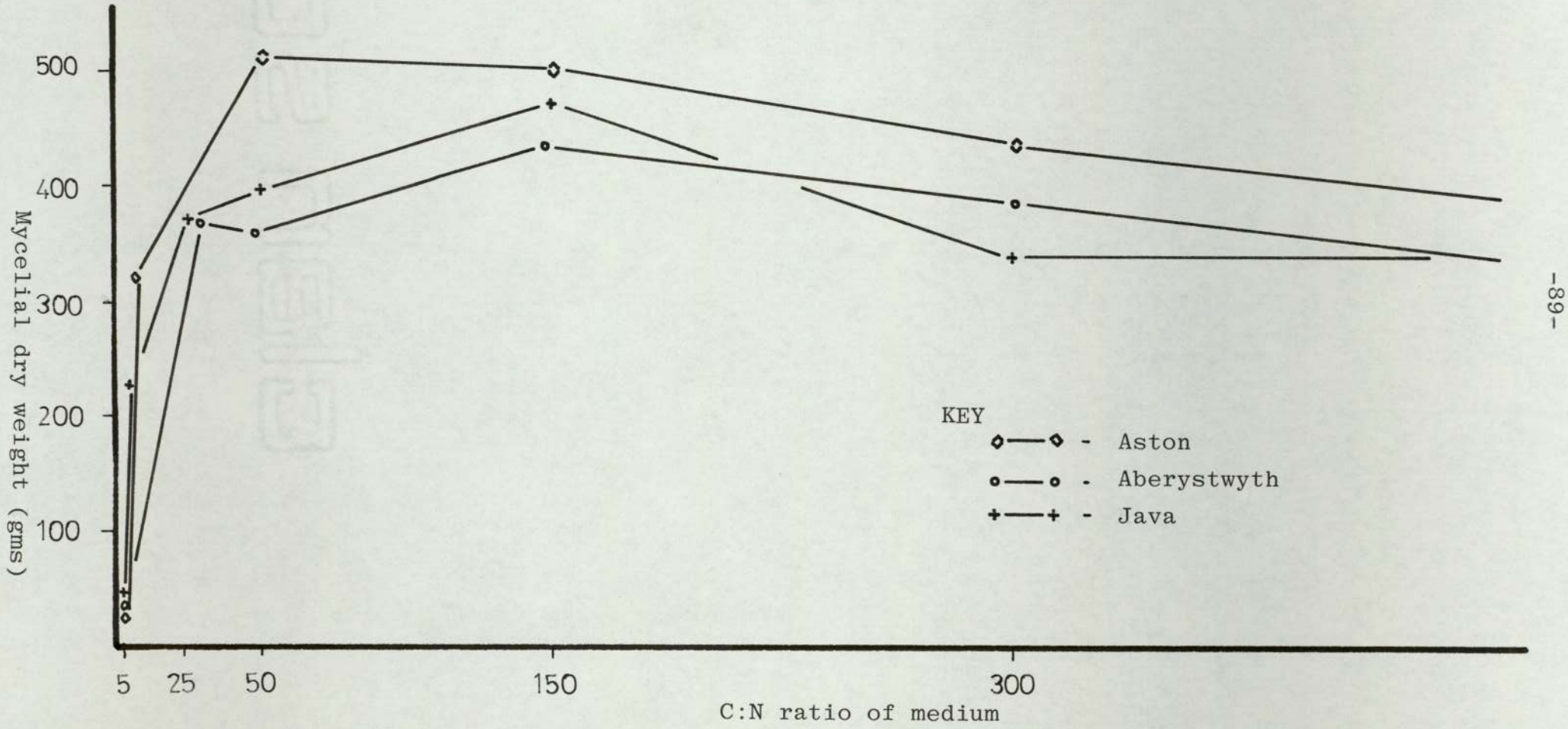


Figure 4.5 Variation of mycelial dry weight production by three strains of C. cinereus at different C:N ratios (expressed logarithmically)

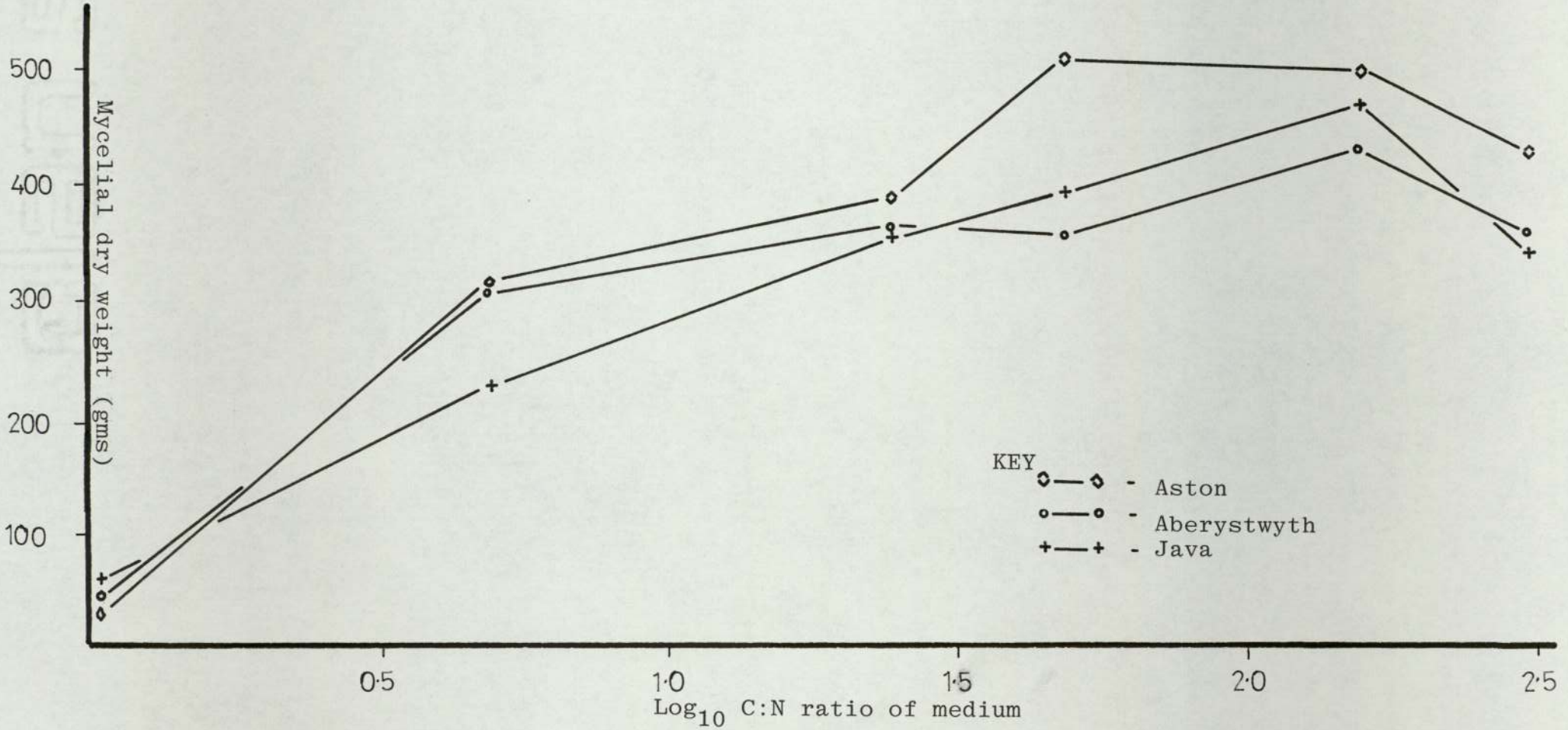


Figure 4.6

To show the mycelial dry weight production and final pH when the three species of C. cinereus were grown on the same medium with varying C:N ratios

Dry Wt. of Mycelium (mgm) / C:N ratio	ASTON	FINAL pH	ABER.	FINAL pH	JAVA	FINAL pH
CONTROL	25.8	7.8	28.7	7.8	52.5	7.9
5:1	320.8	8.2	307.8	8.4	225.6	8.2
25:1	391.5	7.9	368.3	7.9	352.6	8.1
50:1	514.9	7.6	363.0	7.7	391.0	7.8
150:1	501.8	7.4	434.8	7.4	472.4	7.4
300:1	431.2	7.1	364.4	6.9	344.1	7.1
500:1	378.8	7.1	335.7	6.9	352.7	6.9

