This thesis is dedicated to
the memory of my mother
and to my father.

THE DEVELOPMENT OF FILAMENTOUS FUNGI IN SUBMERGED CULTURE

by

COLIN WEBB

submitted in support of an application for the degree of DOCTOR OF PHILOSOPHY

at

The University of Aston in Birmingham

September, 1980.

SUMMARY

During the 24 hour period following inoculation, aggregation of spores and sporelings can have an important effect on the subsequent growth of filamentous fungi in submerged culture. This early phase of growth does not appear to have received much attention, and it was for this reason that the author's research was started.

The aggregation, germination and early growth of the filamentous fungus Aspergillus niger have been followed in aerated tower fermenters, by microscopic examination. By studying many individual sporelings it has been possible to estimate the specific growth rate and germination times, and then to assess the branching characteristics of the fungus over a period of from 1 to 10 hours after germination. The results have been incorporated into computer models to simulate the development of the physical structure of individual and aggregated sporelings.

Following germination, and an initial rapid growth phase, fungi were found to grow exponentially : in the case of A.niger the mean germination time was about 5 hours and the doubling time was as short as 1·5 hours. Branching also followed an exponential pattern and appeared to be related to hyphal length. Using a simple hypothesis for growth along with empirical parameters, typical fungal structures were generated using the computer models : these compared well with actual sporelings observed under the microscope.

Preliminary work suggested that the techniques used in this research could be successfully applied to a range of filamentous fungi.

Colin Webb Ph.D. 1980

KEYWORDS : FUNGI
GROWTH
SIMULATION
AGGREGATION

C O N T E N T S

CONTENTS Continued.

CONTENTS Continued.

CONTENTS Continued.

APPENDICES Continued.

F I G U R E S

FIGURES Continued.

FIGURES Continued.

PHOTOGRAPHS

T A B L E S

ACKNOWLEDGEMENTS

The author would like to thank :

the Science Research Council for providing financial assistance in the form of a research studentship;

members of the Tower Fermenter Research Group for instruction in the methods of microbiology;

Professor G.V. Jeffreys and the staff of the Chemical Engineering department for providing facilities and assistance;

and especially :

Dr. E.L. Smith for his admirable supervision throughout the research;

Miss Maureen-Ann Fox for typing guidance and for her patience.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND TO THE RESEARCH

1.2 THESIS LAYOUT

1·1 BACKGROUND TO THE RESEARCH

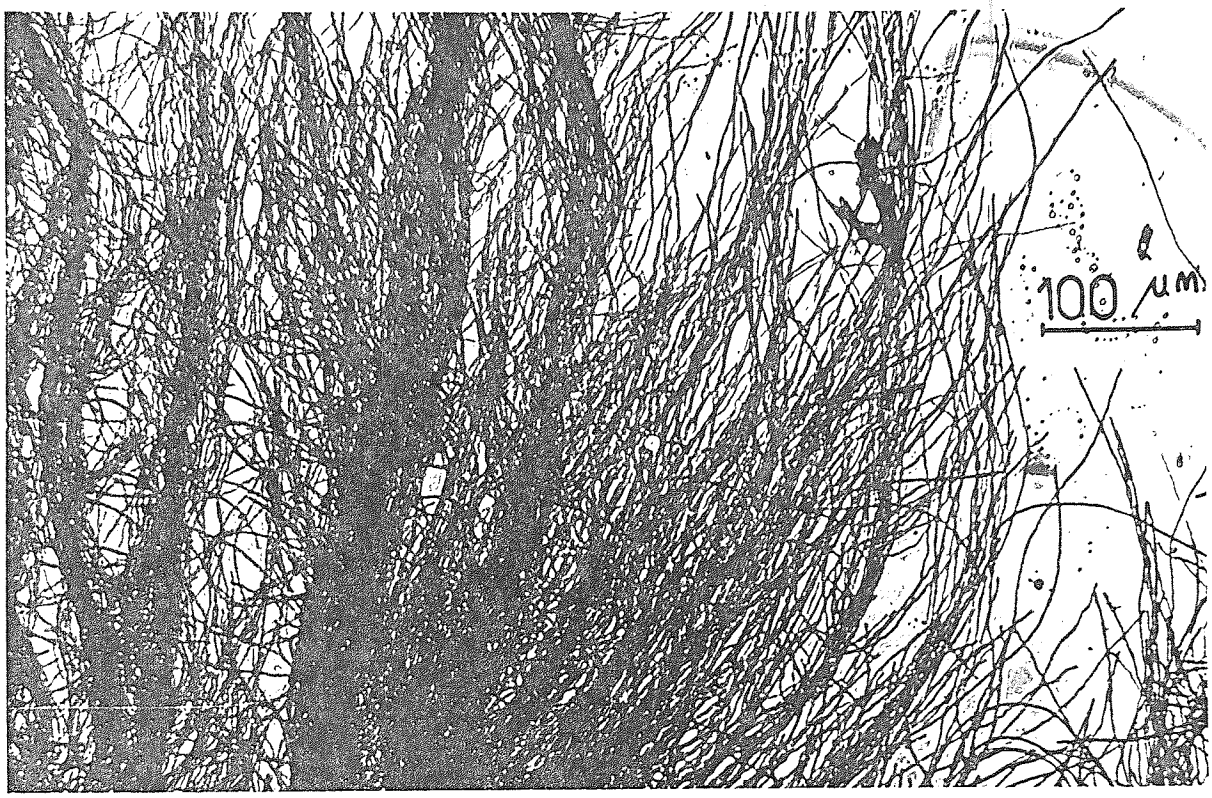
The Tower Fermenter Research Group (TFRG) was set up at the University of Aston in Birmingham in 1970 to study the applications, design and control of tower fermentation systems; the Group is supervised jointly by Dr. E. L. Smith (Chemical Engineering Department) and Dr. R. N. Greenshields (Biological Sciences Department). The tower fermenters used at Aston are simple tubular reactors which can be used for biochemical processes involving bacteria, yeasts or filamentous fungi; the fermenters can be operated aerobically or anaerobically, batchwise or continuously. Recent work within the Group has been concerned with the production of filamentous fungal biomass from carbohydrate wastes.

The broad objective of the author's research was to study the behaviour of microbial aggregates in tower fermentation systems. However, in order to obtain information relevant to the present interests of the TFRG and because of time constraints, the study was restricted mainly to aggregates of the filamentous fungus *Aspergillus niger* grown under aerobic conditions.

The mechanism by which microbial aggregates are formed will clearly have an important effect on their properties and consequent behaviour. Plate 1·1 illustrates how the internal structure of aggregates can vary from loose strands to densely packed mycelium. Since very little quantitative information on this subject could be found in the literature, most of the author's research was concerned with the structural development of such aggregates.

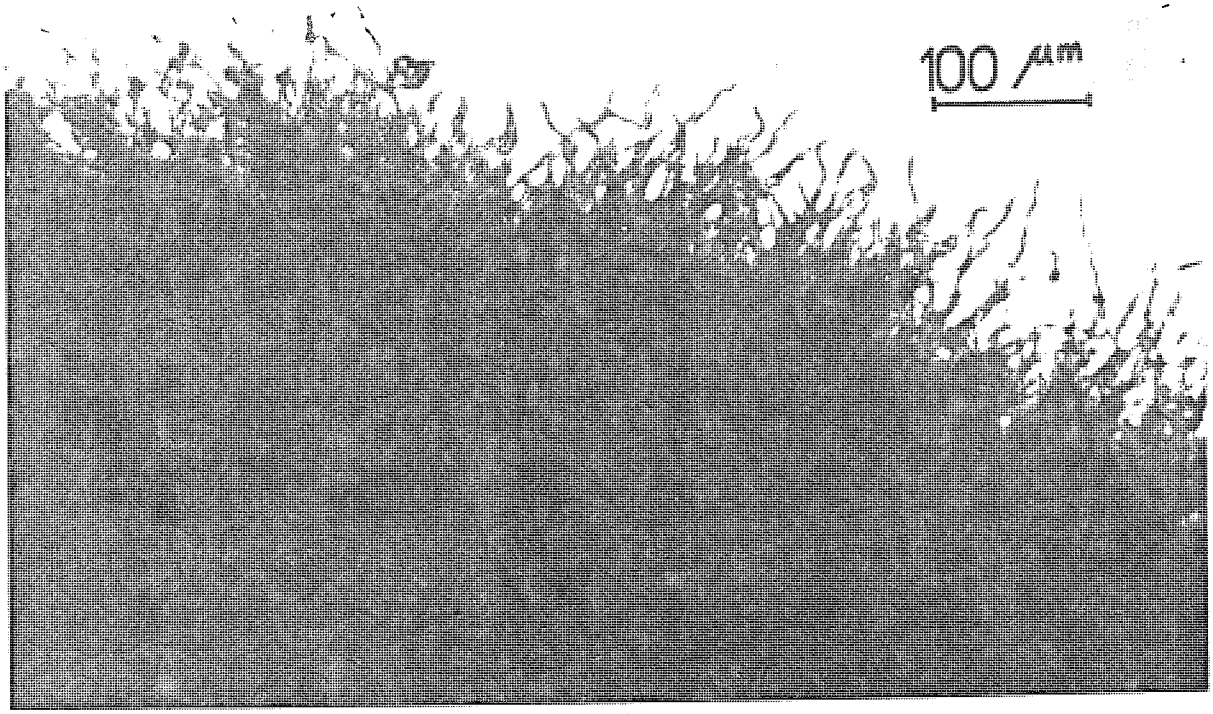
Fig. 1·1 shows some of the factors affecting and affected by aggregate structures. James (1973) was the first member of the Group to study the physical properties of microbial aggregates in tower fermenters and Cocker (1975) has described and classified aggregate morphology in a qualitative way. It is hoped that the author's work will complement this earlier research and so help to provide a fuller understanding of such aggregate systems.

PLATE 1.1 AGGREGATE MORPHOLOGY (A. NIGER)



LOOSE HYPHAE

DENSE PELLET



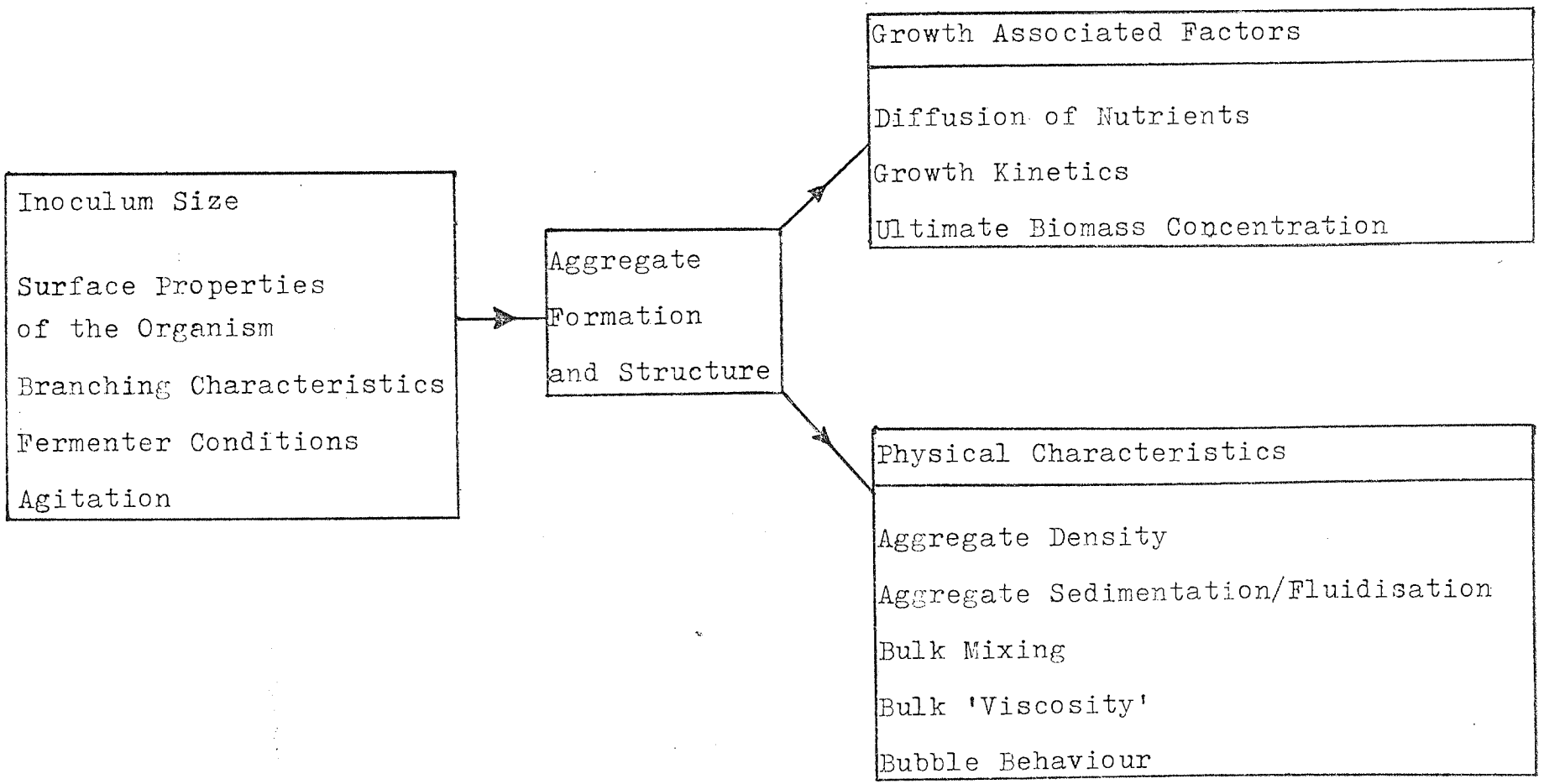


Fig. 1.1 FACTORS AFFECTING AND AFFECTED BY AGGREGATES

1·2 THESIS LAYOUT

The general layout of the thesis is shown diagramatically in Fig. 1·2. Chapter 2 provides general background information on the growth of filamentous fungi and aggregate/colony formation. From this are drawn the major objectives for the research and a revised hypothesis for growth, which is presented in chapter 3. The basis for experimental work is then outlined in chapter 4, and results are presented in chapters 5,6,7. Mathematical models based on the hypothesis and experimental results are given in chapter 8. Results are discussed at the end of each chapter and summarised in the general discussion (chapter 9): the overall conclusions and recommendations for future work are also included in this chapter.

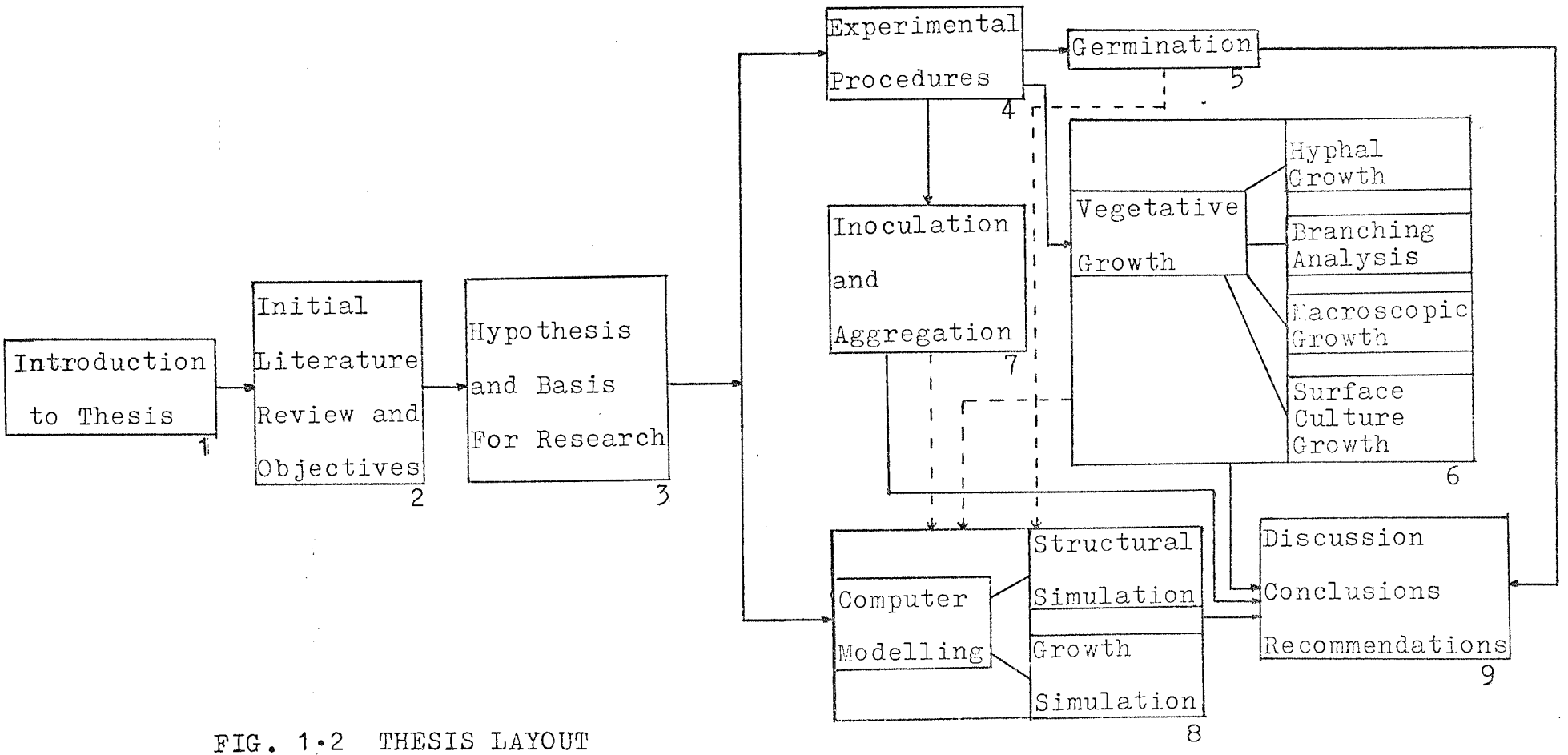


FIG. 1·2 THESIS LAYOUT

C H A P T E R 2

SUBMERGED CULTURE GROWTH AND DEVELOPMENT OF FILAMENTOUS FUNGAL AGGREGATES

2·1 INTRODUCTION

An outline of the growth of a filamentous fungus and its colonial development in submerged culture will be given in this chapter. No mention will be made of the effects of mechanical agitation on growth (for this see Duckworth and Harris, 1949), nor will anaerobic organisms be considered. From this review the objectives of the research will be defined in the discussion section.