Plate 4A Colours of growth media after growth of the ASTON strain

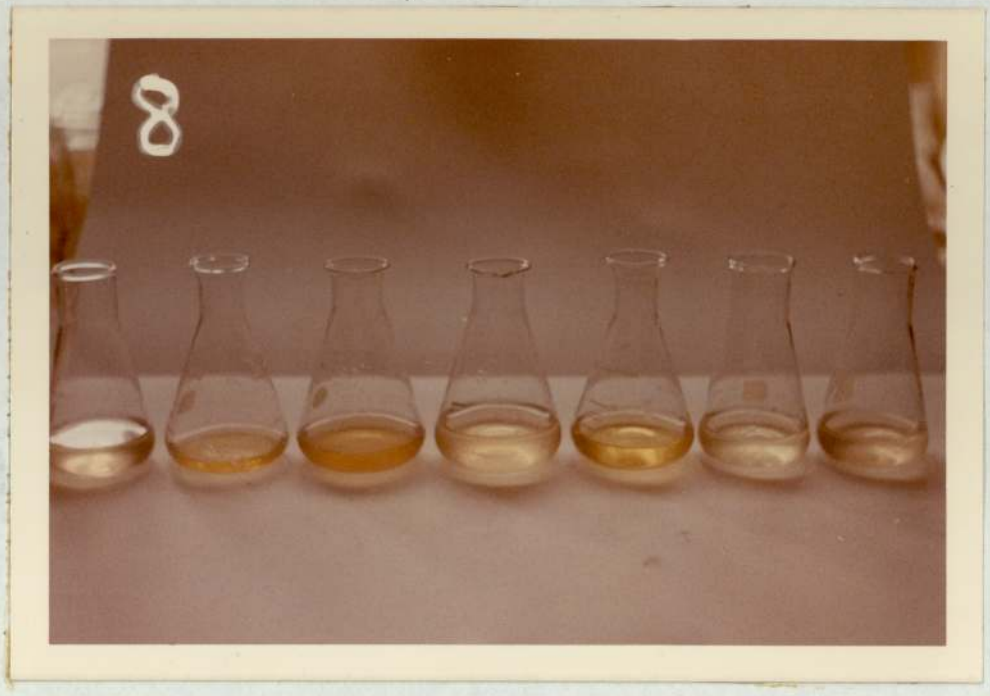


Plate 4B Colours of growth media after growth of the ABERYSTWYTH strain

Plate 4C Colours of growth media after growth of the JAVA strain

was true for inorganic nitrogen sources.

Figure 4.3 contains all of the information from which figures 4.1 and 4.2 were compiled, together with information on the changes in pH within the growth media. This did not change by more than 0.5 pH units from the starting pH of 7.8, except in the following cases. The Aston and Java strains produced pH drops of 3.4 and 2.9 pH units respectively when grown on ammonium tartrate, although the Aberystwyth strain only dropped 0.5 units after producing nearly the same amount of growth as the Java strain. Growth of all strains on ammonium nitrate caused an average drop of 0.8 pH units whilst growth on ammonium phosphate caused an average drop of 2.4 pH units.

Figure 4.4 and 4.5 both show the relationship between the C:N ratio of the growth media and the dry weights of mycelia produced by the three strains of Coprinus cinereus. Both graphs are essentially the same except that in figure 4.5 the C:N ratio is expressed logarithmically to separate the points at low C:N ratios.

Figure 4.4 shows that there is a rapid rise in mycelial production at C:N ratios between 5:1 and 50:1 after which there is a levelling off and a slight drop in mycelial production up to a C:N ratio of 500:1.

Figure 4.6 contains the information from which the last two figures were compiled together with the pH changes within the growth media. There is a very definite trend in

all strains in that low C:N ratios cause a slight rise in pH, at a 50:1 ratio there is no change, whilst higher C:N ratios, up to 500:1 cause progressively larger drops in the pH of the medium.

In addition to these tabular and graphic results, photographs 4A, B and C have also been included. It was noted that after the nitrogen experiments, quite vivid colours had been produced within the growth media. In general the Aston (4A) strain produced very pale pink or yellow colours on all the nitrogen sources whilst the Aberystwyth strain (4B) produced deep red colours on all nitrogen sources. The Java strain (4C), however, produced equally strong yellow colouration in all the flasks. It should be pointed out that these colours did fade quite considerably during the twenty hour period needed to arrange for photographs to be taken and thus the photographs do not illustrate the phenomenon very clearly. Whilst the reason for these colour reactions is, as yet, not fully understood, it was felt that the photographs and comments should be included so that they could be discussed later and possibly form the basis of further research.

4.4 Discussion

Probably the most surprising result of the experiments using different nitrogen sources was the comparative differences in the preferences of the three strains of Coprinus cinereus, especially since the previous two chapters have shown them to be fairly consistent with regard to temperature and pH relationships. As explained in the results section, there appears to be a preference for organic nitrogen in the Java strain, a much less definite preference in the Aston strain and a preference for inorganic nitrogen in the Aberystwyth strain. However, in the introduction to this chapter it was explained that the ability to utilise inorganic nitrogen was probably an adaptive effect and it is felt that the results of these experiments show that this theory may well be correct. The Java strain had been sub-cultured much less frequently (although exact figures are not available), prior to its use in these experiments, whereas the Aston and Aberystwyth strains had been sub-cultured many times before being used. Cochrane (1958) explains that the capacity to utilise nitrate nitrogen can be lost by mutation, but that failure to utilise nitrate is presumed to be absolute. If the Java strain is considered the more natural strain then it would appear that the other two have, through continuous sub-culturing, have adapted to inorganic nitrogen sources, including nitrate. Obviously more research is needed with regard to this phenomenon since this is far from a complete solution due to the fact that many of the sub-culturing was done onto media containing organic nitrogen in the form of

peptone.

Yet another rather surprising outcome of this series of experiments was the high average dry weights of mycelium per flask as compared to those recorded by Fries (1955). The highest recorded dry weight that Fries found was 62.5 mgm when Coprinus fimetarius was grown for five days using asparagine; whereas the lowest weight recorded in this work was 54.5mgm when the Java strain was grown on ammonium nitrate for seven days. Since the same medium was used in both sets of experiments (except for the exclusion of buffers in the present case) the differences in the results must be attributed to a combination of the following reasons:-

a) The species Coprinus cinereus may produce more mycelium in culture than do any of eighteen Coprinus species investigated by Fries, no matter what the nutrients.
b) The buffer system used by Fries may have had some effect on the growth of the species tested.
c) There may have been some fundamental difference in methodology, resulting in the variations.
d) The conditions of pH and temperature as supplied to the strains of Coprinus cinereus may have been comparatively more suitable than those supplied to those species used by Fries.

Although any one of these possibilities would probably not account for the wide differences, a combination of all or some of them could have a summatory effect.

The very small changes in the pH of most of the flasks

supports the theory expressed in the previous chapter with regard to determining the optimal initial pH for this fungus. As expected, the largest drops in pH were experienced when ammonium compounds were used. Tartaric, nitric and phosphoric acids were produced in small quantities when their respective ammonium salts were used, causing a drop in pH. The final pH of the ammonium nitrate series was higher than those of ammonium tartrate and phosphate, but this was probably due to the fact that sufficient ammonium ions had been utilised to allow nitrate reductase to be produced. The nitrate ions were then being utilised, causing the pH to rise.

It appears that Coprinus cinereus, like the Coprinus species investigated by Fries (1955), belongs to the relatively small group of nitrite utilisers. However, this may be due to the fungus' preference for alkalinity which appears to reduce any toxic effects of nitrite (Cochrane, 1958).

On the colour changes within the growth media as shown in the results section are difficult to explain especially since the C:N ratio was the same, 25:1, for each experiment. The most logical suggestion being that they are caused by the release of amino compounds into the media, by the fungus. Although why these amino compounds are strain specific over various nitrogen sources and how they produce such colour changes can only be determined by further research.

Since no previous work has been published on the optimal C:N ratios for the growth of Coprinus cinereus, one had no pre-conceived ideas of what results were to be expected. However, guidelines were obtained from work done on wood destroying and other dung and litter fungi by other workers (Cowling, 1970; Griffin, 1972; Levi and Cowling, 1969; Lodha, 1974; Forbes, 1974). The genus Coprinus comprises terrestrial, lignicolous and coprophilous species, (Webster, 1970) therefore studying the C:N ratios within these environments should give an idea of the range of C:N rations to be used. However, the amounts of nitrogen in the various substrates on which Coprinus and similar species can be found, varies widely; for example 10:1 (tomato foliage) up to 1,250:1 (Sitka spruce heartwood), (Levi and Cowling, 1969).

The optimal C:N ratios from the results of between 50:1 and 500+:1 suggests that Coprinus cinereus is primarily a litter fungus since dung tends to have a lower C:N ratio, (Lodha, 1974). The results also show, however, one factor which is commonly attributed to only wood destroying fungi, the ability of the fungus to utilise substrates deficient in nitrogen, (Cowling, 1970). There seems to be little difference to mycelial production between C:N ratios of 50:1 and 500:1, all three strains being consistent in this respect. This ability to adapt to low nitrogen content is explained by Griffin (1972) and it appears that these fungi are able to re-utilise their own cellular nitrogen by internal translocation.

The effects on the pH of the growth media at different C:N ratios however, are more difficult to explain. At very low C:N ratios ammonia is given off and can be detected by smell. This, then, is the probable cause for the rise in pH at low C:N ratios of 5:1 and 25:1. A ratio of 50:1 showed virtually no pH change as well as heavy growth and therefore this was taken as being optimal. Any rise in the C:N ratio above 50:1 caused an inverse drop in pH. It is unlikely that this drop could be caused by release of amino-compounds into the medium since there is a less than optimal amount of nitrogen in the medium. One possible explanation is that autolysis of the mycelium takes place earlier as the C:N ratio increases, although evidence by Levi and Cowling (1969) suggests that for many Basidiomycetes, a higher C:N ratio retards autolysis.

As noted in the introduction to this chapter, the purpose of these experiments were two-fold. Firstly, the actual work has provided basic data on the nitrogen metabolism of this particular organism which has helped in its characterisation. Secondly the results will be of use in further work by both the author and others working at the B.I.C. in the field of fungal growth and colonisation on non-sterile cellulosic materials.

To summarise the results very briefly:-

a) All three strains of C. cinereus showed an ability to utilise a wide range of nitrogen sources.

b) The Java strain showed a definite preference for organic nitrogen, the Aston a less pronounced preference

and the Aberystwyth strain showed no preference for organic nitrogen but a slight preference for ammonium compounds.

c) There were some interesting, though probably very complex colour and pH changes caused by the growth of the fungi on different nitrogen sources.

d) A C:N ratio of approximately 50:1 using urea and cellulose seemed to be optimal for the Aston strain, whilst higher C:N ratios also produced good growth for all three strains.

CHAPTER 5

The ability of *Coprinus cinereus* to colonise non-sterile wheat straw and resist the growth of other fungi.

5.1 General Introduction

Probably one of the greatest problems facing workers in microbial ecology and physiology is the transition from laboratory conditions to more natural environments. It appears at times as though knowledge of the behaviour of a fungus under sterile, standardised, laboratory conditions can be of only limited use in predicting the behaviour of that same fungus under the plethora of conditions encountered in more natural environments.

The aim of the work outlined in the following text was to determine whether or not the growth optima for Coprinus cinereus as stated in previous chapters, would be relevant when the fungus was grown under non-sterile conditions on a natural substrate. This natural substrate was to be wheat straw, which had already been shown to support the growth of C. cinereus by various workers at the B.I.C. The work to be described was performed as three discreet sets of experiments each of which will be described and discussed separately. This chapter will then be concluded with a general discussion encompassing all three sets of experiments.

5.2 Experimental I

5.2.1 Introduction

Chapters 3 and 4 used the dry weight production of C. cinereus grown on liquid media to determine optimal growth conditions, but to use this criterion with straw as the growth substrate would have introduced large errors as well as being complex and time-consuming to carry out. A much simpler method of assessing the value of the substrate was thus devised, consisting simply of measuring the fungal advance through the straw on a linear basis from a single inoculation point. To achieve this, the straw was placed in glass tubes approximately 30cms in length (see Fig. 5.0), which were supplied with holes from which straw samples could be taken. Obviously this method was difficult to standardise precisely, but it did enable many replicates and many isolations to be taken. In fact the results were surprisingly consistent within each variable. As mentioned, the criterion for assessing growth was to that in chapters 3 and 4, but it must be remembered that in chapters 3 and 4 the growth experiments were carried on for a relatively short period of time and therefore the results obtained did also reflect the rapidity, as well as the amount of growth.

Apart from the radial extension of fungal colonies on agar as described in chapter 2, little evidence of similar work to that described later could be found in the literature. The standard text on linear extension of fungal colonies was performed by Fawcett (1925) in a study to determine if senility became apparent in a constantly growing

and spreading colony. Two citrus disease fungi, Pythiacystis citrophthora and Diplodia natalensis were grown on corn-meal agar which formed a shallow layer in the bases of glass tubes 1.5 metres long. The differences between this system and the one described later are obvious, but two of Fawcett's conclusions are worth noting. Firstly that there did not appear to be any "grand period of growth", rather a steady growth throughout the twenty-seven week experimental period. Secondly that P. citrophthora ceased to move forward after sixteen weeks whereas D. natalensis showed no signs of senility after the full experimental period. The first should prove useful in the compilation and explanation of results whereas the second gives an idea that the problem of senility should not effect the following experiments since an experimental period measured in hours rather than weeks will be used.

In the event of the theoretical growth optima not producing good growth in practice it was decided to fix certain growth optima and permutate others in order to find the best combination.

The temperature was fixed at 35°C for all tests whilst the pH was fixed at 7.8 in all but that straw to which only water was to be added.

The wheat straw used in these experiments had been shown to have a C:N ratio of approximately 50:1 by other workers at the B.I.C. and therefore by addition of water, should have constituted a suitable growth substrate for

C. cinereus. This was used as a control. Another set of experiments was carried out using straw to which a nutrient salts solution was added (see method section) to provide any trace elements and thiamine which may have been lacking in the controls. This straw also had the pH adjusted to 7.8.

Fries (1956) states that a small addition of glucose to any of her media produced rapid initial growth of the Coprinus spp. she used. For this reason a set of experiments was carried out using straw plus nutrient salts at pH 7.8 plus 0.5gms of glucose per 10gm of straw.

Much interest in the fungal colonisation of straw was generated at the B.I.C. when Coprinus cinereus was found to grow very well on straw to which nitrogen had been added. For this reason two sets of experiments were carried out using different concentrations of added nitrogen in the form of urea. The effects of adding nitrogen to natural substrates has been studied by several workers, with a strong emphasis towards wood. Findlay (1934 and Duncan (1960) state that additions of nitrogen, including L-Asparime, to wood resulted in an increase in decay of wood by fungi. However, Schmitz and Kaufert (1936) and Kaufert and Behr (1942) came to exactly opposite conclusions stating that the addition of urea to wood in aconcentration of 1% by weight actually prevents decay. Indeed, they point out that urea is sometimes used as a seasoning and fire protection agent in woods. They also state that urea may be a better source of nitrogen for bacteria rather than fungi,

but that bacteria may well break down the urea and make the nitrogen more accessible to fungi, a view also supported by Menganot (1963).

With regard to the experiments to be carried out, urea had previously been shown to support the growth of C. cinereus, and that there was an indication that an addition of nitrogen to straw did enhance the growth of C. cinereus and the subsequent decay of the straw.

The essence of the experiments carried out is of a complex nature but the aims can be assessed as follows:-

a) To test the predicted growth optima under natural, non-sterile conditions.
b) To guage the use and reproductibility of a novel method of growth assessment.
c) To see what effect the various additions to straw had on the colonisation of the straw by C. cinereus.
d) To test the ability of C. cinereus to colonise a non-sterile substrate and supress the growth of other micro-organisms.

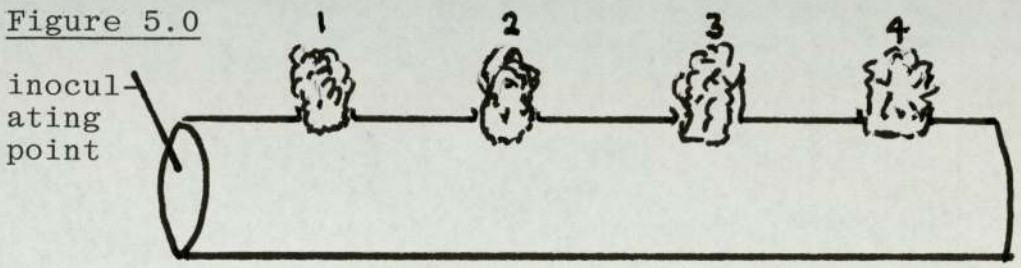
It can be seen that these aims, if satisfied would produce very useful results.

5.2.2 Materials and Methods

Unlike the work carried out by others in similar studies, the object of this particular work was to study the rate of colonisation of straw, rather than the weight loss, by Coprinus cinereus. To this end the apparatus was

designed so that an inoculum could be placed at one point and the extent of colonisation from that point could be measured by means of a metric rule. However, this method proved misleading since it gave no impression of the possible contamination of the straw. Provision for frequent sampling of the straw was therefore made until the apparatus used used was as shown in figure 5.0 and plate 5X.

Figure 5.0



10gms of wheat straw, was weighed and pushed into each of the sixteen tubes in such a fashion that the majority of the stems were arranged longitudinally in the tubes. A nutrient salts solution, as used in previous experiments was prepared, the pH adjusted to 7.8, and divided into sixteen lots of approximately 75mls each. Each of these portions was used to soak the contents of one of the tubes. This was achieved by repeated pourings of the liquid down the tubes. After fifteen repetitions of this procedure, 10gms of straw had taken up between 10 and 20mls of the nutrient solution, which from previous experience had proved to be sufficient for good growth.

The two ends of the tube, and the four sampling holes were then plugged with non-absorbent cotton wool to cut down evaporation rather than prevent contamination since the whole system is non-sterile.

Nitrogen was added to some of the tubes in the form of urea. The required amount of this chemical was dissolved in approximately 4mls distilled water, then this solution was added to the tube via the sampling holes. This procedure was carried out separately for each tube to ensure that the same amount of nitrogen was added to each.

Other tubes received an addition of glucose which was administered in the same way as the urea. The actual amounts of glucose and nitrogen added to the straw is shown in the results section.

The fungal cultures were grown up in petri plates containing 20mls malt extract agar pH 5.3 at 35^{o}C. After thirty hours the colony diameter was equivalent to that of the tubes. Inoculation was by simply pressing one end of the tube into the agar, thus dislodging the fungal mat so that this was now in contact with the straw. The cotton wool was then re-inserted behind the inoculum and the tubes placed in incubators at 35^{o}C. The humidity in the incubator was kept high to avoid drying out of the straw.