2·2 THE GROWTH CYCLE

The growth cycle of a filamentous fungus can be divided into four stages:

1. Spore swelling
2. Germ-tube emergence and growth
3. Vegetative growth
4. Reproduction and sporulation

Spore Swelling - When conditions such as moisture and temperature are right and there are certain substances, such as carbohydrates, present a spore will begin the process of germination by swelling. This swelling is

in fact a spherical growth, involving a rapid increase in the endoplasmic reticulum and numbers of mitochondria. Accompanying these activities during spore swelling is the formation of an inner cell wall, which introduces polarity to the spore and becomes the germ-tube wall on germination (Smith, 1975; Burnett, 1976).

Ekundayo and Carlile (1964) observed the swelling of Rhizopus arrhizus spores to be linear with time, and this has since been found to be true for other species (Trinci, 1971b). The spore diameter may double during swelling; consequently, the tough outer wall of some species becomes stretched and may flake off (Hawker, 1966).

Swelling ceases with the emergence of a germ-tube, and from this time on growth is polar.

Germ-tube Emergence and Growth - Although spore swelling is, strictly speaking, part of germination, as is germ-tube growth, the term used here is restricted to: "the act of protrusion of germ-tube from spore wall" (Manners, 1966).

Germination is responsible for the change from spherical spore to filamentous mycelium by the growth of a vegetative wall, which is a direct extension of the spore wall and which undergoes no compositional change during germ-tube formation (Bartnicki-Garcia, 1968). The germ-tube may emerge from anywhere on the surface of the spore (Hawker, 1966) and is often later accompanied by a second and occasionally a third germ-tube, though rarely any more. Germination occurs over a range of time (see fig. 2·1), often long enough for some germ-tubes to have begun branching before others have emerged from their spores.

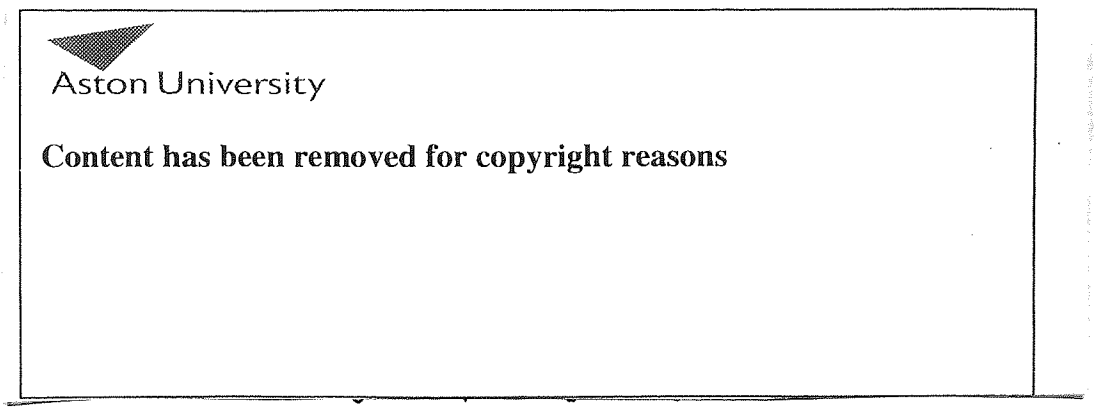


Fig. 2·1 Germination Time-Range (Kier et. al.,1976)

Following germination, growth proceeds by the addition of new cell-wall material at the tip (or apex) of the germ-tube resulting in the characteristic cylindrical shape. This is referred to as apical

growth and was first suggested by Reinhardt in 1892 (Burnett, 1976). Smith (1923) confirmed that all growth occured at the hyphal apex, and in more recent studies Bartnicki-Garcia and Lippman (1969), and Gooday(1971) have shown that the majority of growth is restricted to within 1 micron of the apex. The mechanisms of apical growth are comprehensively reviewed by Burnett (1976).

If growth occurs solely at the apex then it must be dependent upon activities further back along the hypha. This dependency has been demonstrated by Smith (1924) who found that the rate of extension of hyphae is proportional to total length.

To the author's knowledge only one paper, that written by Trinci (1971b), deals specifically with the kinetics of growth of germ-tubes. He showed that growth is initially exponential but soon decelerates (fig. 2·2). He suggested that this was due to the fact that the germ-tubes would draw on the spore's reserves as nutrients, and he explained the deceleration phase as representing the transition from germ-tube growth to vegetative growth. However, Trinci (1974) later suggested that the deceleration

was due only to oxygen limitation and that exponential growth would continue for a considerable time when nutrients were in excess.

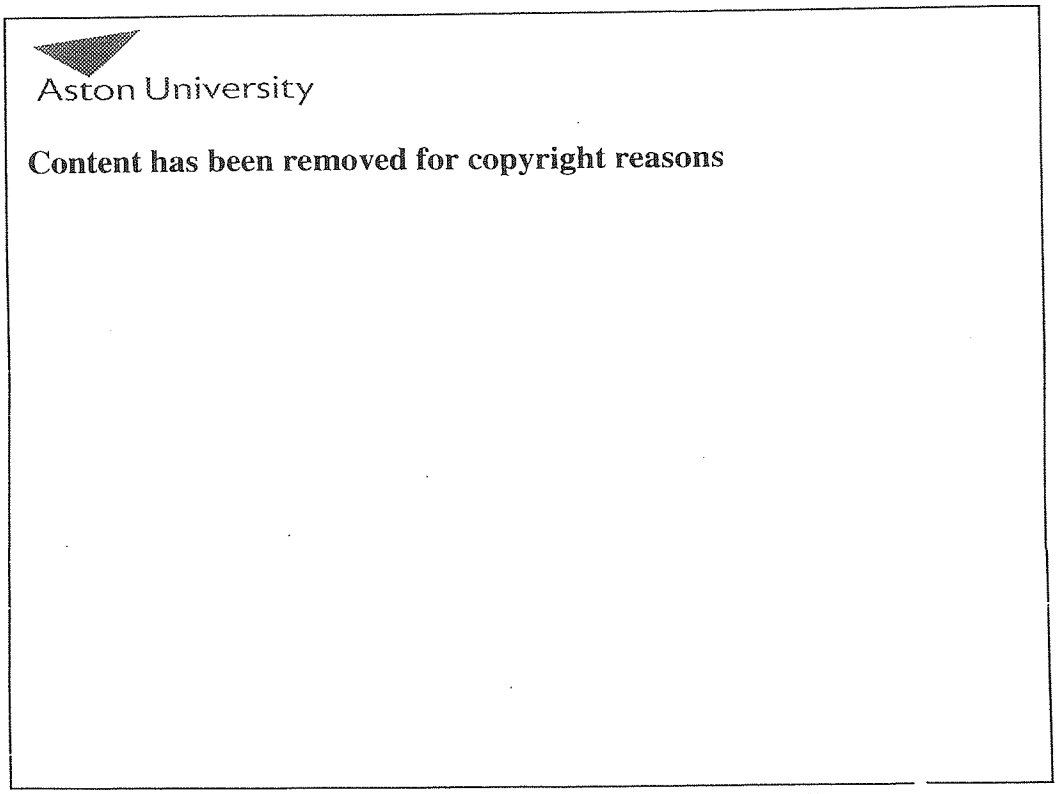


Fig. 2·2 Germ-tube Growth (Trinci, 1971b)

Vegetative Growth - Since the vegetative development of a single sporeling (a spore and its associated hyphae) cannot easily be followed in submerged culture, all of the literature cited in this section refers to work with surface cultures.

As growth proceeds two morphological features become apparent, namely septa and branches. Septa are cross-walls in the hyphae which may or may not allow the passage of protoplasm. The majority of fungi grown in submerged culture have either no septa or they are initially poroid so that the hyphae remain coenocytic. According to Robertson (1965), Butler (1966), and Moore-Landecker (1972), branching of hyphae typically occurs just behind the apex and in acropetal succession, producing a christmas-tree like structure. The branches are referred to as apical, if they are produced by a dichotomous division of the apex, and lateral, if produced from the hyphal wall below the apex. Robertson (1965) states that lateral branches appear at almost right-angles to the parent hypha, grow at a direction towards that of the parent apex, and are produced independently of septa. Trinci (1974), however, proposed a connection between septation and branching: this was based on an observation that vesicles, which are responsible for breaking down the cell-wall and normally accumulate at the hyphal apex, can also accumulate behind septa (Trinci and Collinge, 1974).

The kinetics of hyphal extension were first studied by Smith(1924). He observed that if the sum of the lengths of a hypha and its branches and sub-branches was plotted against time, the resultant curve was exponential during early growth. This could be expressed as;

$$\frac{L}{L_o} = e^{kt} \quad \text{or} \quad \frac{dL}{dt} = kL$$

where, k = constant

L = the total length of hypha + branches

L_o = the total length at t_o

t = time after t_o.

The relationship held for unbranched germ-tubes too.

Although they agreed with Smith's observations of exponential increase of total hyphal length most workers reported that individual hyphae grew at a constant rate (Plomley, 1959; Trinci and Banbury, 1967; Trinci, 1970a; Moore-Landecker, 1972). For this to be true branching has to occur at regular intervals, and the concept of "growth units" naturally follows. Plomley (1959), in a detailed study, likened the growth of filamentous fungi to that of bacteria or unicellular fungi by defining the "growth unit" as being a "free growing hyphal tip associated with a growing mass of constant size" and duplicating itself at a constant rate during growth. Caldwell and Trinci

(1973) extended this idea by defining the "hyphal growth unit" as the ratio of the total length of hyphae to the number of tips. This was found to be constant for given environmental conditions (Trinci, 1973).

Katz, Golstein and Rossenburger (1972) offered a different approach when they observed an exponential rate of elongation in both branched and unbranched hyphae. They proposed that branching occured when the extension rate of the hypha reached a maximum value. Mandels (1965) and Kubitschek (1970), on the other hand, did not agree that exponential growth occurs in filamentous fungi.

As branching increases in intensity, hyphae intertwine creating a complex mycelial structure or colony. This process is usually accelerated in submerged culture by the aggregation of spores, but it can be observed during hyphal development from a single spore (Burkholder and Sinnot, 1945). Colony formation will be reviewed in more detail later (see page 16).

Reproduction and Sporulation - As growth continues and nutrient reserves are depleted, conditions become harsher until vegetative growth ceases. At this point reproduction occurs, resulting in the production of many spores or conidia. These will lie dormant until conditions are once again favourable for growth. Since sporulation and vegetative growth are mutually exclusive (Smith and Galbraith, 1971), reproduction will not occur while growth-limiting factors are absent. Consequently, sporulation in submerged culture, where conditions are homogeneous, is much rarer than in surface culture, where parts of the colony may be sporulating while other parts are still growing. For this reason, the process of reproduction will not be studied further in this research.

2·3 DEVELOPMENT OF COLONIES

When grown in submerged culture many filamentous fungi form pellet-type colonies (Burkholder and Sinnot, 1945). These spherical colonies are closely interwoven mycelial masses, the centres of which are usually large aggregates of spores. Their morphology can vary over a wide range, from loose mycelial strands to densely packed pellets, and is affected by many parameters.

Colony development is best described in two stages, namely colony formation and colony growth.

Colony Formation - The mechanisms of colony formation in submerged culture have recently been reviewed by Whitaker and Long (1973) and by Metz and Kossen (1977). Cocker (1975) has described four ways by which a colony can form; these are presented diagramatically in fig. 2·3. The development of colonies from single spores has also been observed (Burkholder and Sinnot, 1945; Cocker and Greenshields (1977).

Of the processes of colony formation, that beginning with the aggregation of spores appears to be the most common. The basic mechanism of spore aggregation is not clearly understood (Trinci, 1970b) although it does differ markedly from mechanisms leading to the aggregation of yeasts and bacteria (Atkinson and Daoud, 1976). It occurs before germination and as a result of gentle agitation (Foster, 1949). Galbraith and Smith (1969) have associated it with the germination process itself, since when this is inhibited aggregation does not occur. They believed the aggregation process to be caused by changes at the spore surface, which occur at the onset of spore swelling, (see also Martin,

DIAGRAMMATIC REPRESENTATION OF THE AGGLUTINATION AND GROWTH MECHANISMS WHICH PARTICIPATE IN THE FORMATION OF COLONIES

Aston University

Content has been removed for copyright reasons

Fig. 2.3 COLONY FORMATION (Cocker, 1975)

Urubura, and Villanueva, 1973). They were able to discount the possibility of an excreted substance being responsible for aggregation because they found that washing the spores had no effect.

The factors affecting spore aggregation and colony formation are many and often interrelated (Metz, 1976). The more important factors include inoculum size, species and strain, the growth medium, and the physical environment. Whitaker and Long (1973) have summarised in table form those species and strains which form colonies and have reviewed the effects of inoculum size, medium and physical environment. Foster (1949) and Camici et. al. (1952) have reported that colony formation occurs only at low inoculum concentrations, and Foster suggested that this was due to the lack of competition for nutrients. Galbraith and Smith (1969) and Trinci (1970b), however, found that for A. niger and A. nidulans colonies were formed at all spore concentrations. In a recent study, Gabinskaya (1976) reported that colony formation and morphology were affected only by certain nutrients in the medium and were independent of pH, inoculum size and aeration.

Colony Growth and Kinetics - Comprehensive studies of the morphology of submerged culture colonies of A. niger have been presented by Steel et.al. (1954,1955) and Cocker (1975). The wide morphological range has made difficult the study of growth kinetics, resulting in the use of empirical relationships.

Emerson (1950) observed that the rate of increase of radius in both surface and submerged colonies was constant "over a considerable period". Assuming a constant hyphal packing density a consideration of the geometry leads to the following results.

Since, $r \propto t$

then $m^{\frac{1}{2}} \propto t$ for surface colonies,

and $m^{\frac{1}{3}} \propto t$ for submerged colonies,

where, r = colony radius

m = colony mass

t = time.

Dry weight data were collected which supported this "cube-root" law. Emerson's findings were later confirmed by Machlis (1957) and Marshall and Alexander (1960).

By contrast Zalokar (1959), Pirt and Callow(1960) and Borrow et.al. (1964) showed that growth in

submerged culture could follow the exponential growth law:

$$\frac{dm}{dt} = km$$

Pirt and Callow presented diagramatically an analogy between multiplying bacterial cells and branching fungal hyphae to account for this equation.

It was soon realised that the different forms of rate equation for submerged growth were due to the varying morphology of the fungi (Pirt, 1966). In a filamentous form all hyphae are in contact with the medium so that exponential growth is possible; when pellets are formed the central part of the colony may become starved of nutrients as hyphal packing becomes denser, thus limiting the growth. Using this latter concept, Pirt suggested that a pellet, which was not completely penetrated by the rate limiting nutrient, could be considered to be a sphere with an actively growing shell. Although he assumed that the peripheral

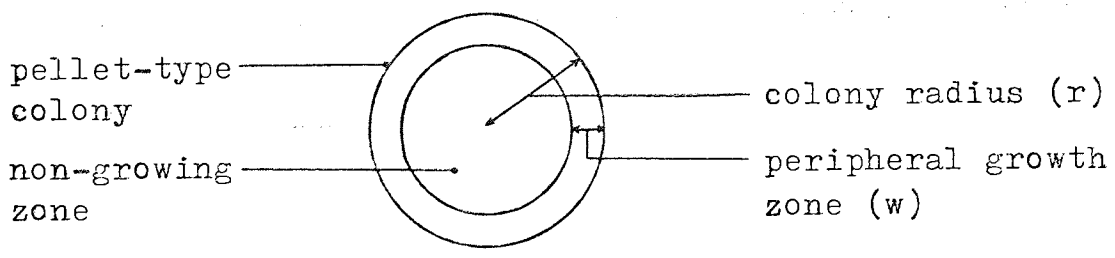


Fig. 2·4 Pellet-type Colony showing Peripheral Growth Zone.

growth zone (see fig. 2·4) was growing exponentially, Pirt was able to show that the total mass of the colony increased according to a cube-root law. Expressing this idea mathematically:

$$\frac{dm}{dt} = km_g$$

Using the approximation

$$m_g \approx 4\pi r^2 \rho w$$

leads to $\frac{dr}{dt} = kw$

and on integration $r = kwt + \text{constant}$.

Since $r \equiv \left(\frac{3m}{4\pi\rho}\right)^{\frac{1}{3}}$, then a cube-root law follows

so that, $m^{\frac{1}{3}} \propto kwt$

where, w = width of the peripheral growth zone

m_g = active mass, contained in the peripheral growth zone (fig. 2·4).

Pirt calculated that w should be about 0·08 mm for tightly packed pellets where the limiting nutrient is oxygen and where diffusion is the rate controlling step. Morris (1972) has observed a 0·2 mm peripheral growth zone, and Choudhary and Pirt (1965) have found that exponential growth of pellets is possible: these results suggest that the extent to which the hyphae are packed can influence growth kinetics.

In a kinetic study of fungal pellets grown in shake flasks, Trinci (1970b) noted that growth

included both morphological extremes observed by Pirt. He demonstrated that there are three phases during growth: initially, very filamentous structures, which grow exponentially, are formed; then these structures slowly become more dense until cube-root kinetics applies; and eventually autolysis of the pellet centres occurs.

2·4 DISCUSSION AND FORMULATION OF OBJECTIVES

It is apparent even from a brief examination of the literature that most of the quantitative work on the growth of filamentous fungal hyphae is confined to surface culture. This is understandable when one considers that individual hyphae cannot readily be kept under constant observation in submerged liquid culture. However, in view of the differences between the two modes of growth caution must be adopted in using the results from surface culture studies to predict behaviour in submerged culture.

In a surface culture, on agar or unstirred liquid, there are usually three hyphal forms present; submerged hyphae that are totally surrounded by the substrate, surface hyphae that are exposed to substrate

on one side and air on the other, and aerial hyphae that are surrounded only by air (fig. 2·5).

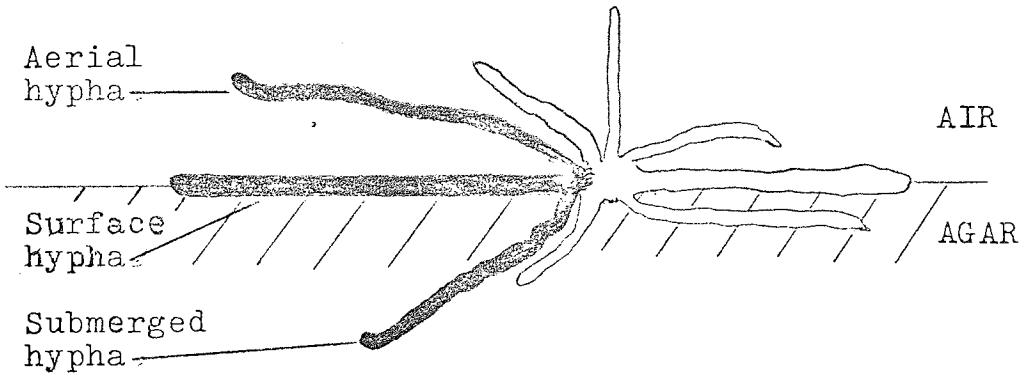


Fig. 2·5 Hyphal Growth on Solid Medium

The system is further complicated by the fact that nutrients can only move by diffusion, and hence local depletion may lead to sporulation in one part of the colony while other parts are still growing in unrestricted fashion. This is not the case with submerged liquid culture where all hyphae are totally surrounded by nutrients and where mixing ensures that local concentration gradients are minimised.

One should not be too surprised if the kinetics of growth in the two systems differ. Intuitively one might expect growth in submerged culture to be "autocatalytic" since the whole surface area of a hypha is available for the transfer of nutrients. As growth proceeds the increasing surface area leads to an increasing intake of nutrients, which in turn leads to a larger increase in surface area.

Expressed mathematically

$$\frac{dS}{dt} \propto S \quad \text{or} \quad \frac{dS}{dt} = kS \quad \begin{matrix}(\text{assuming constant}\\ \text{hyphal diameter})\end{matrix}$$

where, S = the surface area.

This theory might apply also to the submerged hyphae of a surface culture, but cannot apply to the aerial hyphae, the surface of which is not directly in contact with nutrient. If, for the sake of discussion, it is assumed that half the hyphae are at the surface, then the net rate is

$$\frac{dS}{dt} \propto \frac{S}{2} \quad \text{or} \quad \frac{dS}{dt} = \frac{kS}{2} \; ;$$

and hence one would expect the constant of proportionality (the specific growth rate) for surface culture to be approximately half that for growth in submerged culture. Indeed, in the only microscopic study of hyphal growth in moving liquid culture known to the author, Machek and Fencl (1973) have presented data which show a specific growth rate of 0·41 h^{-1} for A. niger: the maximum specific growth rate measured for A. niger on solid medium is 0·27 h^{-1} (Trinci, 1971b). CLEARLY, FURTHER STUDY OF SUBMERGED HYPHAL GROWTH IS DESIRABLE.

Considering colony growth, it is evident from the literature that a wide variety of morphologies are obtainable in submerged culture and that the rate of growth is affected by morphology. In most studies of the kinetics of such growth, dry weight measurements are used to assemble data. By the time meaningful measurements can be made (say 24 hours after inoculation) the morphology may have already influenced growth kinetics: in such cases, the potential maximum growth rate is never measured.

Pirt (1966) introduced the peripheral growth zone model in an attempt to explain the observation of cube-root kinetics during growth of dense (or large) pellet structures (see fig. 2·4, page 20). Growth is likely to be exponential until the radius equals the width of the peripheral growth zone, but further consideration of the Pirt model suggests that cube-root growth kinetics are not likely to be observed until the ratio of colony radius to width of the peripheral growth zone is about 10.

When $r < w$,

$$\frac{dm}{dt} = km \quad \text{or} \quad \frac{dr}{dt} = \frac{kr}{3}$$

and there is exponential growth;

when $r > w$,

$$m_g = \frac{4}{3}\pi \left[r^3 - (r-w)^3\right] \rho$$

so that $$\frac{dm}{dt} = \frac{4}{3}\pi\rho\, 3r^2 \frac{dr}{dt} = \frac{4}{3}\pi\rho k \left[3r^2 w - 3rw^2 + w^3\right]$$

or, $$\frac{dr}{dt} = k\left[w - \frac{w^2}{r} + \frac{w^3}{3r^2}\right].$$

Now, only when $r > 10w$

$$4/3\ \pi\rho \left[3r^2 w - 3rw^2 + w^3\right] \approx 4\pi\rho r^2 w$$

so that $$4/3\ \pi r^2 \rho \frac{dr}{dt} = k\, 4\pi\rho r^2 w$$

or, $$\frac{dr}{dt} = kw \quad \text{and} \quad \frac{dm^{\frac{1}{3}}}{dt} = kw.$$

This model is still of only limited use since, even if the hyphal packing density is uniform throughout the colony, w cannot be readily calculated or measured in submerged culture, although Trinci (1971a) has measured values for surface cultures. For the extreme case where growth is limited by oxygen diffusion into a pellet, which is as densely packed as cell tissue, w can be estimated; in other cases, w will depend on the morphology. Without a better understanding of morphology at the microscopic level, it will clearly be difficult to evaluate w. ALL THIS SUGGESTS THAT IT IS THE MORPHOLOGY WHICH NEEDS TO BE MODELLED IN ORDER TO PREDICT GROWTH.

As a result of the above considerations it was decided that the two primary objectives for the research should be:

1. to study quantitatively the growth of filamentous fungal hyphae in submerged liquid culture, and

2. to develop a model to simulate the morphological development of submerged culture colonies.

C H A P T E R 3

HYPOTHESIS FOR FILAMENTOUS FUNGAL GROWTH

3.1 INTRODUCTION

3.2 THE HYPOTHESIS

3-1 INTRODUCTION

It is apparent from the previous chapter that there exists, in the literature, a degree of uncertainty concerning some aspects of the growth of filamentous fungi. There is, for example, little quantitative information regarding germination time distributions or germ-tube growth, and data for vegetative growth does not resolve whether individual hyphae extend at a linear rate or exponentially. It is desirable, therefore, at this stage, to present those concepts which the author has adopted as the basis for the research.

The hypothesis for growth will form the basis on which the experimental programme is planned, and will also provide the concepts used in the computer modelling. Testing the hypothesis will, of course, be one of the objectives of the research.

It must be pointed out that although the following is essentially the original hypothesis, as based on a review of the literature, slight modifications and ammendments have been made as the result of direct experimental observation, or from new ideas arising from experimental results. The hypothesis refers particularly to the growth of *A. niger* but it is hoped that it will also apply to other filamentous fungi.

3·2 THE HYPOTHESIS

Considering the growth of a population of spores, development can follow four paths.

1. All spores could germinate and grow synchronously.
2. All spores could germinate together and then proceed to grow at different rates.
3. Spores could germinate over a range of time but once germinated grow at the same rate.
4. Spores could germinate over a range of time and growth proceed at different rates.

Once germinated, conditions of growth are equal for all individuals in the culture and hence no variation should be expected. However, it is clear from any sample of individuals that synchronous germination and growth does not occur. The variation is most likely to arise from differences in the individual characteristics of the spores, causing germination to occur over a range of time. Thus assumption (3), that spores germinate over a range of time and growth proceeds at the same rate, is the one preferred and used by the author. The following hypothesis is now proposed.

Once dispersed in a well mixed aqueous suspension, the spores of a filamentous fungus, such as A. niger, will begin to swell, and aggregate as a consequence of physical collision. The degree of aggregation will depend upon the initial concentration of spores in suspension and the length of time over which aggregation occurs, (ie. from inoculation to germination). The germination phase will occur over a fairly wide range of time, perhaps several hours, and initial growth will be assisted by a spore's reserves. Only spores on the outside of the aggregate will germinate, the inner spores being deprived of certain nutrients.

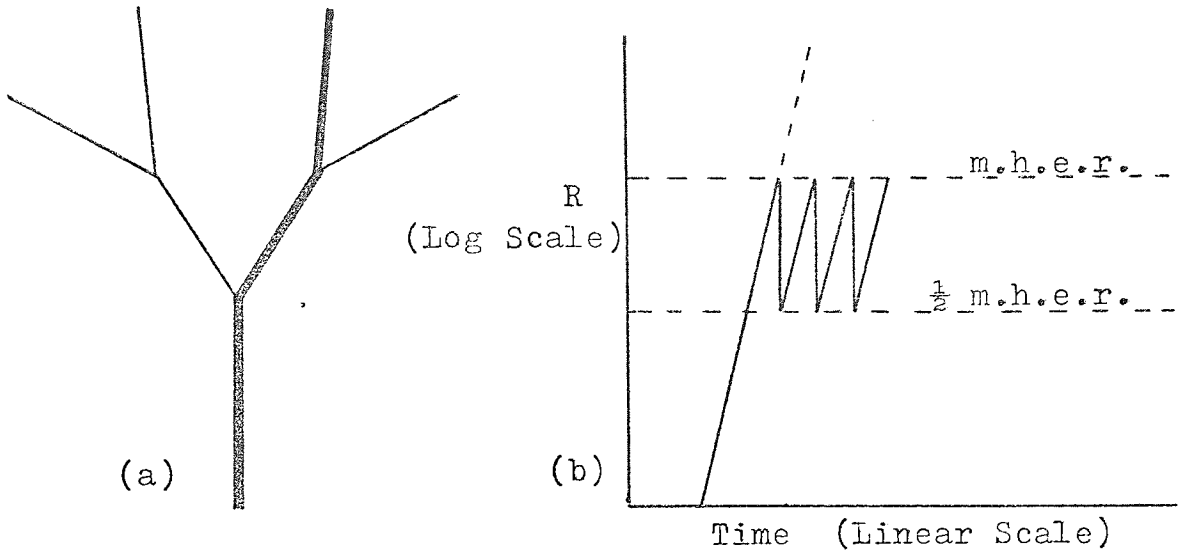
When growth becomes totally dependent on the external environment, ie. during vegetative growth, each individual sporeling will increase in size according to the same specific growth rate (which is a function of the conditions). While it is surrounded by a non-limiting excess of nutrients, a hypha will absorb a constant amount of nutrient per unit area per unit time, and hence its growth rate will be proportional to the surface area available for the transfer of nutrients. As growth continues, however, the lowering concentration of nutrients around a hypha may result in a falling rate of absorption. Growth rates may be further reduced by the decrease in effective surface area caused by hyphal entanglement and dense colony formation.

All growth occurs at the hyphal apex so that during unrestricted growth the extension rate will be constantly increasing. There will come a time, if this mode of growth continues, when the synthesis of new cell wall material at the apex can only just balance the input rate of nutrients (the maximum hyphal extension rate), resulting in a splitting of the apex - referred to as apical branching (fig. 3·1). Both limbs of the apical branch will then begin to grow at half the maximum hyphal extension rate.

Growth may continue in this fashion with apical branches being formed at regular intervals, but normally septa will be formed along hyphae, which may then become blocked. This will result in the slowing down of the apex extension rate and a build-up of nutrients behind the septum causing the formation of a lateral branch. The lateral branch will grow at right-angles to the parent hypha and will begin at a rate based on the length of hypha below the septum. The segment of hypha above the septum will continue contributing to growth at the main hyphal apex, while the segment below will now contribute to growth at the branch apex (see fig. 3·2). The overall effect will always be an exponential increase in total hyphal length per unit time.

Shortly after germination the formation of a second germ-tube may often be observed. This provides an extra apex at which growth can occur and consequently has the same effect on the rate of elongation of the main hypha as does the formation of an apical branch. Growth will be equally divided between the two apices until a septum is formed which blocks one from the other.

FIG. 3·1 APICAL BRANCHING

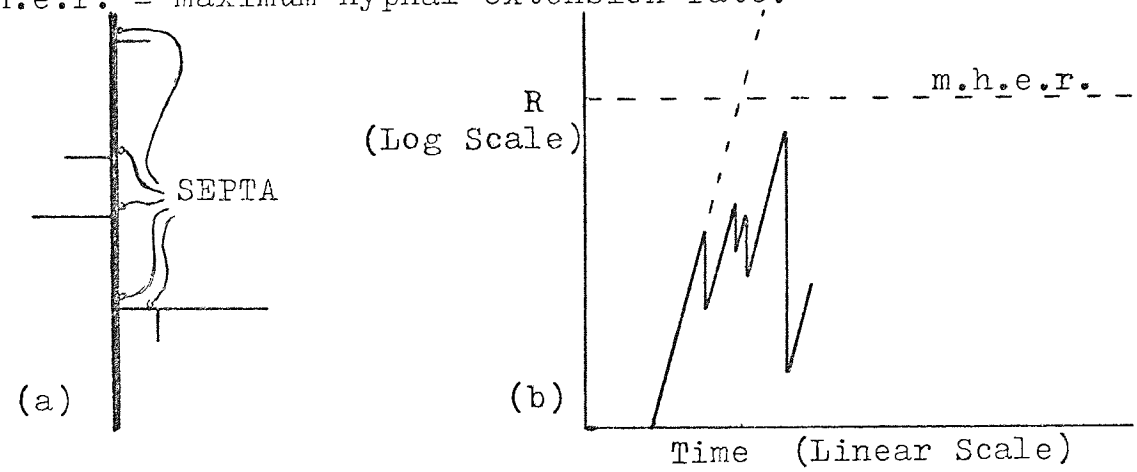


(a) Regular formation of apical branches in non-septate mycelium. The thick line represents the main hypha.

(b) Effect of apical branching on growth rate. R is the extension rate of :-

——— the main hypha (excluding branches)
- - - - - the whole mycelium

m.h.e.r. = maximum hyphal extension rate.



(a) Lateral branching showing positions of septa along hypha. The thick line represents the main hypha.

(b) Effect of lateral branching on growth rate. R is the extension rate of :-

——— the main hypha (excluding branches)
- - - - - the whole mycelium.

FIG. 3·2 LATERAL BRANCHING

C H A P T E R 4

EXPERIMENTAL EQUIPMENT AND PROCEDURE

4.1 INTRODUCTION

To achieve submerged culture growth of filamentous fungi a certain degree of agitation is required, otherwise growth occurs at the bulk air/liquid interface. Mechanical agitators are often used for this purpose; the high shear rate in the neighbourhood of the agitator largely preventing aggregation of the mycelium. Alternatively, agitation may be achieved by the passage of air bubbles through the liquid. This is the method used in tower fermenters, the gentler agitation providing adequate mixing but at the same time conditions suitable for aggregate or pellet formation. The latter method was used in this research because of the availability of equipment and knowledge of its use within the TFRG at Aston.

Wherever possible, throughout the research, the same experimental apparatus and operating procedure and conditions have been used; so, to minimise repetition later, these will all be described here. Where different procedures are followed, these will be introduced in the relevant chapters.