At periods of twenty-four hours the distance of the well defined fungal front from the initial inoculum was measured at several points around the circumference of the tube. At the same time small pieces of straw were taken from the sampling holes. These pieces of straw were cut into eight using sterile scissors and forceps. Four of the pieces were placed on malt extract agar pH 5.3 whilst the other four were placed on malt extract agar pH 7.8.

These isolates were incubated at 35°C for forty-eight hours after which time they were examined and any resulting fungal colonies identified. Due to this relatively short incubation time, the relative amounts of growth of the resulting fungal colonies reflects the extent of their colonisation on the isolate. The amount of growth was therefore recorded on an arbitrary scale from one to four as shown in table 5.2.

5.2.3 Results

These are expressed in figures 5.1 to 5.7, and plates 5.1 to 5.7. It is suggested, however, that due to the quantitative and qualitative nature of these results, a full appreciation can only be gained by viewing all of the figures as a whole. With this in mind, a brief description of each figure is given, followed by a synopsis of the general results.

The linear advance of the fungal fronts along the tubes is shown in tabular form in figure 5.1 and from this data, the graph, figure 5.2 was constructed. It can be seen that the graph only contains data up to ninety-six hours after inoculation of the tubes due to the fact that all distinct fungal fronts disappeared before the next reading, at 160 hours, was taken. From the graph it appears that there is little significant difference between the tubes as regards speed of colonisation until the latter stages of the experiment, when the actual fronts disappeared anyway. It does seem however, that the straw treated with only the nutrient salts solution promoted slightly quicker colonisation. All

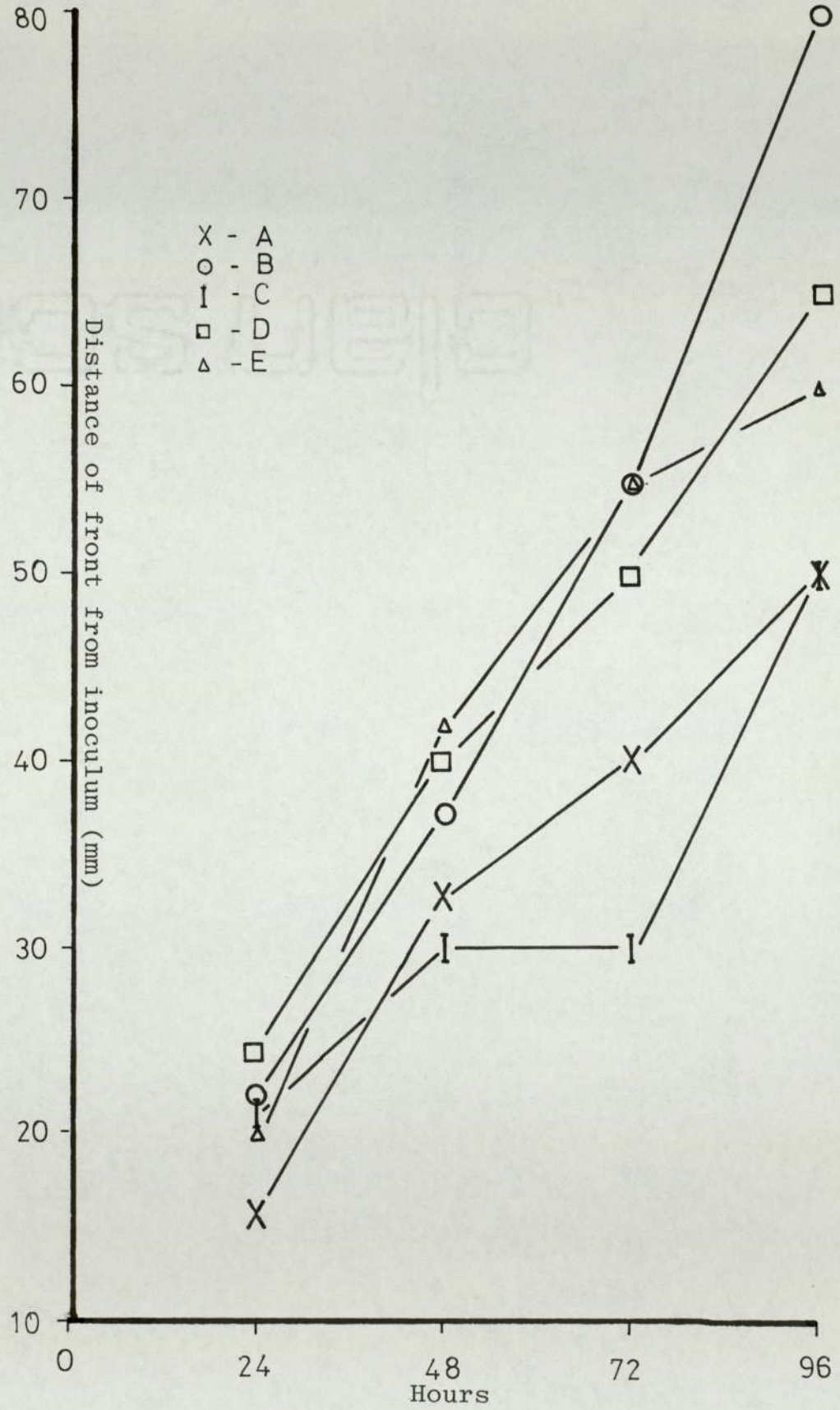
Figure 5.1 Rate of colonisation of various non-sterile wheat straw treatments by Coprinus cinereus

* = ammonia smell

TIME OF MEASUREMENT AFTER INOCULATION (hrs.)	DISTANCE OF FUNGAL "FRONT" FROM INITIAL INOCULUM (mm)				
	A CONTROL	B NUTRIENT SALT SOLUTION	C AS "B" +0.4gm N /10gm straw	D AS "B" +0.1gm N /10gm straw	E AS "B" +0.5gm glucose /10gm straw
24	16	22	21	24	20
48	32	37	30*	40	42
72	40	55	30	50	55
96	50+	80++	50+++	65++++	60
	NO DEFINITE FRONTS NOW VISIBLE				
160	Light growth of Coprinus at both ends of tube. Heavy growth of A. fum. all over.	As in "A" but slightly more A. fum.	Heavy growth of Coprinus all over with no visible sign of contaminants.	As "C" but possibly heavier growth of Coprinus.	Heavy growth of Coprinus in patches with heavy growth of A. fum.

Figure 5.2 Graphical interpretation of Figure 5.1

Rate of colonisation of various non-sterile wheat straw treatments by Coprinus cinereus



of the tubes showed a fairly regular rate of colonisation except that to which 0.4gms of nitrogen had been added. Between twenty-four and forty-eight hours no forward spread of the mycelium took place but by the time the seventy-two hour reading had been taken, growth at a steady rate had resumed. It is very important however, to note that at this stage there was a striking smell of ammonia from these tubes between twenty-four and forty-eight hours which was not present in tubes of the other treatments.

As mentioned previously, after ninety-six hours, definite fungal fronts disappeared and therefore the 160 hour readings in figure 5.1 are purely qualitative. It was also noticeable at the ninety-six hour stage that there were differences in the density of growth between treatments. These differences have thus been incorporated into figure 5.1 on an arbitrary scale of '+' signs where + means slight growth and ++++ means very dense growth.

Figures 5.3 to 5.7 show which fungi were isolated at the different isolation points along each tube at the various time intervals. A detailed description of these figures would be superfluous since they are self explanatory. However, there are several important points upon which emphasis should be laid.

Possibly the most interesting result of this isolation work was the fact that only two fungal and one bacterial species were recorded; especially in view of the non-sterile nature of the experiment. The bacteria was identified as

Figure 5.3 Organisms isolated from straw as described in Figure 5.1. Non-sterile wheat straw at 35°C

CONTROL	Hours after inoculation	pH of Malt Agar	REGION FROM WHICH SAMPLE WAS TAKEN			
			1	2	3	4
ORGANISMS ISOLATED	24	5.3	A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)
		7.8	A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)
	48	5.3	C. cinereus (3)	C. cinereus (1) A. fumigatus (1)	C. cinereus (2)	C. cinereus (1) A. fumigatus (1)
		7.8	C. cinereus (3)	C. cinereus (2) A. fumigatus (1)	C. cinereus (2)	C. cinereus (1) A. fumigatus (1)
	72	5.3	AS ABOVE	AS ABOVE	C. cinereus (2) A. fumigatus (2)	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	C. cinereus (2) A. fumigatus (1)	AS ABOVE
	96	5.3	C. cinereus (2) A. fumigatus (2)	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	C. cinereus (2)	AS ABOVE	AS ABOVE	AS ABOVE
	160	5.3	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE

Bracketed figures indicate relative amounts assessed subjectively.