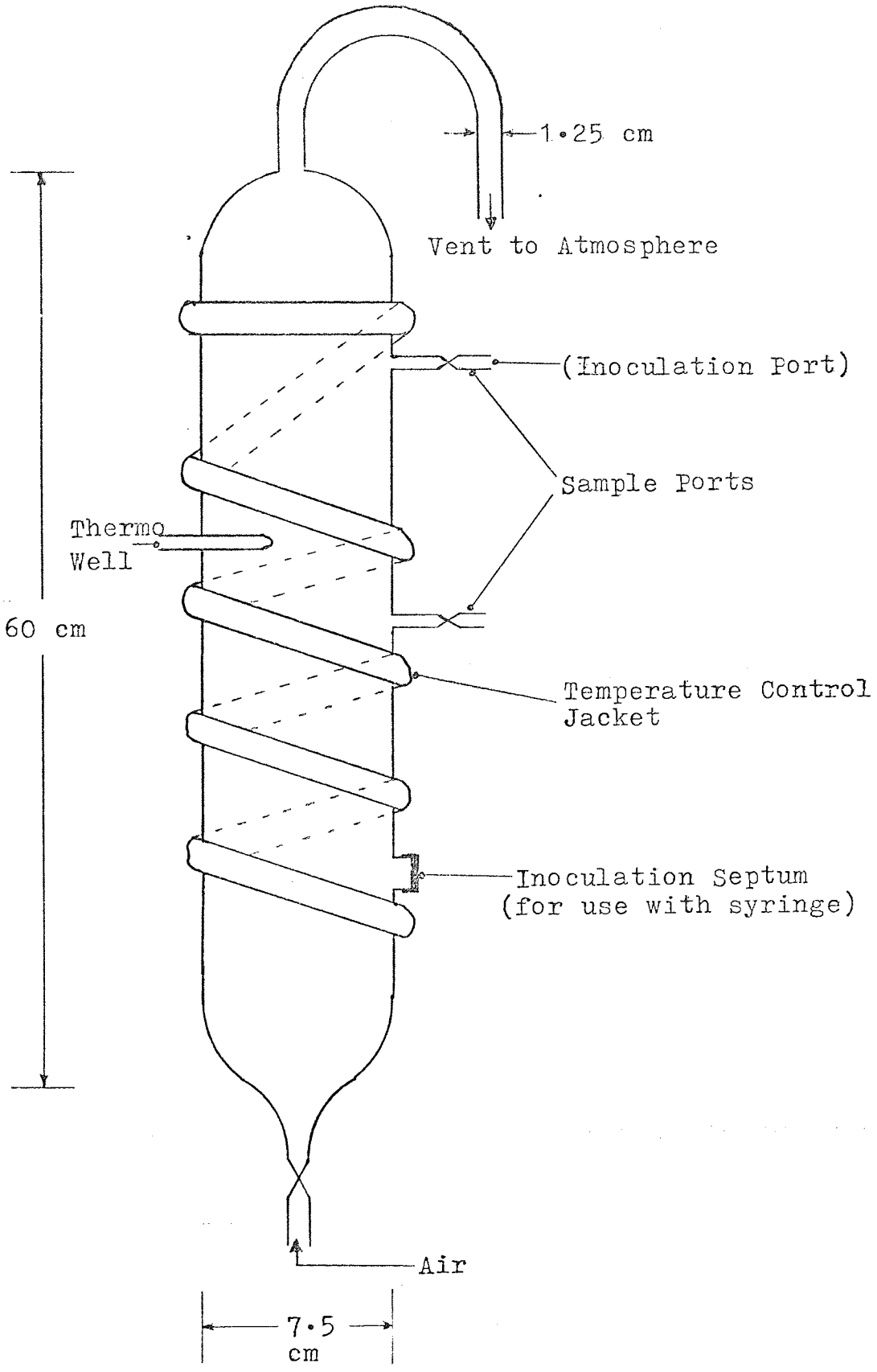
4·2 THE TOWER FERMENTER

Detailed descriptions of the design and uses of the tower fermenter are given elsewhere (Greenshields and Smith, 1971; Smith and Greenshields, 1974) but a brief summary is necessary.

The fermenter, a vertical tubular vessel (fig. 4·1), contains the liquid medium and micro-organism. Air, which is previously sterilised by passing through an in-line filter (Whatman, Gamma-12) is fed to the base of the tower and serves to agitate the contents as well as provide oxygen for microbial metabolism. In this way the tower behaves as a well mixed reactor. Temperature control is effected by a thermocirculator (Churchill) pumping water through a jacket of thin-walled tubing. The fermenter may be operated batchwise or continuously by feeding fresh medium to the base.

For most of the author's work a tower fermenter of 3 litre capacity and having a diameter of 7·5 cm was used in batchwise operation. Use of an air distributor was found to be unnecessary, so air was simply fed to the base of the tower.

FIG. 4.1 THE TOWER FERMENTER



Preparation of the Fermenter and Medium - Before each experiment the fermenter was sterilised by passing steam through it for 24 hours. The steam was fed to the top of the tower and released through the air inlet; all sample ports were also left open. At the end of the steaming period air was supplied at a low rate before disconnecting the steam. This ensured that a positive pressure was maintained in the tower. Following disconnection of the steam all sample ports were closed. At this point hot medium ($80^{o}C$) which had been autoclaved at $120^{o}C$ for 15 minutes was introduced into the tower via a sterile connection at the lower sample port. The aeration rate was then set at the required level for the experiment, the temperature controller set to the optimum for growth, and the fermenter left for a further 12 hours to stabilise before inoculation.

4·3 INOCULATION APPARATUS

The normal laboratory practice of inoculating the fermenter using a sterile syringe and needle was unsuitable for much of the research, since large numbers of spores had to be used. To ensure high inoculum concentrations and also to reduce risk of infection the apparatus in fig. 4·2 was used.

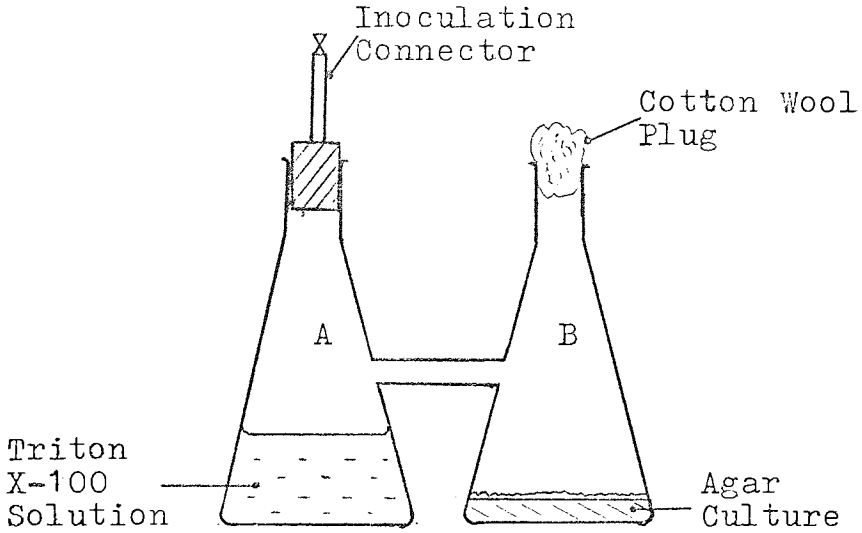


Fig. 4·2 Inoculation Apparatus

The preculture was grown on 50 cm^3 of nutrient agar in flask B. 100 cm^3 of Triton X-100 solution (Sigma Chemicals Ltd.) in flask A was then transferred to B in order to wash spores from the preculture. The spore suspension was returned to A and then to the fermenter by means of the connector tube. Since the whole apparatus, complete with agar and Triton X-100 solution, was sterilised before starting the preculture, asepticity was maintained easily. This method also overcame the problem of blockage of syringe needles by hyphal fragments or agar and had the additional advantage that the apparatus could be used over and over again.

Inoculum Preparation - Spores scraped from a fresh sub-culture of the organism were used to inoculate the nutrient agar in the inoculation apparatus. This preculture was then incubated for a period of 10 - 12 days (see chapter 5) at 30°C, by which time a thick mat of spores was produced. Shortly before the experiment spores were washed from the mat with a 0·01% v/v (ie. one drop per 100 cm^3) Triton X-100 solution and shaken for one hour. The surface active Triton X-100 ensured maximum removal of spores from the preculture. After shaking, the suspension was transferred to the fermenter as described above.

4·4 GENERAL EXPERIMENTAL PROCEDURE

Having prepared the fermenter and inoculum, conditions were checked and inoculation carried out. Time was recorded and samples taken at the desired intervals.

Organisms - These were: Aspergillus niger strain A38; Penicillium chrysogenum; and Mucor sp. All were taken from the Culture Collection of the Fermentation Laboratory, Department of Biological Sciences, University of Aston in Birmingham. Master cultures were kept at 0°C and replaced at monthly intervals. Sub-cultures were kept at 30°C and renewed weekly.

Medium - The substrate was a non-limiting growth medium of composition:

Sucrose	2·75 %
Ammonium sulphate, $(NH_4)_2.SO_4$	0·6 %
Sodium dihydrogen orthophosphate, $Na.H_2.PO_4$	0·05 %
Yeast extract	0·05 %
Potassium chloride, K.Cl	0·025 %
Magnesium sulphate, $Mg.SO_4$	0·01 %
Calcium chloride, $Ca.Cl_2$	0·005 %
Tap water	96·51 %

Operating Conditions - The fermenter was operated at 31°C, the optimum temperature for growth of A.niger in submerged culture. Air was supplied at a superficial velocity of 1·5 cm/s (approximately 1·4 v.v.m.), sufficient to supply the oxygen requirements of the organisms and to keep the fermenter contents well mixed.

Inoculation - It was found that growing precultures on 50 cm^3 of nutrient agar in 250 cm^3 conical flasks provided enough spores to inoculate the fermenter to a concentration of at least 10^6 spores/cm^3. A small amount of the inoculum was kept in order to determine its concentration, and shortly after inoculation a small sample was taken from the fermenter to check this.

The Haemocytometer - Spore concentrations were determined using this instrument, which provides a very small, known volume within which the number of spores may be counted under a microscope (see fig. 4·3). A cover slip is pressed into position on the slide, such that Newton's rings are visible, and the sample is allowed to fill the volume by capillary action. When counting the number of spores within each square of the grid, those overlapping either the top or right edge are included while those overlapping the other two edges are not.

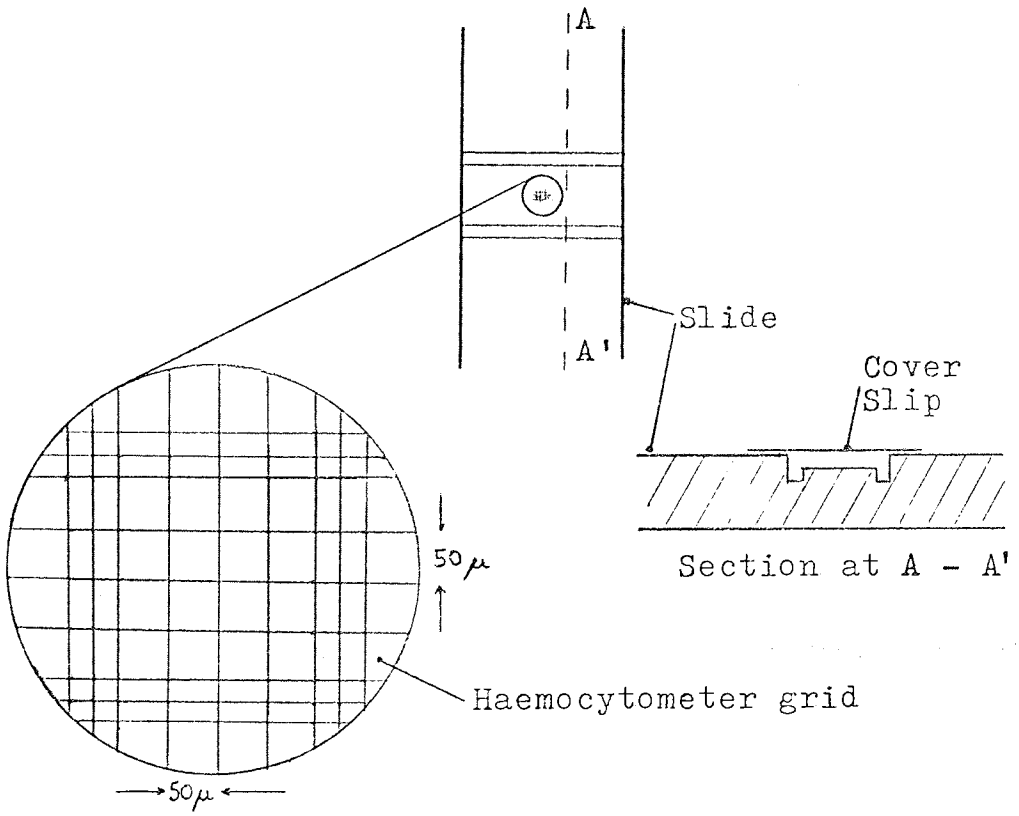


Fig. 4·3 The Haemocytometer

Sampling - Samples were taken from the fermenter via the lower sample port at regular intervals depending on the type of experiment. Approximately 10 cm^3 was first run off to ensure the sample was not from the still zone of the port, and, after sampling, the port was rinsed with alcohol to prevent infecting organisms entering the fermenter. Samples were either analysed immediately or fixed with 40% w/v formaldehyde solution.

C H A P T E R 5

G E R M I N A T I O N

5·1 INTRODUCTION

5·2 EXPERIMENTAL PROGRAMME

5·3 RESULTS, OBSERVATIONS AND DISCUSSION

5·4 CONCLUSIONS

5.1 INTRODUCTION

The germination phase of growth includes the activity between the initial lag period and the onset of vegetative growth. It therefore covers the swelling phase, the period of germ-tube emergence and the initial growth of the germ-tube (Allen, 1965).

It is evident from the literature that the swelling phase involves a linear increase in diameter of the spore and, microbiologically, is a very significant part of the growth cycle. Nevertheless, it is felt that its effect on the engineering of processes can be ignored, and so no experimental work was carried out with the main aim of studying spore swelling.

In order to quantify and model the growth of fungi it is necessary to know when, and over what time range, germination occurs. Such information must be obtained experimentally. It is also necessary to know what proportion of spores in an inoculum actually germinate.

Germ-tube growth is difficult to define and isolate from other phases of growth and so it was not studied separately. In data analysis and modelling it has been viewed as part of the vegetative growth stage.

The main aim of this chapter will therefore be to establish a generalised germination function for use in the subsequent growth model.

5·2 EXPERIMENTAL PROGRAMME

Experiments were performed to determine the time distribution of germination and the effect of spore age on germination.

The procedure set out in chapter 4 was followed and the fermenter was inoculated to a concentration of the order of 10^6 spores/cm^3. All conditions were as described in chapter 4. Small samples were taken from the fermenter every 30 minutes until germination began and then at intervals of 10 minutes. The samples were analysed by counting 100 single spores under the microscope and recording whether each was either unswollen or swollen or germinating. This was continued until germination was complete, ie. no change was observed in the proportion of germinated spores.

5·3 RESULTS, OBSERVATIONS AND DISCUSSION

During the experiments, spores invariably started to swell at 3·5 hours after inoculation, and germination began at around 5 hours. They were seen to swell from

a mean diameter of 2·8 microns (standard deviation 0·25 microns from 33 measurements) to a mean of 6·9 microns (standard deviation 0·65 microns) prior to germination. No intermediate measurements were made to check whether swelling was linear. Germination under normal conditions took place between 5 and 8 hours and was distributed about a mean of 6·5 hours. Fig. 5·1 shows a typical germination curve obtained from the results, and fig. 5·2 indicates that the distribution was normal.

Yanagita (1957) reported that the germination of A.niger in submerged culture started at 5 hours and continued until 9 hours after inoculation. Unfortunately, he did not include a germination time distribution, and the only one that could be found is that for Geotrichum candidum (Kier et.al., 1976). This organism germinates after only one hour and over a range of only 1·5 hours, but the mean germination time is at the mid-point of the range as is the case for the results in fig. 5·1.

The proportion of spores germinating was affected by the age of the spores used for inoculation. Inocula were usually prepared from spores that had been grown on a nutrient agar for 10 days, but for these experiments spores were harvested after as little as 5 days incubation or as much as 28 days.

FIG. 5·1 GERMINATION TIME DISTRIBUTION

(Data from experiment G3)

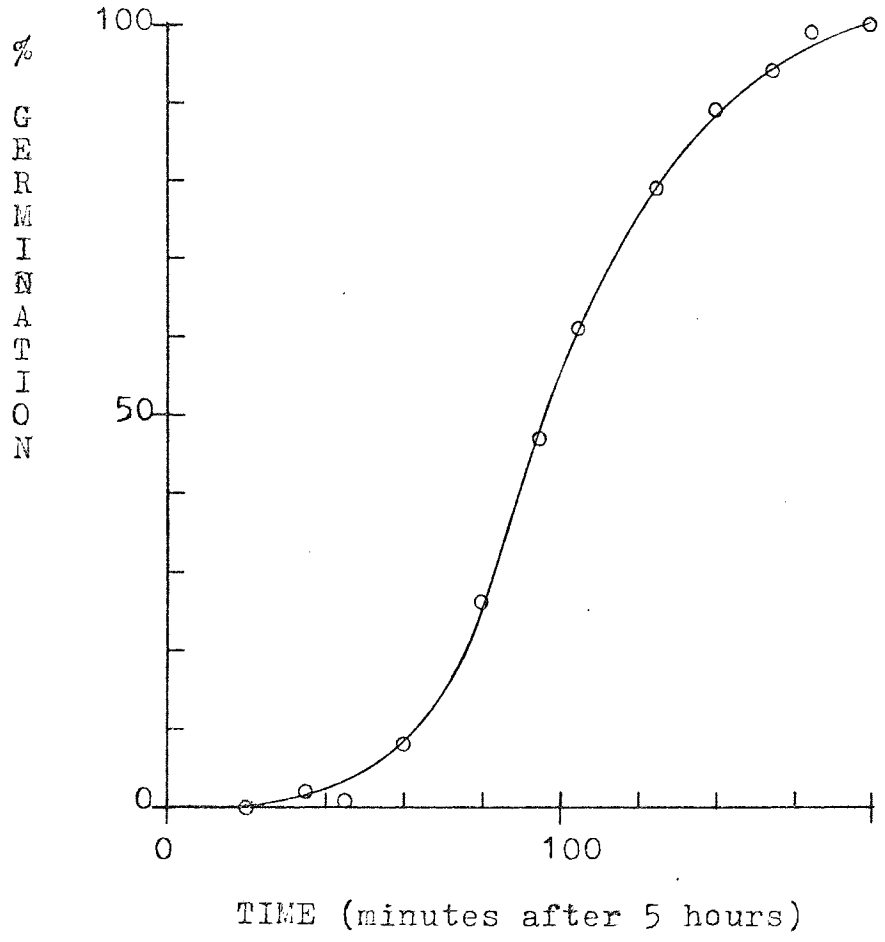
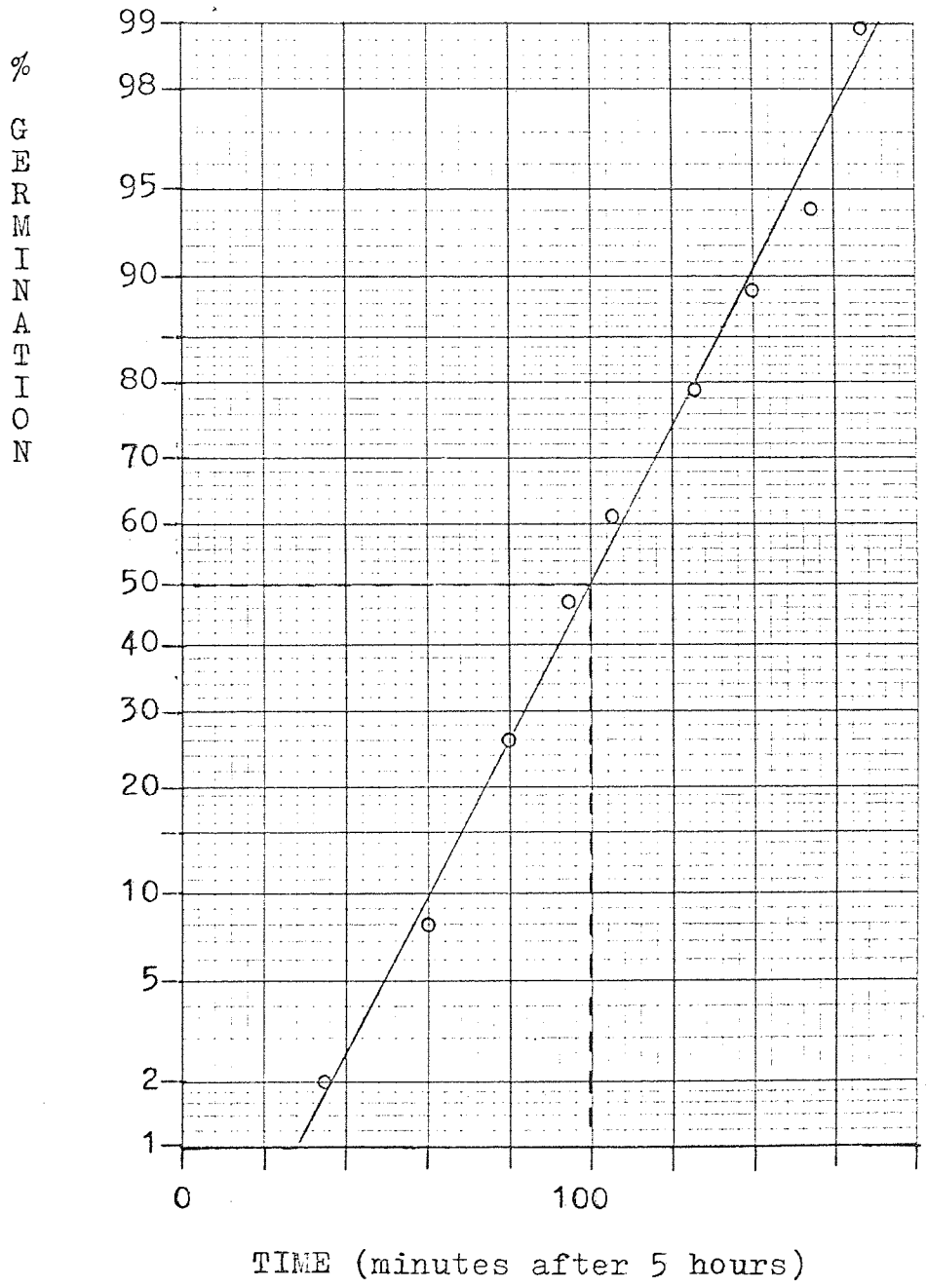


FIG. 5·2 GERMINATION TIME DISTRIBUTION

(Data from G3 plotted on 'normal probability' scale)



The results (fig. 5·3) showed that 10-12 days was the optimum incubation period and that at this age 100% germination was achieved amongst unaggregated spores. Approximately 40% of the unaggregated spores had not germinated by 8 hours when cultures both older and younger than 10-12 days were used. With older spores germination did not continue after 8 hours, and when the data were corrected for 65% germination (by multiplying by 100/65) a similar curve to that in fig. 5·1 was obtained. The younger spores, though, did not show the same trend. Germination began as normal at 5 hours after inoculation but progressed slowly; it was still occuring by 8 hours and continued for at least an hour afterwards.

Meyrath and Suchanek (1972) have noted that viability decreases with age and advise the use of fresh spores for inocula. However, they do not mention the effects of using an immature preculture. The longer germination range experienced with the young spores is possibly due to the wider range of spore sizes and relative maturity.

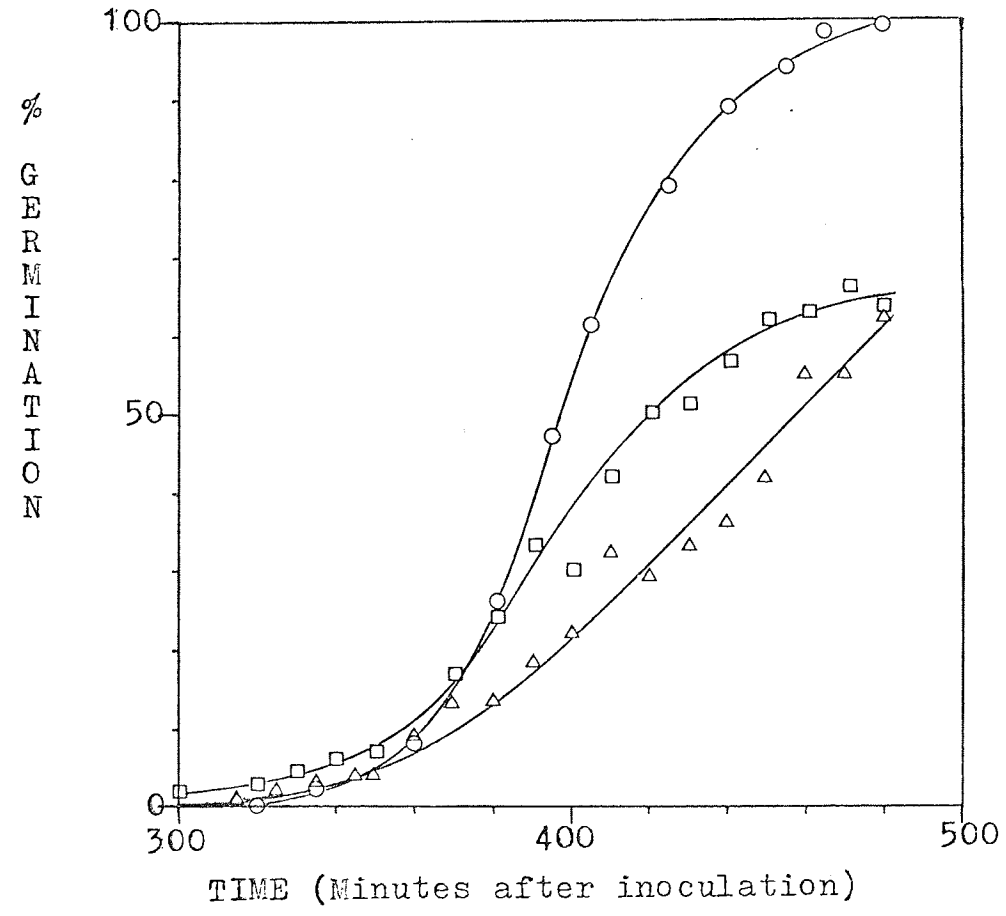
Fig 5·3 THE EFFECTS OF SPORE AGE ON GERMINATION

Data :-

△- 5 day old culture (experiment G4)

○- 12 day old culture (experiment G3)

□- 27 day old culture (experiment G5)



The main factor affecting the proportion of spores which germinate is not apparent from the above results, which are for unaggregated spores only. Aggregation prevents the germination of many spores, and only those at the surface of the aggregate appear to germinate. This was observed many times during the course of the research: it was verified by changing the aeration rate just after germination had started, and so causing aggregates to break up and release many ungerminated spores into the medium (Plate 5·1). Although these spores had in some way been previously prevented from germinating, they later produced germ-tubes.

Obviously this effect on germination must be included in any model of growth. The proportion of spores germinating in an aggregate will depend on its size but may be assumed to be those spores in the outer layer only. Now

$$N_{ss} = \frac{4(d_a - d_s).(1 - \epsilon)}{(d_s)^2} \qquad (5\cdot1)$$

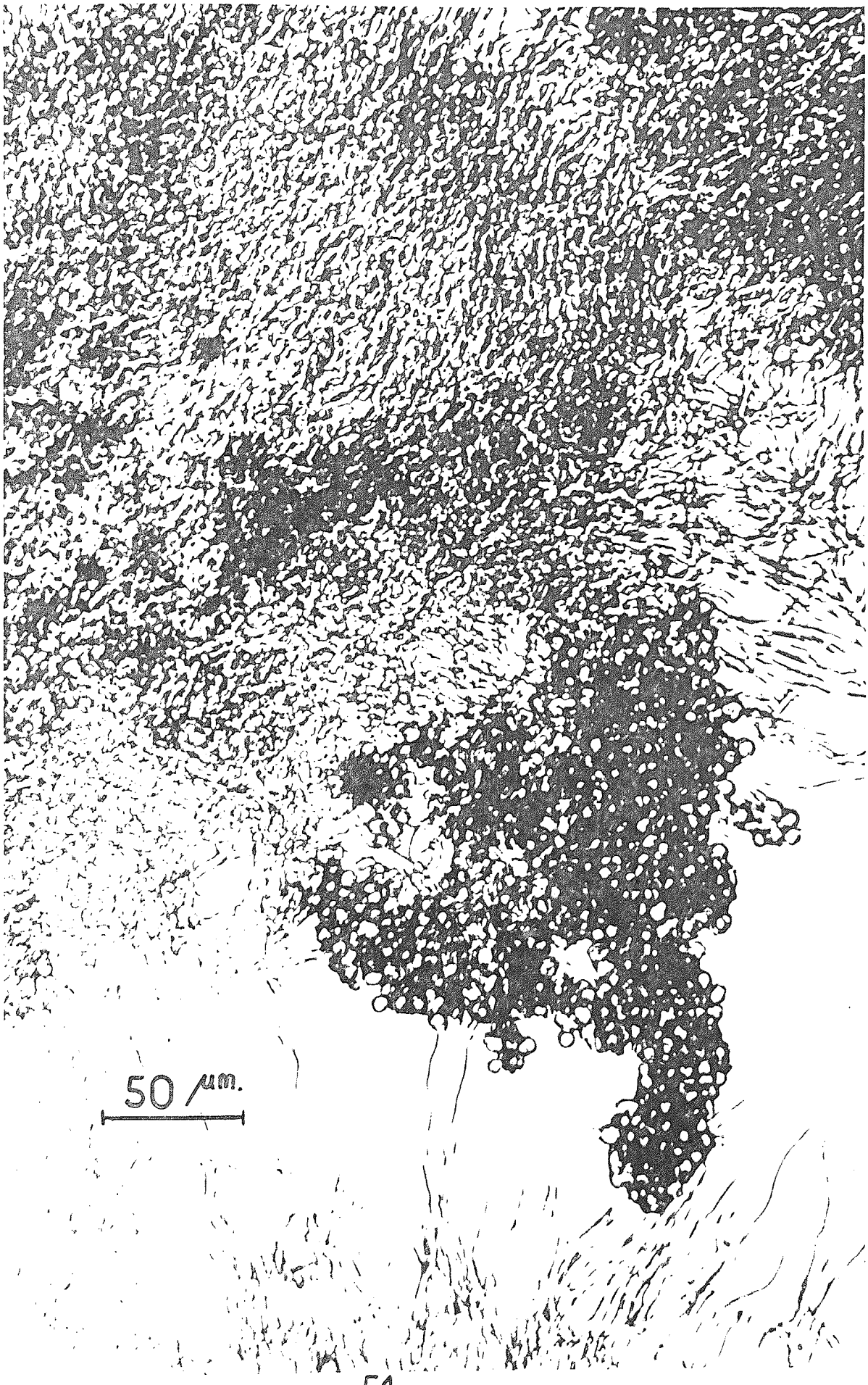
where, N_{ss} = number of spores at the surface of an aggregate (see fig. 5·4)

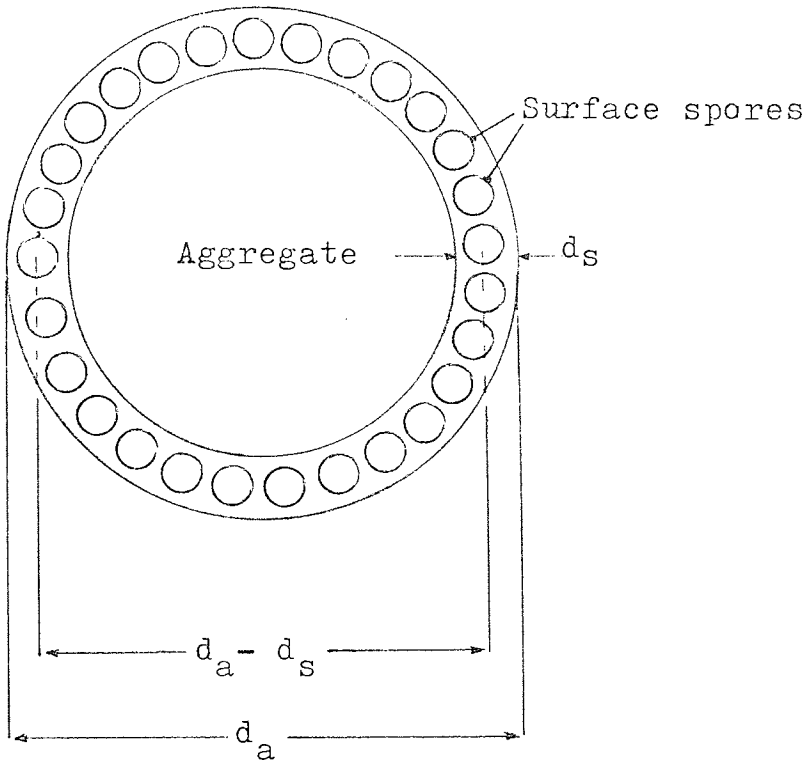
d_a = aggregate diameter

d_s = spore diameter

ϵ = surface voidage of spores

PLATE 5·1 UNGERMINATED SPORES WITHIN AN AGGREGATE





Spore cross section area = $\frac{(d_s)^2 . \pi}{4}$

Area of aggregate (at dotted line) = $\pi . (d_a - d_s)^2$

Fig. 5·4 Spores at the Surface of an Aggregate

The proportion of spores from an inoculum that actually germinate is further affected by washout from the fermenter. This is discussed in chapter 7, and so it is sufficient to say here that up to 50% of the inoculum may be lost in this way, resulting in a drastic reduction in spore concentration.

5·4 CONCLUSIONS

Germination begins at about 5 hours after inoculation and 1·5 hours after the onset of spore swelling. During the swelling phase a spore will more than double in diameter but stops swelling when a germ-tube is produced. The exact time at which germination occurs is randomly spread over the three hour period 5 - 8 hours, with a mean time of 6·5 hours. If fresh, mature spores are used for the inoculum, 100% germination may be achieved, though this is affected by the concentration of spores in the inoculum and by aggregation. With as many as 50% of the inoculum being washed out of the fermenter and over 70% of aggregated spores unable to germinate (see section 8·2·3) the actual percentage germination is very low.

CHAPTER 6

VEGETATIVE GROWTH

6.1 INTRODUCTION

Most of the research was concerned with vegetative growth, since the microscopic growth of filamentous fungi in submerged culture has received little attention to date. First, a technique for studying growth of hyphae in submerged culture had to be developed. This was then used to collect growth data for modelling purposes. The need for information about germ-tube formation and branching characteristics was also taken into account when planning the experiments.

Results using the technique were then compared with results from dry weight and surface culture measurements. The experimental and analytical techniques were then used in studying the growth of filamentous fungi other than *A. niger*.

6.2 EXPERIMENTAL PROGRAMME AND PROCEDURE

Various experiments were performed in order to develop the technique, which was then used to gather data for modelling purposes. It can be argued that data from the more usual methods of assessing growth, ie. by means of dry weight measurements or surface

culture observations, are easier to analyse; for this reason experiments were performed to compare these with the author's technique to see if either could replace it.

The Technique for Study of Submerged Growth - Flowers and Williams (1977) have pointed out that for studying the early growth of microbes in submerged culture, cell counting and turbidimetric techniques are of most use provided that the organism is extremely well dispersed. Although filamentous organisms are generally badly dispersed, turbidimetric methods have been used to study early growth of Streptomycetes (Flowers and Williams, 1977) and Geotrichum candidum (Trinci, 1972; Kier et.al., 1976). There is no direct method for measuring early growth in submerged culture (Calam, 1969), and, apart for the above exceptions, most work has been restricted to cultures on solid media. This mode of growth enables the use of time-lapse photography (eg. Trinci, 1969), since the subject remains at a fixed point for the duration of the study. The nearest approach to time-lapse photography possible in submerged liquid culture studies involves taking many photomicrographs of individual 'sporelings' (young germinated spores) from samples taken at regular

intervals. Each sample must come from the same inoculum and be growing under the same environmental conditions. These requirements indicate the need for a large volume fermenter and preclude the use of shake-flasks: for this reason the aerated tower fermenter was chosen. The tower, described in chapter 4, lacks mechanical parts which might cause hyphal fracture and mycelial fragmentation, and its aspect ratio (height to diameter) ensures uniform mixing and good temperature control.