Figure 5.4 Organisms isolated from straw as described in Figure 5.1 (continued)
Non-sterile wheat straw at 35°C

Nutrient Salts Solution	Hours after inoculation	pH of Malt Agar	REGION FROM WHICH SAMPLE WAS TAKEN			
			1	2	3	4
ORGANISMS ISOLATED	24	5.3	C. cinereus (1) A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)
		7.8	C. cinereus (2) A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)	A. fumigatus (1)
	48	5.3	C. cinereus (2) A. fumigatus (1)	C. cinereus (1) A. fumigatus (1)	C. cinereus (1) A. fumigatus (1)	C. cinereus (1) A. fumigatus (1)
		7.8	C. cinereus (3)	C. cinereus (1) A. fumigatus (1)	C. cinereus (1) A. fumigatus (1)	C. cinereus (1) A. fumigatus (1)
	72	5.3	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
	96	5.3	C. cinereus (3)	C. cinereus (3)	C. cinereus (2)	C. cinereus (2) A. fumigatus (1)
		7.8	C. cinereus (3)	C. cinereus (3)	C. cinereus (3)	C. cinereus (1) A. fumigatus (1)
	160	5.3	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE

Figure 5.5 Organisms isolated from straw as described in Figure 5.1 (continued)
Non-sterile wheat straw at 35°C

NUTRIENT SALTS ±0.4gm N/10gm straw	Hours after inoculation	pH of Malt Agar	REGION FROM WHICH SAMPLE WAS TAKEN			
			1	2	3	4
ORGANISMS ISOLATED	24	5.3	C. cinereus (4)	A. fumigatus (1)	0	0
		7.8	C. cinereus (4)	0	C. cinereus (1)	0
	48	5.3	C. cinereus (4)	C. cinereus (2) A. fumigatus (1)	C. cinereus (1) A. fumigatus (1)	C. cinereus (1)
		7.8	C. cinereus (4)	C. cinereus (1)	C. cinereus (2)	C. cinereus (1)
	72	5.3	C. cinereus (4)	C. cinereus (2)	C. cinereus (2)	C. cinereus (1)
		7.8	C. cinereus (4)	C. cinereus (2)	C. cinereus (2)	C. cinereus (1)
	96	5.3	AS ABOVE	AS ABOVE	AS ABOVE	C. cinereus (2)
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	C. cinereus (2)
	160	5.3	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE

Figure 5.6 Organisms isolated from straw as described in Figure 5.1 (continued)
Non-sterile wheat straw at 35°C

NUTRIENT SALTS +0.1gm N/10gm straw	Hours after inoculation	pH of Malt Agar	REGION FROM WHICH SAMPLE WAS TAKEN			
			1	2	3	4
ORGANISMS ISOLATED	24	5.3	C. cinereus (3) A. fumigatus (1)	0	A. fumigatus (1)	0
		7.8	C. cinereus (3)	C. cinereus (1)	0	0
	48	5.3	C. cinereus (3)	C. cinereus (1)	C. cinereus (1)	0
		7.8	C. cinereus (3)	C. cinereus (1)	C. cinereus (1)	0
	72	5.3	C. cinereus (3)	C. cinereus (2)	C. cinereus (2)	C. cinereus (2)
		7.8	C. cinereus (3)	C. cinereus (1)	C. cinereus (2)	C. cinereus (2)
	96	5.3	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
	160	5.3	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE

Figure 5.7 Organisms isolated from straw as described in Figure 5.1 (continued)
Non-sterile wheat straw at 35°C

NUTRIENT SALTS +0.5gm glucose/ 10gm straw	Hours after inoculation	pH of Malt Agar	REGION FROM WHICH SAMPLE WAS TAKEN			
			1	2	3	4
ORGANISMS ISOLATED	24	5.3	C. cinereus (3) A. fumigatus (1)	A. fumigatus (3)	A. fumigatus (3) C. cinereus (1)	A. fumigatus (1) C. cinereus (1)
		7.8	C. cinereus (3) A. fumigatus (1)	A. fumigatus (3)	A. fumigatus (1) C. cinereus (1)	C. cinereus (2) A. fumigatus (1)
	48	5.3	C. cinereus (3)	C. cinereus (1) A. fumigatus (1)	AS ABOVE	AS ABOVE
		7.8	C. cinereus (3)	C. cinereus (2) A. fumigatus (1)	AS ABOVE	AS ABOVE
	72	5.3	C. cinereus (3) A. fumigatus (1)	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	C. cinereus (3) A. fumigatus (1)	AS ABOVE	AS ABOVE	AS ABOVE
	96	5.3	C. cinereus (3)	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	C. cinereus (3)	AS ABOVE	AS ABOVE	AS ABOVE
	160	5.3	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE
		7.8	AS ABOVE	AS ABOVE	AS ABOVE	AS ABOVE

a gram-positive Staphylococcus, but no further identification to determine the genus was undertaken. The control straw (to which only distilled water was added) gave fairly predictable results in that although C. cinereus was isolated from all parts of the tube eventually, it never managed to suppress the growth of Aspergillus fumigatus (Fr.) which was nearly always isolated with it.

Although the straw to which nutrient salts had been added produced the furthest extension of the front of the Coprinus colony, it also produced quite heavy growths of A. fumigatus. However, C. cinereus does appear to have suppressed the growth of A. fumigatus as far as isolation point three after ninety-six hours.

The straw to which nitrogen was added gave essentially the same results, whether 0.1gm nitrogen or 0.4gm nitrogen were added, with respect to fungal isolations. A. fumigatus persisted slightly longer when 0.4gm nitrogen was added whilst the only other significant difference was that the Coprinus growth was denser on the straw to which 0.4gm nitrogen was added, in particular around the first isolation point.

An addition of 0.5gm glucose to the straw gave essentially the same results as the control in that C. cinereus and A. fumigatus grew in unison. The major difference being that the density of growth of the two species was higher in the straw to which glucose had been added.

To summarise, the results were as follows:-

a) Only two fungi, namely Coprinus cinereus and Aspergillus fumigatus, and one bacterium, were isolated in the experiments.

b) Only when the straw was treated as in C or D was C. cinereus able to wholly suppress the growth of A. fumigatus.

c) The Coprinus colony proceeded along the tubes fastest when only nutrient salts were added to the straw, although the Aspergillus growth was not totally suppressed.

d) Addition of glucose to the straw merely increased the growth of both species over the control.

With regard to plates 5.1 to 5.7, it is unfortunate that the colour reproduction in these prints is not of a sufficient quality to distinguish easily between colonies of A. fumigatus and C. cinereus. Since both fungal colonies are tinted blue in the photographs, only their apparent texture gives an idea of their identity. As a guide, the fungus in plate 5.3 is Coprinus and gives an impression of being like cotton wool. Plates 5.1, 5.2 and 5.4 have varying degrees of the cotton wool appearance mingled with the more granular appearance of Aspergillus.

Plates 5.5 to 5.7 show the overall appearance of the straw when extracted from the tubes. These plates are intended to show the extent, rather than species, of fungal growth in tubes A, B and C compared with that in tube C.

Plate 5.1 Straw from tube B (water only added)

Plate 5.2 Straw from tube A (nutrient salts added)

Plate 5.3 Straw from tube C (o.4gm nitrogen added)

Plate 5.4 Straw from tube D (0.5gm glucose added)

Plate 5.5 Straw from tube C compared to control straw from tube B.

Plate 5.6 Straw from tube C compared to straw from tube A. (Nutrient salts only, added)

Plate 5.7 Straw from tube C compared to straw from tube D. (Glucose and nutrient salts added).

5.2.4 Discussion

The results of these experiments are discussed in the order in which they appear in their summarised form.

Firstly, it would seem at first sight extremely surprising that only two fungal species were isolated from a non-sterile substrate such as straw. These results are even more unusual when one considers the large number of fungal types isolated from straw by previously mentioned workers, especially Chang and Hudson. One would, of course, expect to find C. cinereus on many isolates due to the experimental conditions and the quite high level of inoculation. The high frequency of isolation of A. fumigatus may well be a characteristic of the B.I.C. laboratories. The straw used in the experiments had been present, in sacks, in the laboratory area for several months prior to use. Since A. fumigatus has been shown to be prevalent in the atmosphere around these laboratories, the straw may well have aquired a heavy inoculum of spores of this fungus. Added to this the fact that both C. cinereus and A. fumigatus are vigorous growers, and that the incubation temperature was almost optimal for both species (and higher than normal mesophilic temperatures) it seems highly probable that any other fungal propagules in the straw were swamped out during the first few hours of incubation. This would therefore result in heavy growth of one or both of the two species on each sample of straw used for isolation which would again produce rapid growth on the isolating medium and swamp out any other fungi present. The fact that the

Coprinus could prevent A. fumigatus from establishing itself in some cases is in itself an extremely important outcome of this work. This point becomes even more important if this work were to proceed towards a system of producing animal feedstuffs or feed additives due to the possible health hazards encountered where heavy growths of A. fumigatus are present.