The experimental procedure followed for the growth experimnts has been outlined in chapter 4. It was found by experiment that an inoculum concentration of about 10^6 spore/cm^3 was required in order to provide a suitable number of sporelings for microscopic study. Small samples, about 10 cm^3, in volume, were extracted from the fermenter at hourly intervals and fixed immediately by the addition of small amounts of a 40% w/v aqueous solution of formaldehyde. Microscopic analysis of samples was normally carried out within seven days of the experiment.

Photomicrographs of individual sporelings were made in each case by scanning a slide containing a little of the sample, as shown in fig. 6.1.

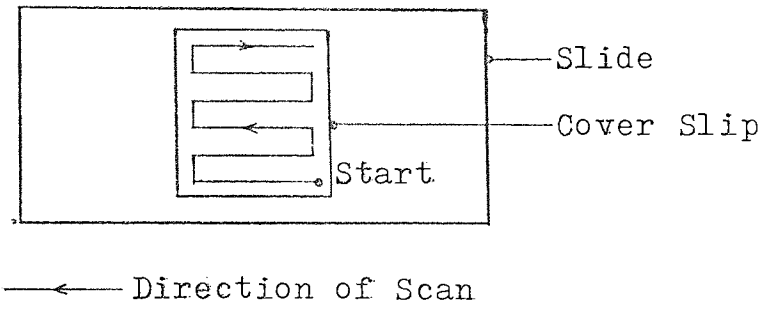


Fig. 6.1 Sample Scanning Procedure

The first 35 or so individual sporelings encountered were photographed in order to get a representative set, and any sporelings in contact with others were noted and passed over. A photomicrograph of a slide micrometer (Graticules Ltd.) having 10 micron divisions was also taken with each sample.

The image from each developed photomicrograph was projected onto the back of a glass screen, ground on one side and kept at a fixed distance from the projector (final magnification x2300 or sometimes x4600). Measurements of total hyphal length (THL) were then made by tracing the hyphal image with the geared wheel of a map-measuring instrument. The measurements were scaled to actual size using the projected image of the slide micrometer. The lengths, position, and type of branches (apical or lateral) were also recorded. Note that spores were not included in the length measurements.

Dry Weight Work - The usual method of assessing growth in submerged culture involves making dry weight measurements of samples, starting approximately 24 hours after inoculation. As with other experiments the procedure for the dry weight work was that set out in chapter 4, with 100 cm^3 samples being taken hourly from 24 hours onwards. The samples were washed and filtered onto previosly dried and weighed papers (Whatman, no.3), and then they were dried at 90°C for at least 24 hours until a constant weight was obtained.

A further experiment (BF12) was performed in which samples were taken hourly from 8 hours onwards. In this case, fresh medium was used to maintain a constant volume within the fermenter; the dry weight measurements were adjusted accordingly.

Surface Culture Work - For this work a microscope with heated stage (Reichardt Ltd.) was used. Small amounts of the medium (see chapter 4) were solidified by the addition of agar agar (Oxoid Ltd.) in a petri dish. This was inoculated with a drop of spore suspension and the base inverted over the circular microscope stage, which was kept at 31°C (see fig 6·2).

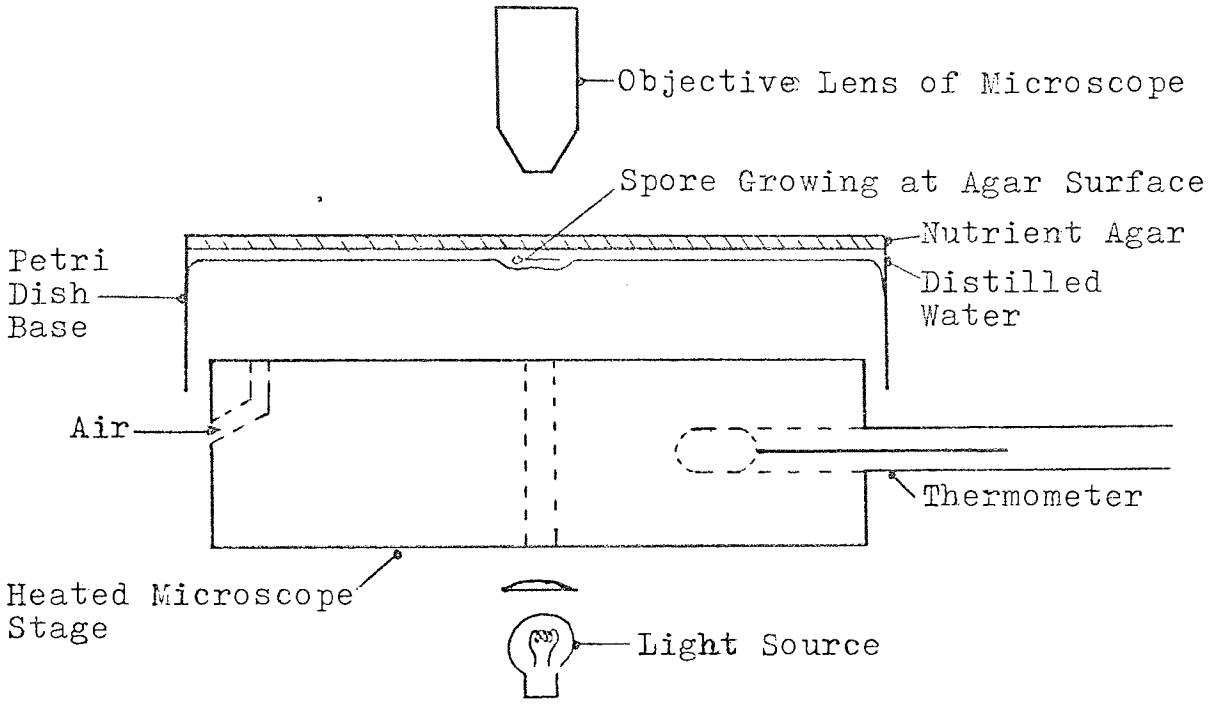


Fig. 6·2 Arrangement for Surface Culture Work

Air movement was by means of natural convection through a hole in the side of the stage. At the same time the agar was kept wet by periodic addition of distilled water: this aided microscopic observation and also enabled submerged culture conditions to be partially simulated.

The petri-dish base was arranged such that a single spore could be observed through the microscope. Photomicrographs of the spore were taken at 15 minute intervals during growth, and the data were analysed in the same way as those obtained in the submerged culture work (see above).

6·3 RESULTS, OBSERVATIONS AND DISCUSSION

6·3·1 The Overall Growth of Aspergillus Niger

The decision to measure only the growth of individual sporelings was reached because of the following points. Firstly, it was noticed that germination of aggregated spores was apparently synchronous within each aggregate (see Plate 6·1), although the extent of growth at any time varied from aggregate to aggregate. Secondly, when two or more spores were in contact there was often uncertainty as to which spore was associated with particular hyphal segments.

Probably the most prominent feature of the initial data was the remarkably wide size range in each sample (see Plate 6·2). Total hyphal length (THL) measurements frequently covered a near ten-fold range. However, making the assumption that, once germinated, all sporelings grow at the same specific growth rate (see chapter 3), it was possible for analysis to be carried out. Fig. 6·3 shows typical size distributions from samples.

PLATE 6·1 SYNCHRONOUS GROWTH

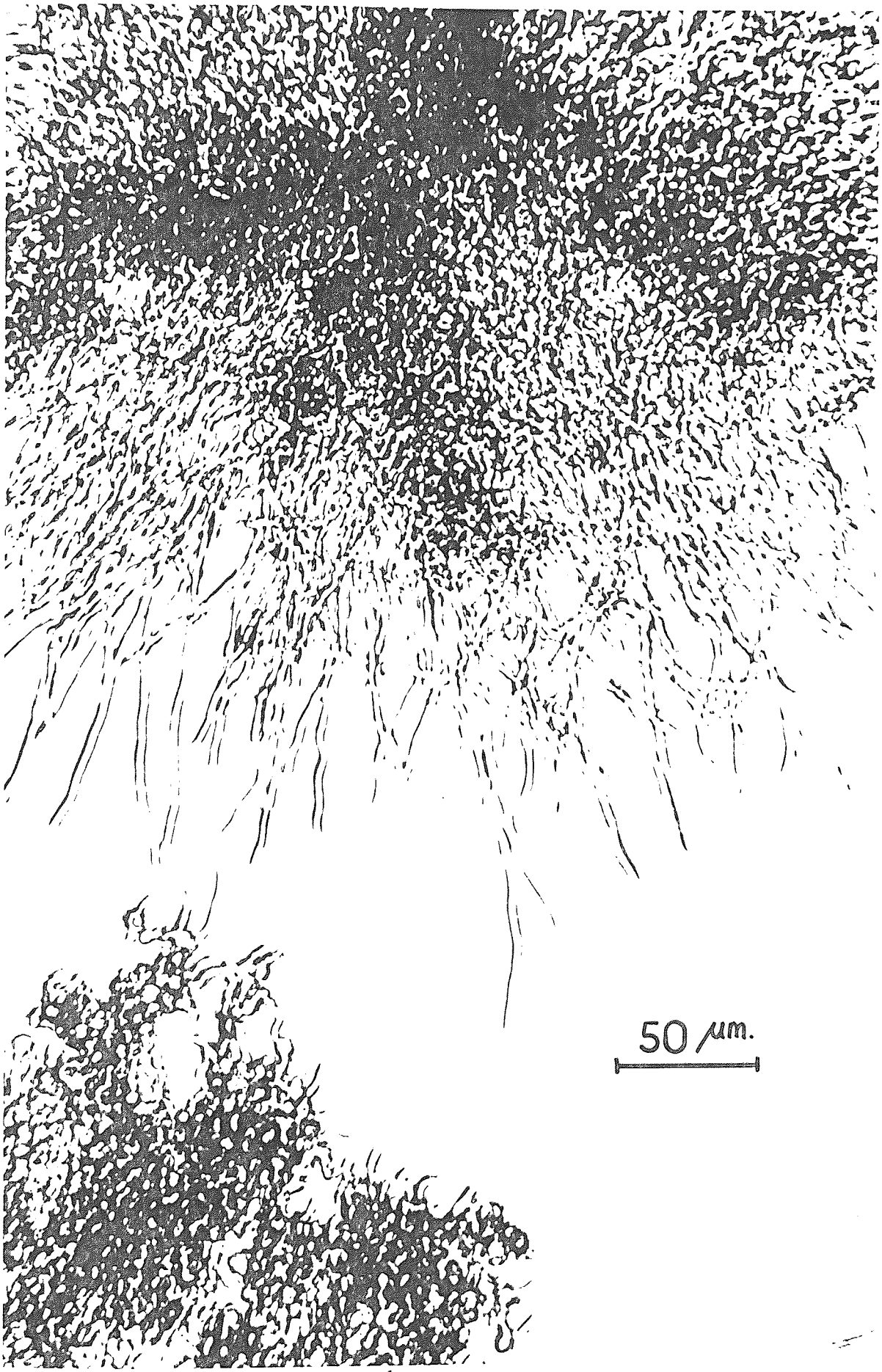
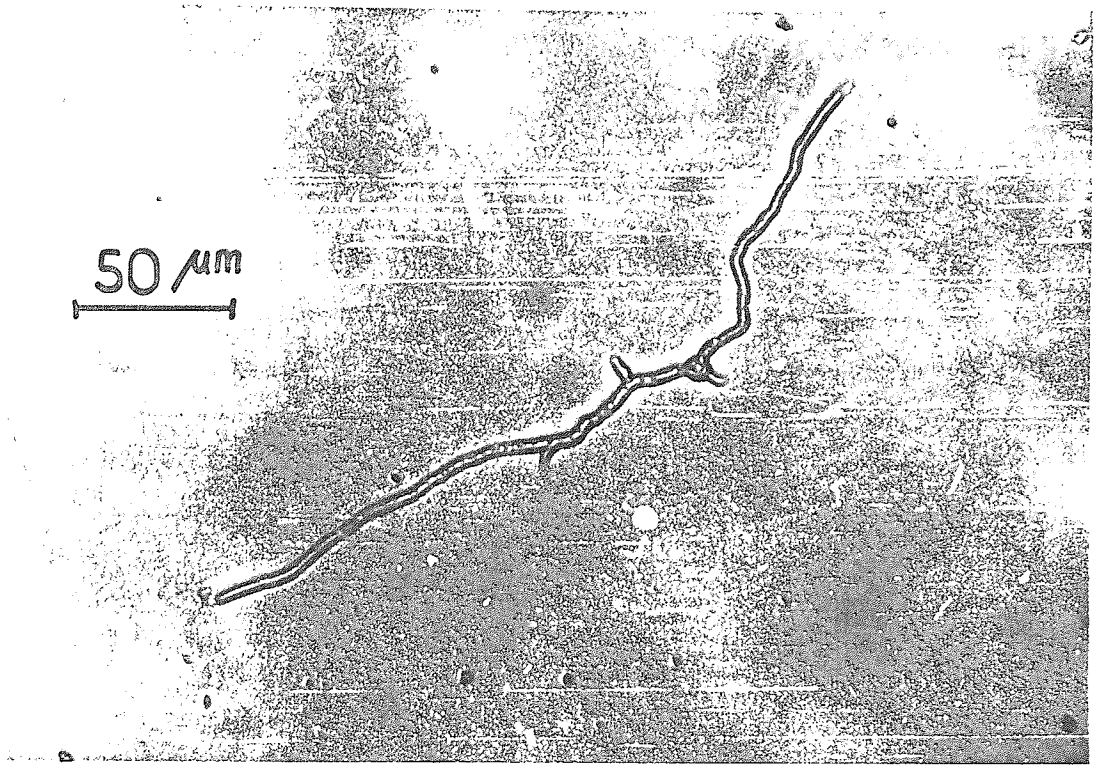


PLATE 6·2 SIZE RANGE WITHIN A SAMPLE



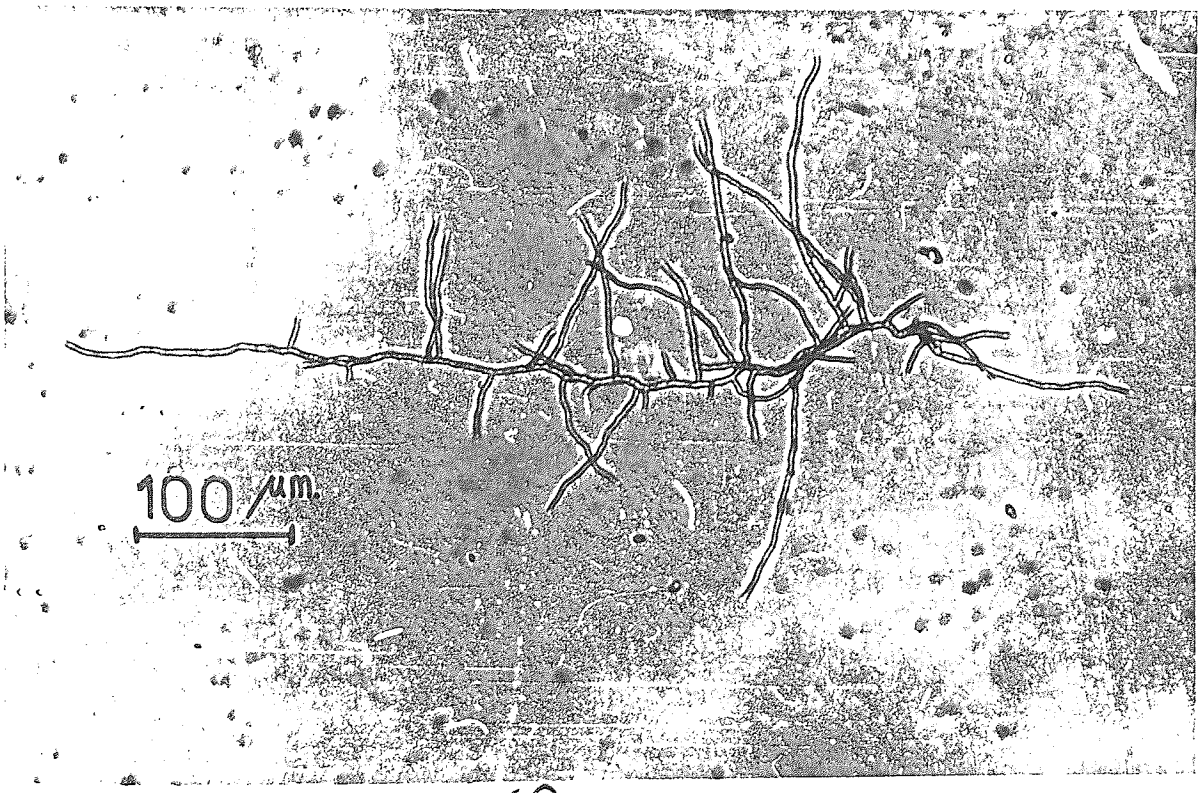
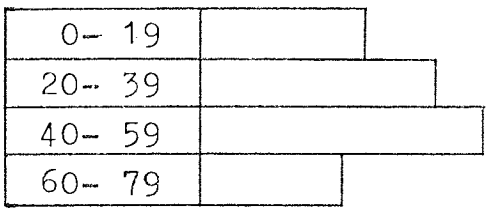
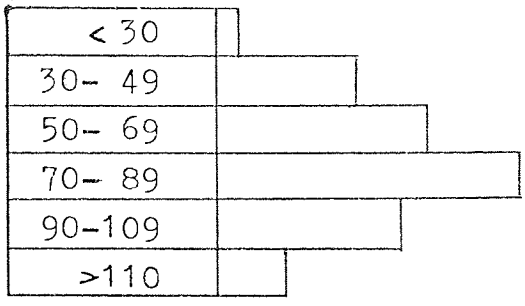


Fig. 6.3 SAMPLE SIZE DISTRIBUTIONS FROM EXPERIMENT BF5

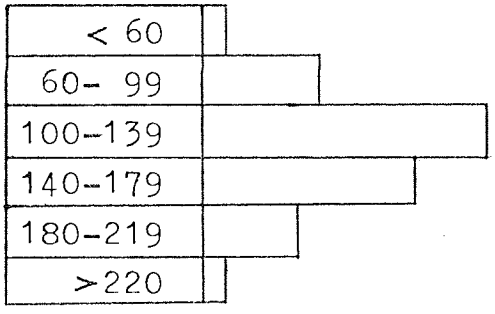
(Total Hyphal Length ranges, measured in microns)



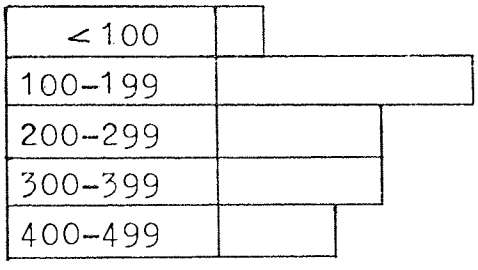
Sample 1 (mean = 37 μm)



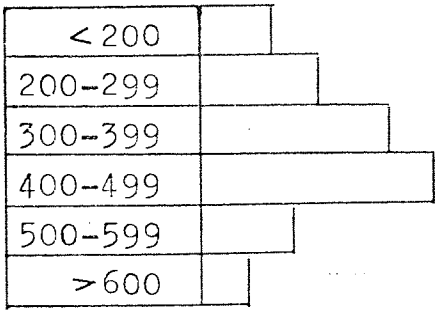
Sample 2 (mean = 78 μm)



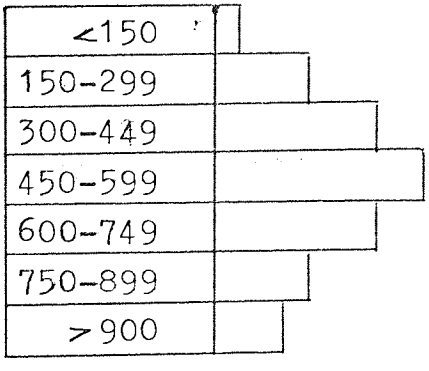
Sample 3 (mean = 137 μm)



Sample 4 (mean = 260 μm)



Sample 5 (mean = 391 μm)



Sample 6 (mean = 540 μm)

From the 35 or so data from each sample the THL of the largest individual and the mean THL were recorded; Table 6.1 shows three such sets of data. Measurements could not be accurately made after 17 hours due to the complexity of the hyphal structures, and even at 15 or so hours it was often difficult to ascertain whether growth was from a single spore.

Sample Time	BF5		BF6		BF12	
	largest THL	mean THL	largest THL	mean THL	largest THL	mean THL
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	32	-	-	-
8	-	-	74	-	-	-
9	61 (117)	37	137	-	-	-
10	180	78	252	134	-	-
11	268	137	410	230	261	145
12	444	260	718	430	391	162
13	670	391	1146	792	731	375
14	956	540	1537	1016	1061	491
15	1702	793	2821	1455	1670	816
16	1902 (2362)	982	-	-	-	-
17	3795	1569	-	-	-	-

Data in brackets are from a later scan of the sample but were not included in the analysis.

UNITS - THL in microns, Sample Time in hours after inoculation.

Table 6.1 Total Hyphal Lengths Measurements

Fig. 6·4 shows growth to be extremely rapid during the initial few hours following germination (cf. Trinci, 1971b), but from about 9 hours there was a linear relationship between the logarithm of the largest THL per sample and time (figs. 6·4 and 6·5). The relationship between mean THL per sample and time was not so clear.

Returning to fig. 6·4, the data represent the maximum growth of A.niger under the author's experimental conditions. By extrapolating the solid line to the point corresponding to the time of earliest germination (5 hours) a fictitious hyphal length (L_g) of 25 microns results. This idea is based on the exponential growth equation

$$L = L_o . e^{\mu.(t-t_o)} \qquad (6·1)$$

Germination times (t_g) for all other individuals were then calculated by putting equation 6·1 in the form

$$t_g = t_s - \frac{\ln(L/L_g)}{\mu} \qquad (6·2)$$

where, t_s = sample time (Hours after inoculation)

L = THL of individual (microns)

μ = Specific growth rate, represented by the gradient of the solid line (1/hours)

L_g = 25 microns for A.niger

Fig. 6·4 MAXIMUM GROWTH LINE - A.niger

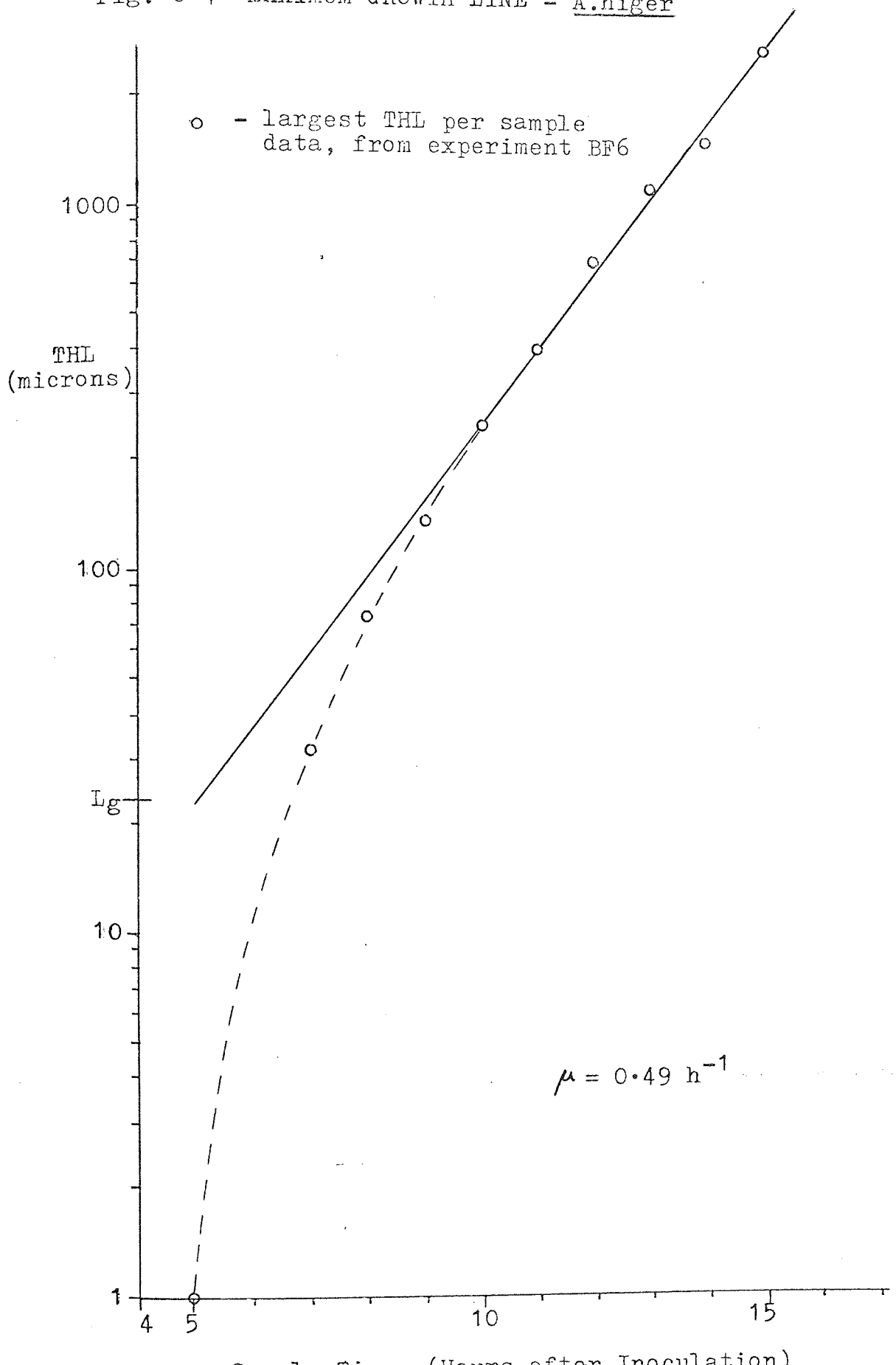


Fig. 6·5 MAXIMUM GROWTH LINE - A.niger

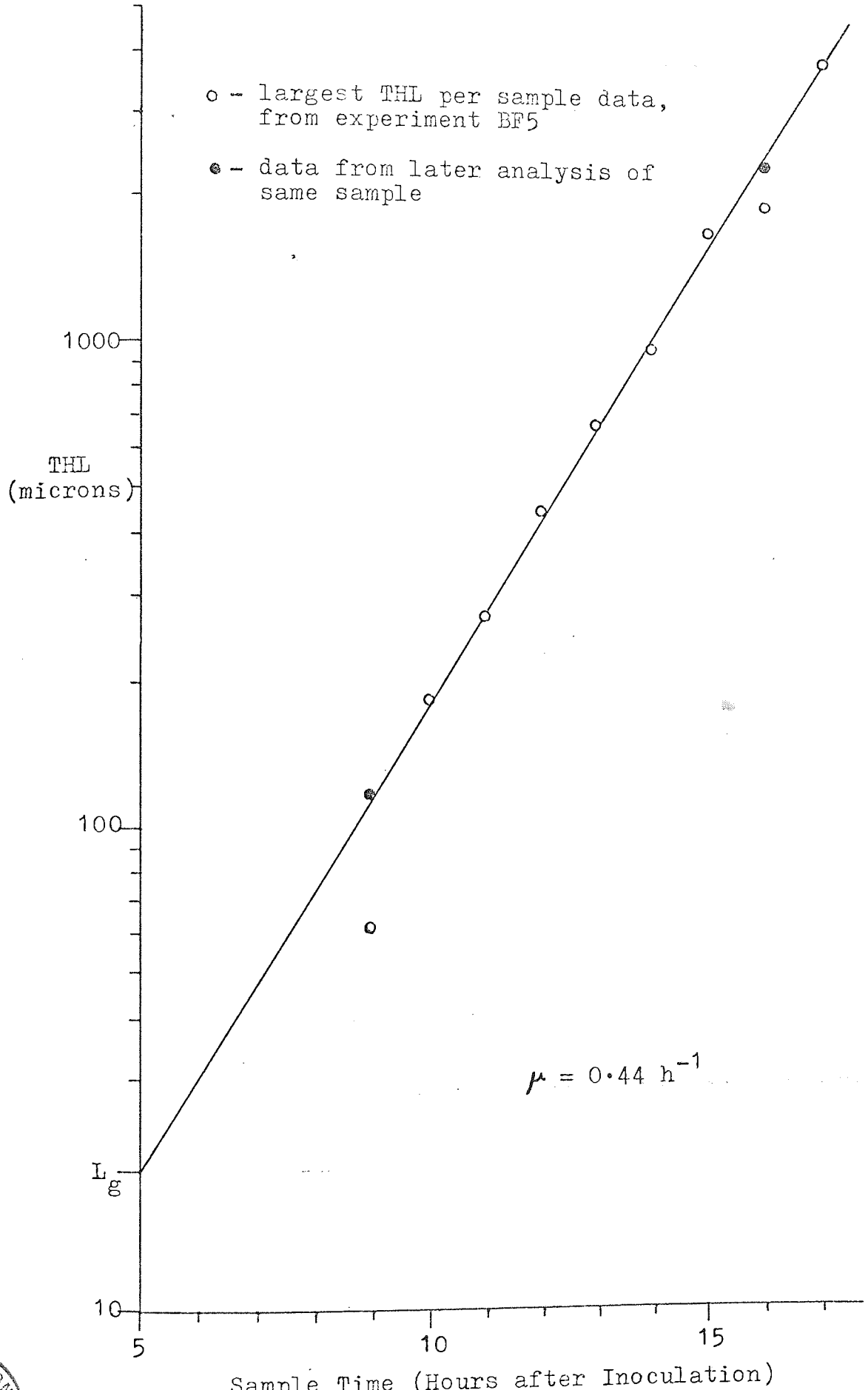


Fig. 6·6 shows a germination time distribution resulting from this analysis, compared with experimental data from chapter 5.

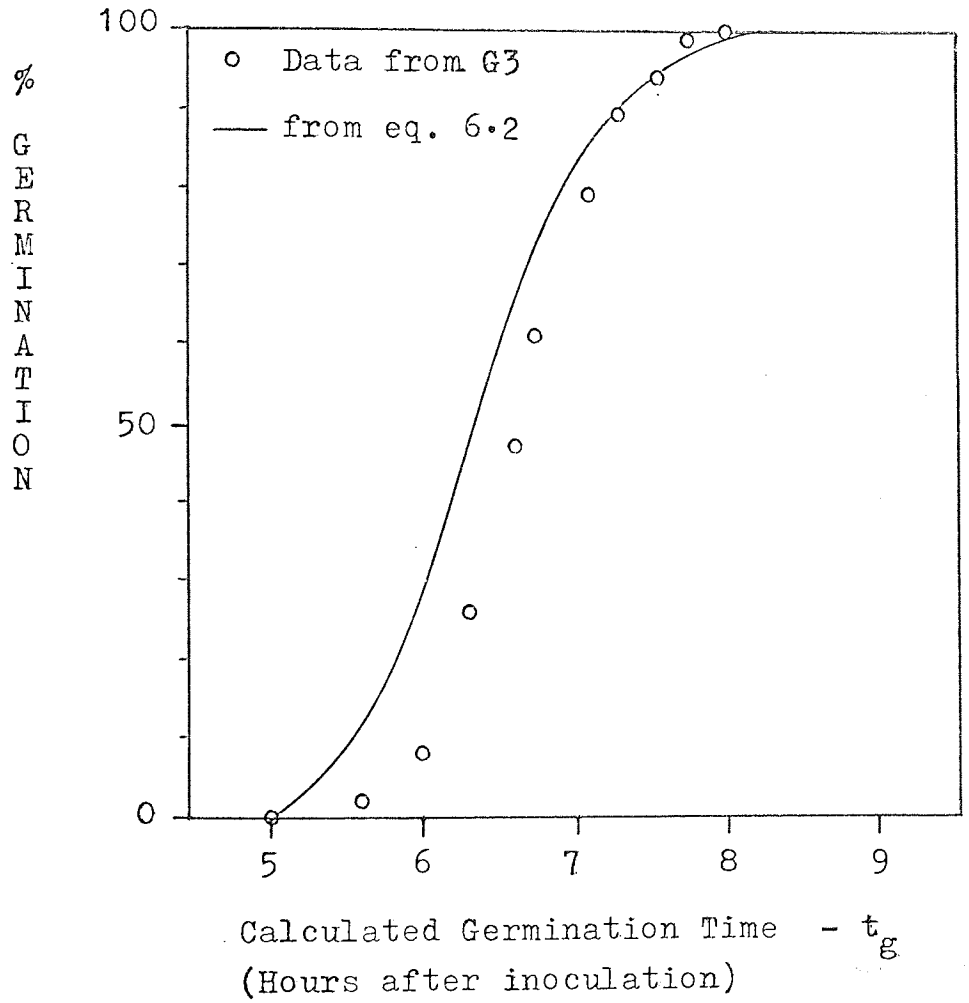


Fig. 6·6 Germination Time Distribution

Starting at the calculated mean germination time and L_g, and using a slope equal to the specific growth rate, semi-log plots were drawn through the data based on mean THL values. These graphs are presented on figs. 6·7 and 6·8, which also show linear regression lines. The same value for L_g was used in each case.

Following this analysis the age of each individual could be calculated $(t_s - t_g)$ and data regrouped according to age. This made it possible to study branching and secondary germ-tube formation.