With regard to the second conclusion, it can be seen that the tubes containing straw to which nitrogen had been added resulted eventually in total supression of A. fumigatus by C. cinereus. It would thus appear that C. cinereus has a lower optimal C:N ratio than A. fumigatus. The effects of providing a low C:N ratio result in either rapidly increased growth rate of C. cinereus or drastically reduced growth rate of A. fumigatus or, more probably, both. An inspection of the results from the previous chapter may cast a doubt upon the validity of the first option. Those results showed that the optimal C:N ratio for C. cinereus was approximately 50:1 which is close to the C:N ratio of the straw prior to the additions of urea. These additions of urea brought the C:N ratios of the straw substrate down to approximately 10:1 when 0.1gm N was added and approximately 3:1 when 0.5gms N was added. Figures 4.4 and 4.5 show that C:N ratios as low as these could produce little growth. However, there is here, a variable in that the criteria for determining C:N ratio optimality are different, in the one case the fungus is grown on liquid media and assessed for dry weight production, whilst in the other it is grown on a solid substrate and assessed for its mycelial

spread throughout that substrate. Further, one would expect that availability of N in the straw may be somewhat less than it was in solution. This is, then, an example of the previously mentioned pitfall of attempting to directly correlate laboratory studies with practical work. In point of fact, this discussion regarding the two sets of C:N ratio work may have been a little over-simplified due to variations in carbon and nitrogen types and availabilities between the two experiments, but this again only lends credence to the view that laboratory work may sometimes only provide a rough estimate of reactions in more natural conditions.

A further slight complication arises from conclusion (c) which shows that the straw to which only nutrient salts was added was subjected to a rapid colonisation by C. cinereus. This, however, is probably due to the well charted ability of Basidiomycetes in general to produce an extensive exploratory mycelium. Further evidence for this is the fact that the Coprinus was not able to obtain a sufficient supply of the correct nutrients to establish itself and prevent A. fumigatus producing extensive growth. It would appear then that the addition of extra nitrogen to the straw, although costly and reducing the speed of colonisation by C. cinereus, is a worthwhile practice in that it gives C. cinereus the opportunity to swamp out other species.

The addition of 0.5gms of glucose to the straw would obviously have raised the C:N ratio, but only by a comparitively small degree, and therefore, according to previous statements should have slightly favoured the growth of

A. fumigatus. However, probably much more relevant was the fact that the glucose merely provided an instant supply of easily available energy which was equally utilisable by both fungi. Thus both fungi were able to produce fairly lush early growth, preventing each other from becoming dominant.

With regard to those aims of these tests set out in the introductory section of this chapter, it would appear that all have been satisfied. The effects of chemical additions to straw was studied as was the ability of C. cinereus to suppress fungal growth of other species. It is true that the bacterium was not suppressed, but this seemed to have little effect on the growth of Coprinus. Indeed some workers believe that the two may exist in some form of physiological or nutritional relationship, although it is not intended to discuss or investigate this point within this study.

The predicted growth optima did seem to hold fairly well in practice whilst the novel method of growth assessment was simple to use and gave many rapid results which were relatively constant within each variable although statistical methods were not applied to the results.

5.3 Experimental II

5.3.1 Introduction

Although the experiments in section 5.2 of this chapter were useful in their own right, they also stimulated further thoughts on the colonisation of straw by C. cinereus. It was felt that much more work could be done but that concentration should be centred upon endeavouring to answer the following major questions:-

a) In previous experiments why was Aspergillus fumigatus the only contaminant isolated from the straw?
b) Would initial and maintained sterility of the straw affect the growth and colonisation by C. cinereus?
c) What would be the effect on the growth and colonisation by C. cinereus if one or more of the growth optima were not implemented into a non-sterile substrate?

Each of the questions is dealt with in turn in the above order.

5.3.2 Contamination

It was originally postulated that the straw which was used previously, had been kept in the confines of the B.I.C. which may have exposed it to continual showers of A. fumigatus conidia. The atmosphere within the B.I.C. had been sampled on several previous occasions by the author and A. fumigatus had always been isolated. This must obviously have been due to the general interest within the B.I.C. on thermophilic and thermotolerant fungi since most workers at some time had kept A. fumigatus in culture.

However, other species, notably Trichoderma viride and various Penicillia species were also isolated regularly from the air.

It was felt that the simplest way of testing the hypothesis was to use freshly cut straw which had not been stored in the B.I.C.

The method employed was identical to that described in section 5.2.2. Samples were taken as before but no incidence of A. fumigatus was recorded, nor, indeed was any other fungal species isolated.

These results appear to confirm the hypothesis proposed earlier, but the fact that no fungal species were isolated from the straw which did not yield C. cinereus was surprising. Earlier theories suggested that where C. cinereus or A. fumigatus were isolated, they grew with such vigour on the isolating media that even if other species were present, they would easily be masked. These results seem to suggest that either the fungal spora of straw is negligible, or that those species present are unable to grow on the isolating media (i.e. malt extract agar pH 5.3 and 7.8). Both theories would seem unlikely for obvious reasons and more work seems necessary. This will be discussed later. However, as previously stated, one must reach the rather tentative conclusion that the incidence of A. fumigatus was due to storage in the B.I.C.

5.3.3 The Effects of Sterility

Although, ultimately, a sterile process was not envisaged, it was felt that a comparison with growth under sterile conditions could give a valuable insight into any possible detrimental effects of using a non-sterile procedure. Any reduction in the efficiency of growth and/or colonisation of the straw resulting from non-sterility could only arise from competition from other microbial species. Previous work had shown that contamination, if present at all, was on only a low level, but it was felt necessary to discover if this did reduce efficiency. Thus several experiments were carried out in an attempt to make a direct comparison between sterile and non-sterile systems.

To date only the linear spread of the fungal front had been used as a function of growth, and although this method had been successful, it was felt that to obtain a more complete idea of the overall efficiency of the system, another growth criterion would need to be investigated to complement the results from the linear spread measurements. As discussed in section 5.2.1 dry weight determinations can be time consuming and liable to large errors, it was nevertheless decided that if a large number of dry weight measurements were taken some meaningful results would be obtained, which, together with the results for linear spread, would enable the relevant questions to be answered.

One factor enabling large numbers of dry weight experiments to be carried out was the fact that interest was centred upon only one of the straw treatments used in section

5.2.2. The introduction of 0.1 gram of nitrogen (in urea) to 10 grams of straw appeared to assist the colonisation of C. cinereus, as well as producing very heavy growth. Thus since only one treatment was involved, large numbers of replicates could be carried out.

For the linear spread measurements, the tubes used previously were set up as described in section 5.2.2, one half of the tubes containing straw sterilised at 110lb/in^2 for twenty minutes. The urea and nutrient salts solution was filter sterilised to prevent decomposition of any of the chemicals. Inoculation was again as described in section 5.2.2 and measurements of the fungal front were taken at twenty-four hours, forty-eight hours, four days, ten days and fifteen days.

For the dry weight determinations, 100 pickle jars were used, each containing approximately 10 grams of wheat straw. Each jar, filled with straw, was then dried at 50^{o}C for forty-eight hours. This was a relatively low drying temperature, but any higher temperature would have affected any fungal spores on the straw. After the drying process, each jar was weighed and numbered. Fifty jars were then supplied with 15mls of nutrient salts solution at pH 7.8, containing enough urea to furnish approximately 0.1gm of nitrogen to each 10 grams of straw. Each jar was then inoculated with a 10mm plug of the Aston strain of C. cinereus and incubated at 35^{o}C. The other fifty jars were steam sterilised at 110^{o}C for twenty minutes, after which time they received the same treatment as the previous jars.

All jars were provided with fairly loose fitting foil lids to prevent contamination whilst allowing a reasonably free air flow. It was also found necessary to admit sterile distilled water on several occasions into most of the jars to maintain a high humidity. Since high number of replicates were required, jars were sacrificed on only two occasions; twenty-five jars from each batch at the four day period and the other twenty-five from each batch of the fifteen day period. To analyse results, each jar was then re-dried, this time at 100°C for thirty-six hours and re-weighed. This gave a weight loss figure, but to arrive at a more meaningful percentage weight loss one further procedure was needed. Since the actual amount of straw in each jar had not been weighed it was necessary to clean, dry and weigh each numbered jar and subtract this weight from the original jar plus straw figure.

The results of the linear measurements are shown in figure 5.3.3.i:-

Figure 5.3.3.i. To show the effect of sterility on the colonisation of wheat straw at 35°C by C. cinereus

	Distance of fungal front from inoculum in the times (days) shown				
	1	2	3	10	15
Sterile	24.1	40.4	66.0	No discreet fronts visible	
Non-sterile	22.3	40.3	65.1		

Each figure is representative of three readings around

the circumference of five tubes, and it is immediately clear that if there was any contamination in the non-sterile tubes (none was detected) it did not affect the colonisation of the straw. One other interesting feature to note is the close correlation between these results and those from section 5.2 when a similar non-sterile experiment was run.

With regard to the results from the dry weight readings, it was felt unnecessary to reproduce each individual reading. Instead, a condensed version, each figure being an average of twenty five readings, is shown in figure 5.3.3.ii:-

Figure 5.3.3.ii To show the effect of sterility on the weight loss of wheat straw at 35°C, due to the growth of C. cinereus.

	% weight loss of the straw at the intervals (days) shown	
	4	15
Sterile	7.8 ± 1.7	31.3 ± 1.8
Non-sterile	8.0 ± 1.7	28.4 ± 1.9

Again it would appear that any differences between the two treatments are insignificant, although, here again no contamination was observed.

In conclusion, it would be expected that heavy contamination would result in the efficiency of a fungal growth system being lowered. In the case of the non-sterile system being used it would appear that under the conditions

incorporated any contaminants were not able to flourish and hence reduce efficiency. However, much more work is needed and this will be discussed in chapter 6.

5.3.4 Effects of Altering the pH

As outlined in the introduction is was decided to discover the effects of not implementing one of the suggested growth optima into a non-sterile system. Other workers at the B.I.C., engaged on related topics had shown interest in this aspect, especially with regard to the effect of varying the pH of the system. It was felt that if any contaminating organisms were present, they may well have been prevented from growing in previous experiments by the prohibitively high pH. Further, if the pH was kept lower, those organisms, if present, would stand a better chance of becoming established.

Another aim of varying the pH would be to thoroughly test the suggested optimum pH of 7.8. As mentioned on several occasions the correlation between results obtained on sterile artificial media and non-sterile, natural substrates is sometimes very tenuous and these experiments provided an opportunity to test the correlation.

Lastly, one further aim could be fulfilled. As explained in chapter 3, the arguments over the efficacy of buffers for fungal growth systems seem never ending, but in this case it was decided that in order to fulfil the major aim of these experiments, at least an attempt at buffering should be made.

The procedure was as follows:-
One hundred pickle jars were filled with approximately 10gms of wheat straw; these were then dried at 50°C for forty-eight hours, numbered and weighed.

The basic nutrient salts solution as used in previous experiments was prepared and divided into four portions. Each portion was treated with either dilute HCl or dilute NaOH to give the following pH range:- 4.0, 6.0, 8.0 and 8.6. The latter being buffered with carbonate/bicarbonate buffer and the other three with the appropriate amounts of citric acid/di-sodium hydrogen phosphate buffer mixture.

The pickle jars containing straw were divided into four groups of twenty-five, each sample of straw being soaked with 15mls of the solution at one pH. Finally, each sample of straw was provided with a urea solution to give 0.1gms of nitrogen/10gms of straw, also a 10mm plug of C. cinereus grown on malt extract agar, and incubated at 35°C for fifteen days. The loose fitting lids as used previously were again employed, as was the distilled water at periodic intervals to maintain humidity.

Small samples of straw were extracted at random from the jars on days two, four, ten and fifteen. The pieces of straw were incubated on malt extract agar at the same pH as that sample from which it was taken.

On day fifteen any remaining fluid in the jars was pooled within each pH group, and the final pH of the solut-

ions recorded.

Finally the percentage weight losses for each jar were determined as in section 5.3.3.

The results of the isolations were quite simply that no other fungi except C. cinereus were isolated up to day four. On days ten and fifteen however, several samples from all pH's showed small numbers of Ascomycete fruit bodies, later identified as those of Chaetomium globosum. These fruit bodies could also be seen in several of the jars, apparently at random, growing on the straw chiefly near the top of the jars.

The results of the pooled pH readings were as follows:-

Figure 5.3.4.i pH changes of buffered nutrient soln. after fifteen days incubation on straw.

initial pH	4.0	6.0	8.0	8.6
final pH	7.6	7.5	7.5	8.0
pH change	+3.6	+1.5	-0.5	-0.6

Finally the results from the dry weight readings are shown in figure 5.3.4.ii,

Figure 5.3.4.ii Dry weight changes of straw after fifteen days incubation

Initial pH	4.0	6.0	8.0	8.6
% loss in dry weight	13.0 $\pm$ 1.4	12.0 $\pm$ 1.9	27.9 $\pm$ 1.5	23.1 $\pm$ 1.5

Dealing with the isolation results firstly, it again appears strange that no other organisms were isolated from the straw even at lower pH. Even more surprising is the occurrence of C. globosum on samples at any pH and its seems obvious that much more work is needed to elucidate exactly what is responsible for these seemingly inconsistent results. This will be discussed in chapter 6.

The results on pH analysis seem to confirm doubts about using buffers since in all cases there has been a movement towards the pH 7.5 area, a phenomenon charted on sterile media in chapter 3.

A simple analysis of the dry weight results reveals that an initial pH of 8.0 certainly gives significantly more growth (expressed as a greater weight loss) than higher or lower initial pH and this, together with the pH analysis would seem to give credence to the suggested optimum pH of approximately 7.8. Although lower and higher pH's eventually arrived at around pH 7.5 their growth, as judged by weight loss, was still not as great. It was suggested that this could be due to the initial consideration of the organism to alter the pH to a suitable degree. The fungus had shown this ability in the experiments outlined in chapter 3 and it appeared to be performing in the same way. Several more jars were set up as before but this time the pH was monitored, by sacrificing several jars at four, ten and fifteen days. The results are shown in figure 5.3.4.iii,

Figure 5.3.4.iii

Initial pH		4.0	6.0	8.0	8.6
pH change at	4 days	+4.2	+2.2	-0.0	-0.3
the intervals	10 days	+3.6	+1.7	-0.4	-1.0
shown	15 days	+3.6	+1.5	-0.5	-0.6

Thus it can be seen that the further away from the optimum, the more violent the pH change. This changes the moderates slightly until no matter what the initial pH, the final pH of all the solutions becomes relatively closely matched.

Again more work is needed to determine exactly how these pH changes are brought about, especially in view of the fact that buffers are present, but the precise nature of the mechanism is beyond the scope of the work in hand.

5.4 Experimental III

5.4.1 Introduction

One of the aims of this study, as outlined in the introductory chapter, was to study the growth of Coprinus cinereus under non-sterile conditions, on straw. This has been dealt with to date, in purely physiological terms, by studying the rate and extent of colonisation by the fungus under different physiological conditions. There are however, several other physical factors which could affect this colonisation rate. Probably the most important of these are:-

a) Aeration of the straw,

b) The moisture content of the straw,

c) The physical condition and position of the inoculum.

The first two of these require a depth of work which, at this stage, is beyond the scope of this present study, whereas the third is well within the possibility of study in terms of both time and apparatus. It was therefore decided to study this small, yet important, aspect of fungal colonisation.

5.4.2 Position of Inoculum

Although previous results had shown that rapid colonisation of non-sterile straw could be achieved by a single point inoculation, it was suspected that even better results could be achieved by distributing the same amount of inoculum throughout the straw. The type of inoculation used in the previous chapter did enable the progress of the fungal

front to be measured easily, but it did restrict the spread of the colony to a unilateral direction. The alternatives to this type of inoculation were:-

a) The same amount of inoculum could be minced up and 'tossed' with the straw before the two were incorporated into the tubes.

b) The inoculum could be cut into several large pieces, each one being placed at intervals throughout the straw.

To test the result of such treatments, two simple experiments were carried out in the following manner.

Firstly, fine, 10gm portions of wheat straw were weighed out and each portion soaked in a nutrient salts solution as explained in the previous chapter. Cultures of the Java strain of C. cinereus were grown up a malt extract agar in petri dishes. Each plate contained 20mls of agar and was incubated at 35°C for thirty hours. By this time the colony diameters were approximately equivalent to the diameter of the glass tubes. A fungal disc, equivalent to the diameter of the tubes, was cut from each petri dish. Each disc was then marked with a spatula, mixed with the straw, then both were tossed in a large beaker for several minutes to allow thorough mixing. Non-sterile techniques were used throughout.

The mixtures of straw and fungus were then placed in the glass tubes and 0.1gm of nitrogen in the form of urea was added in solution to each tube. The tubes were then incubated at 35°C in a high humidity incubator.

Secondly, five, 10gm portions of straw were weighed out and soaked as before. Each portion was then pushed into a glass tube to which was added the same amount of nitrogen, in the same form, as in the previous test. The cultures of C. cinereus were produced similarly, but after discs had been cut out, these discs were quartered, each quarter being added to the straw by way of the sampling holes. Again, non-sterile techniques were used and the tubes were incubated in the same incubators.

After sixty hours the tubes were inspected visually, photographed, and samples of the straw were taken from each sampling point. These samples of straw were plated out onto malt extract agars at pH 7.8 and 5.3.

The results were both highly consistent and conclusive:-

a) On none of the isolations did any fungus, other than C. cinereus, show up.
b) Both methods resulted in rapid colonisation of the straw. The rate of colonisation being greater than when single point inoculation was used.
c) It was visibly obvious that by quartering the inoculum, a much heavier colonisation results. Plates 5X and 5Y show this point excellently.

It would appear then that although mashing the inoculum obviously spreads it further throughout the straw, this does not result in very heavy growth. Possibly the mashing process causes some high degree of hyphal damage or physiological trauma. This seems to cause a lag phase in

Plate 5X

Showing the growth of C. cinereus on non-sterile wheat straw using mashed inoculum.

Plate 5Y

Showing the growth of C. cinereus on non-sterile wheat straw using sliced inoculum.

colonisation which, however, does not prevent the fungus suppressing the growth of other species in a relatively short time.

By preserving some of the integrity of the inoculum colony in larger segments no such physiological trauma or lag phase appears. Very rapid, dense growth results after a short time and it is felt that this simple, non-sterile, yet effective of inoculation may be suitable on a much larger scale, possibly within actual bales of straw. Baling of straw is an expensive process requiring both time and machinery, therefore any process whereby these bales would need to be destroyed to allow fungal growth would not be readily acceptable. It is obvious that problems like these are a relatively larger burden to any process where initial scepticism of the process itself will be high. A simple procedure, whereby bales could be dipped for a period in nutrient salts and nitrogen solutions could be used. The inoculum could be introduced at several points using some simple penetrating apparatus.

Obviously, the development of any process involving the work from this thesis is, at this stage, hypothetical and beyond the scope of this study. It is thought, however, that suggestions such as these should be noted at this stage in the event that they may be useful for further reference.

5.5. General Discussion and Conclusions

Much work has been performed in endeavouring to determine whether or not those growth optima suggested in previous chapters would hold true when C. cinereus was grown on a non-sterile, natural substrate. In doing so, several techniques have been used and many replications produced in an effort to provide meaningful results. A brief outline of the major conclusions reached will be given:-

a) Coprinus cinereus does grow well on damp straw at 35°C and pH 7.8.

b) This growth is enhanced when a small amount of nitrogen is added.

c) If this nitrogen is added in the form of urea, C. cinereus is able to utilise it very well.

d) Little, though inconsistent, contamination occurs when optimal conditions are employed in non-sterile conditions.

e) Initial and maintained sterility of the straw did not seem to increase the rate nor the amount of colonisation by C. cinereus.

f) Failing to incorporate one growth optimum, viz pH did affect the amount of growth on non-sterile straw although this did not seem to lend the straw to being contaminated.

g) C. cinereus appears to be able to alter the pH of non-sterile substrates to between 7.5 and 8.0 within which range has its own optimum pH.

h) Large inoculations of C. cinereus at several specific points throughout the straw, gives much more vigorous

growth than shreading the inoculum to give greater spread.

i) Much more work is needed to elucidate the many questions which have arisen. These will be dealt with in chapter 6.

C H A P T E R 6

General discussion and suggestions for further research

6.1 Discussion of Preceeding Work

At the outset of any study such as this one must outline certain aims to provide guidelines along which it is hoped work will progress. The aims for this particular study were summarised in section 1.7 and only work directly relevant to fulfilling these aims has been carried out. On occasions points have arisen both in line with and tangential to the main theme, but due to the time limiting factor involved these have merely been noted in the text and discussed in section 6.2.

Probably the easiest method of determining the relevance of any study is by dealing with each of its aims in turn, to evaluate the extent to which each has been fulfilled.

Firstly a straight forward study of the growth of C. cinereus under various physiological conditions in pure culture was planned as the basis from which all other work could stem. The major physiological characteristics of the fungus, namely, temperature, pH, source of nitrogen, C:N ratio, were studied using a variety of growth measuring criteria. As explained in chapter 1 the intent of this work was to endeavour to predict or at least suggest a combination of physiological conditions which would produce optimal growth. With regard to this latter point, three strains of C. cinereus were used in this section of the study to reduce the chance of extreme results. It is felt that this part of the work was quite successful, although

one was constantly trying to come to terms with the meaning of the word growth in relation to the overriding theme. The relative importance of extent of growth and speed of colonisation was difficult to evaluate, since the two need not necessarily result from any one set of physiological conditions. The problem is further complicated since for further work on non-sterile material it was felt that an initially high speed of colonisation was needed, followed by terminally heavy growth. However, because various methods of measuring growth, viz colony diameter, dry weight production and later spread of the fungal front, were used, it is felt that a balance between extent of growth and speed of colonisation was automatically arrived at.

On a more specific note, growth optima were suggested for all three strains of the fungus which helped to fulfill the second aim. The only major works on the physiology of Coprinus generally are by Fries (1955 and 1956) where C. cinereus is not mentioned as such; and by Hedger in his Ph.D. thesis (1972). Two additional strains have been used in this study and therefore on two counts the second aim of complementing current knowledge of the genus and the species has been fulfilled.

The majority of chapter 5 is devoted to a study of the growth of C. cinereus on non-sterile straw, growth being determined objectively by dry weight loss and colony spread and subjectively by visual analysis.

Virtually inseperable from this work was the constant

awareness that results from sterile, pure culture work on synthetic media were being applied to a non-sterile, natural substrate. Although efforts were made to provide evidence that the predicted optima would apply (by sterilising straw and altering the pH), the fourth aim many nevertheless have been rather neglected. It is true that the combination of optima used did produce quick initial colonisation and terminal heavy growth, but this combination cannot safely be called definitive since the toal permutations of varying growth conditions were not studied on straw.

Heading on from this the next aim was to provide some of the necessary knowledge required to control the organism. Such was the extent of monitoring of the growth on sterile media that given a set of physiological conditions, one could predict with a degree of certainty how the fungus would respond. However, as explained above such monitoring on straw was not performed adequately and hence at this stage one must depend largely on the results from sterile work to predict the behaviour of C. cinereus on non-sterile substrates.

As previously explained, any novel foodstuff, be it intended for livestock or human consumption must be fully tested and the production process able to provide a consistent product. This is especially important where microorganisms are involved. Since this latter necessity requires one to have a thorough knowledge of the organism used, the work completed to date has supplied the basis upon which further knowledge can be built. Hence the final aim

has been satisfied.

On the whole it would appear that taking the fulfillment of aims as a criterion of success, this study has been worthwhile. However, it must be borne in mind that the aims were very broadly based and for each to be studied to its conclusion would have taken more than the time available. Since ground work regarding each of the aims was required, concentration was centred on this aspect.

6.2 Suggestions for further Research

As mentioned earlier at several times points arose which would have been interesting to follow up, but which were not necessarily directly concerned with the work in hand. A list of ideas for such 'peripheral' research follows:-

a) It may be useful for the exact cause of the drop in pH of various media during autoclaving, to be determined. By doing so, one may obtain a more complete analysis of the nutrients available to the organism subsequent to the autoclaving process. This point may be even more important when dilute acids or alkalis are added to media to adjust the pH.

b) The colour changes noted in chapter 3 were probably caused by metabolites excreted by the fungi but their exact nature could well prove useful to establish in case they contain any toxic radicals.

c) The differing preferences for nitrogen sources between the three strains of C. cinereus must lie in their respective enzyme systems. It would be interesting to elucidate the genetic basis of these differences in what otherwise seem physiologically very closely related strains.

Whereas the above points may not bear any direct relevance to the study, there were other interesting points which it is felt should be explored to gain a more complete picture of the organism and the occurrences throughout its growth. These are listed below:-

1) Probably the most severe self-criticism of chapters 2 and 3 was that a total temperature and pH range was not explored. This was neglected in favour of determining optima rather than cut-off points.
2) Throughout much of the work a nutrient salts medium was used to which was added various primary growth requirements. However, it may well prove extremely valuable to assess the need for each of the constituents of the salts medium, especially in the non-sterile work. Economies could well be made if it is found that some ingredients are superfluous. Thiamine was added to the solution in all cases, but a full study of the vitamin requirements of the fungus was not performed.
3) One of the more interesting features throughout the study has been the dramatic pH changes after growth of C. cinereus. One feels sure that a more in depth study of this, both for C. cinereus and other fungi, would prove greatly interesting and informative.
4) With regard to the growth of C. cinereus on non-sterile wheat straw there are several areas for further study. Probably the one causing the greatest contention is the rather inconsistent results regarding contamination. A complete study of the fungal flora of wheat straw is suggested together with a study of the growth of such organisms alone and with competition with C. cinereus using the conditions employed in this study.
5) As previously mentioned a criticism of the experiments on non-sterile straw was the fact that conditions were not varied sufficiently to be able to predict the behaviour of the fungus. Another effect of varying conditions

would be to confirm or otherwise, those optima predicted from sterile work.

6) Yet further economies could be made if a thorough study of the optimal water content and humidity of the straw/growth area were performed. This was not strictly monitored in this study and may well have a strong bearing on the growth of the fungus.

7) That the fungus grows on the straw is immediately evident, but exactly from which fraction of the straw and from which anatomical regions of the straw the fungus derives nourishment is largely unknown. Several efforts were made by the author, but time did not permit the various techniques to be explored fully. A study of this type may show what effects the fungus has on the rigidity and hence the digestibility of the straw. This may also involve studying the ligninolytic properties of C. cinereus, which is in itself a complex problem due to the many forms of lignin as previously explained.

8) Since straw is baled to facilitate storage, any process involving the breaking of bales may sound unattractive. Having already studied the best state of the fungus for inoculation, it would prove profitable to examine way of inoculating large batches of straw. Again it is envisaged that this would be a complex study since many factors, including differential wetting and drying, heating and cooling and possibly aeration would be involved. However, if a method of growing C. cinereus on bales of straw could be found, one feels the work could be deemed worthwhile.

As can be seen from the above, this study has been reasonably successful in its own right, but has also fulfilled one other major function of any preliminary work programme, that of suggesting several ideas for further related research.

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