Fig. 6·7 GROWTH LINE BASED ON CALCULATED MEAN GERMINATION TIME - A.niger

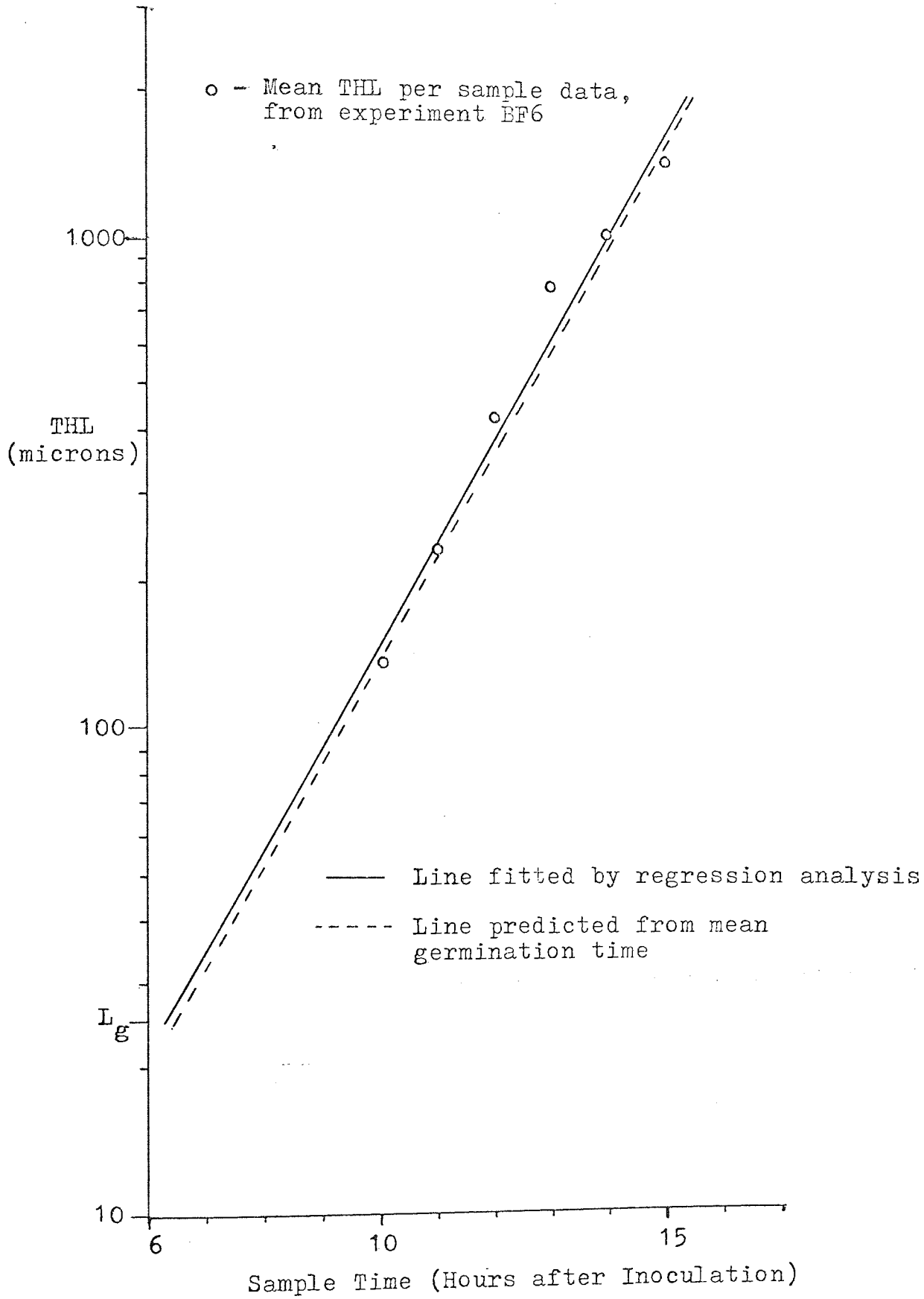
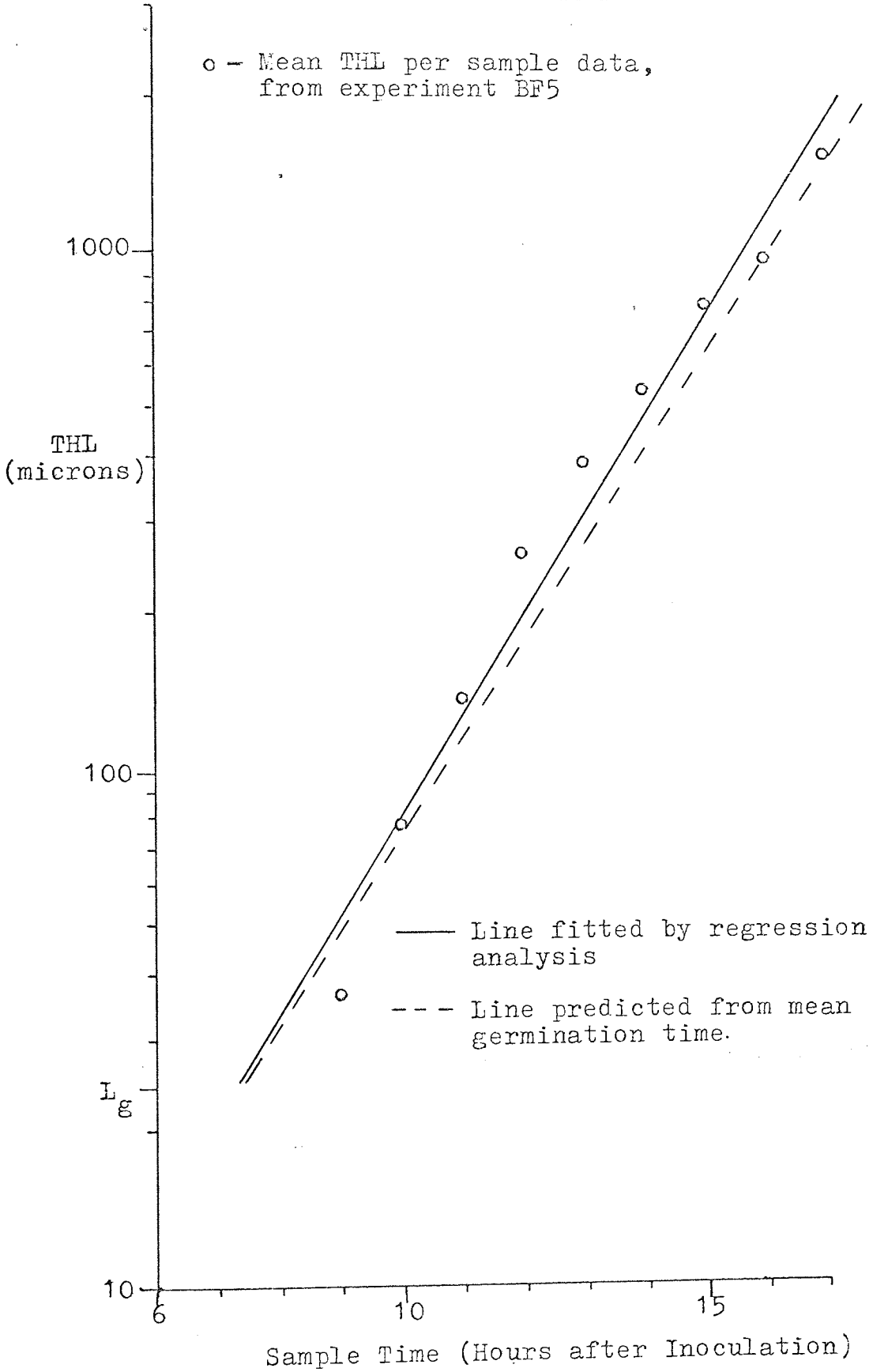


Fig. 6.8 GROWTH LINE BASED ON CALCULATED MEAN GERMINATION TIME - A.niger



Discussion - The wide variation in sporeling sizes observed with all samples from the fermenter probably explains why so few studies have been made of hyphal growth in submerged culture. Smith (1924) was faced with a similar problem when studying the growth of surface cultures: "The results were at first bewildering in their apparent irregularity ..... and this although they were on the same preparation, distant from one another not more than a couple of millimetres, and under conditions of growth as identical as one could hope to achieve." As in the present study, once variations were accounted for in terms of a spread of germination times data became much easier to handle. Of course, with submerged culture there was the additional problem that one could not study the growth of any particular individual. However, by selecting the largest sporeling from a sample one has essentially chosen the individual which germinated first. Similar selection from the other samples yields sporelings with approximately the same germination time, so that a picture emerges of the change in size of such sporelings: this is shown well in figs. 6·4 and 6·5.

The period of rapid elongation observed before the onset of exponential growth (see fig. 6·4) is clearly related to the germ-tube growth phase. At germination the spore can be thought of as bursting under the increased pressure associated with swelling, thus pushing out the newly formed germ-tube which is able to utilise the spore's reserves until vegetative growth takes over. It is interesting to contrast this phase of growth with its counterpart on the idealised curve often used to describe fungal growth (see fig. 6·9).

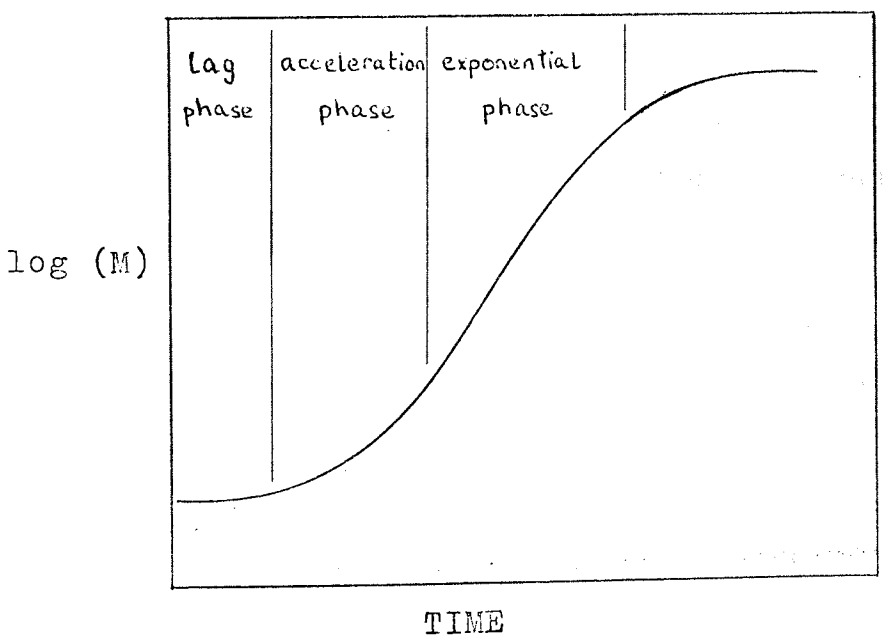


Fig. 6·9 Idealised Growth Curve (from Mandels, 1965)

Following the lag phase, growth is shown to accelerate from zero to an exponential rate, whereas in fig 6·4 growth is shown as starting suddenly at germination and then decelerating to an exponential rate.

That submerged culture growth is exponential during the early stages is fairly widely accepted and is supported by the results of the experiments presented in this chapter. The results also show that the specific growth rate for submerged culture is extremely high when compared with values based on surface culture measurements (Trinci, 1971b). This is discussed in section 6.4.

The germination times obtained using equation 6·2 above provide a good basis for assessment of the analysis technique and also of the hypothesis behind it. As can be seen from fig. 6·6, data calculated from the results of experiment BF6 produced a very similar distribution to that corresponding to the actual data of experiment G3. These two particular sets of data have not been chosen for any specific reason and exemplify results obtained in other experiments. Their similarity clearly suggests that

the hypothesis that all individuals grow with the same specific growth rate provided a reasonable basis for analysing the photomicrographic data. Additional support for the hypothesis is provided by the way that predicted lines, based on calculated mean germination times, fitted data for the mean THL per sample. Obviously, because of the method of sampling and data analysis some scatter in the results is to be expected: this is reflected in the differences apparent in fig.6·6 and also in figs. 6·7 and 6·8 between actual data points and predicted lines.

6·3·2 Secondary Germ-tube and Branch Formation

General Observations - During the first few hours of growth all sporelings developed a second germ-tube and sometimes a third.

As growth continued branches were formed. These tended to be mainly lateral branches and were usually situated closer to the spore than to the hyphal apex. They were approximately the same width as the main hypha and grew initially at right-angles to it. Occasionally, such a branch formed close behind the main hyphal apex and could be seen to have taken over as the leading hypha (see fig. 6·10).

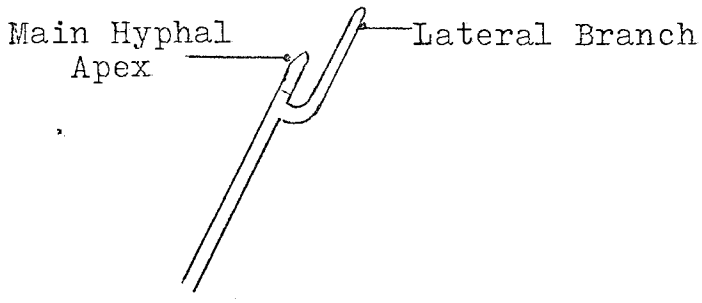


Fig. 6·10 Lateral Branch Taking Over as Leading Hypha

The presence of septa along hyphae was often associated with lateral branching. A branch would be positioned typically just upstream of the septum (assuming flow is towards the apex), and quite often there would be a short 'empty' section of hypha just downstream of it (see fig. 6·11).

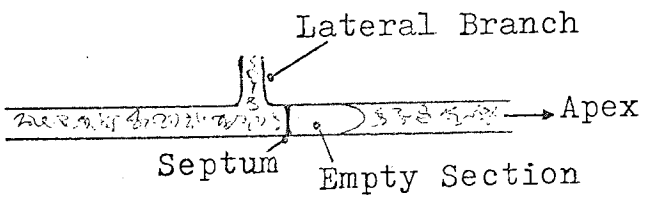


Fig. 6·11 Lateral Branching and Septation

It is impossible, from analysis of the photomicrographs, to say definitely whether septa appeared before branches or visa versa, but the indication is that septa were

laid down first. Quite often two septa were seen with no branch between them, but it was rare to find two branches which were not separated by a septum. A highly significant correlation between septation and branch initiation has recently been reported by Fiddy and Trinci (1976a,b). Plate 6·3 shows typical lateral branching and septa.

Occasionally, at later stages of growth, apical branches were observed. These tended to grow identically and at about 20^{o} to either side of the parent hypha (see Plate 6·4): septa were not associated with apical branching. Because of the low numbers observed, apical branches could not be included in the branching analysis which follows.

Following numerical analysis of the data (see section 6·3·1), it was possible to regroup sporelings by age rather than sample time. This made it possible to directly compare sporelings of a similar age and so carry out analysis of secondary germ-tube formation and branching patterns.

PLATE 6·3 SEPTATION AND LATERAL BRANCHING

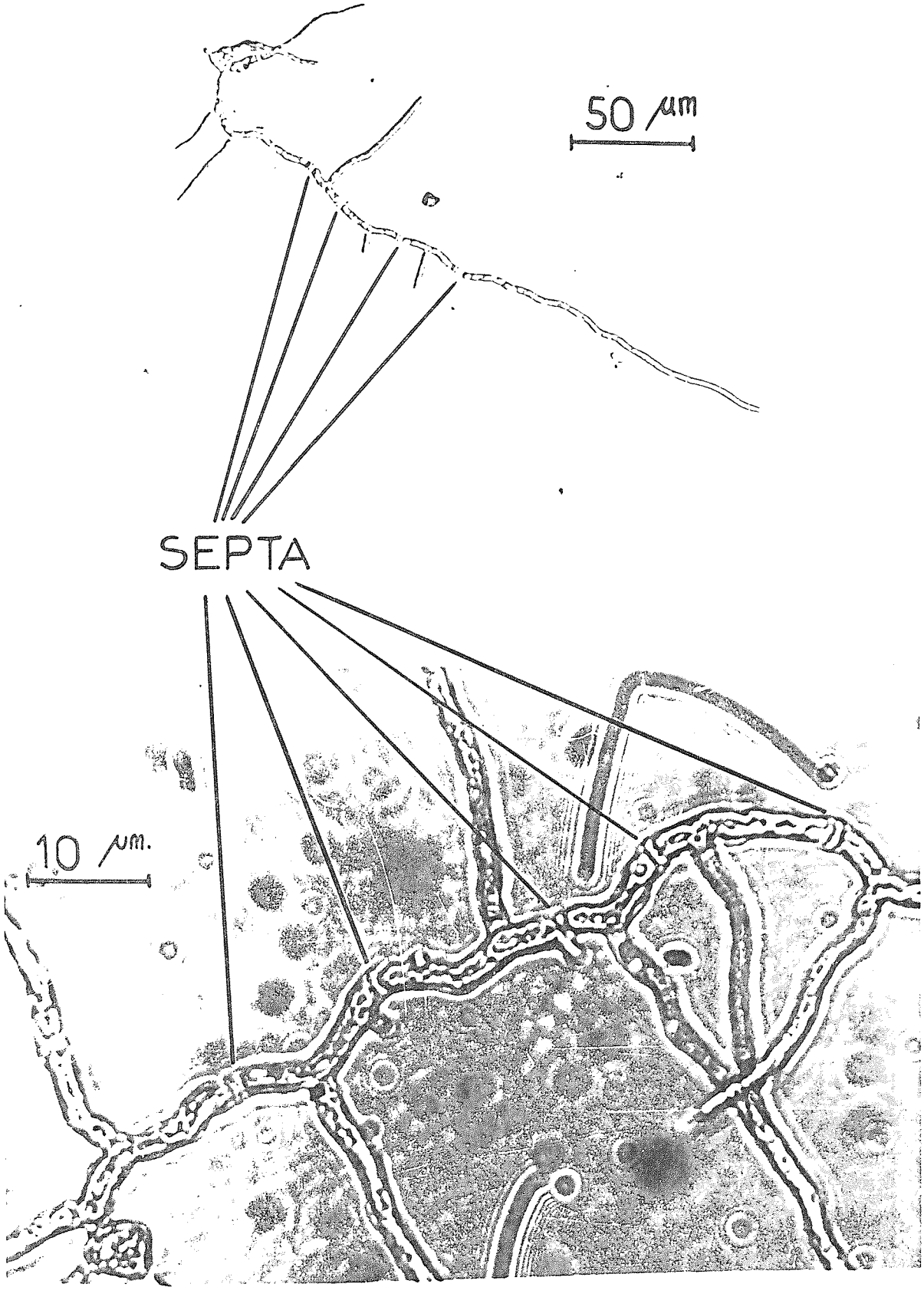
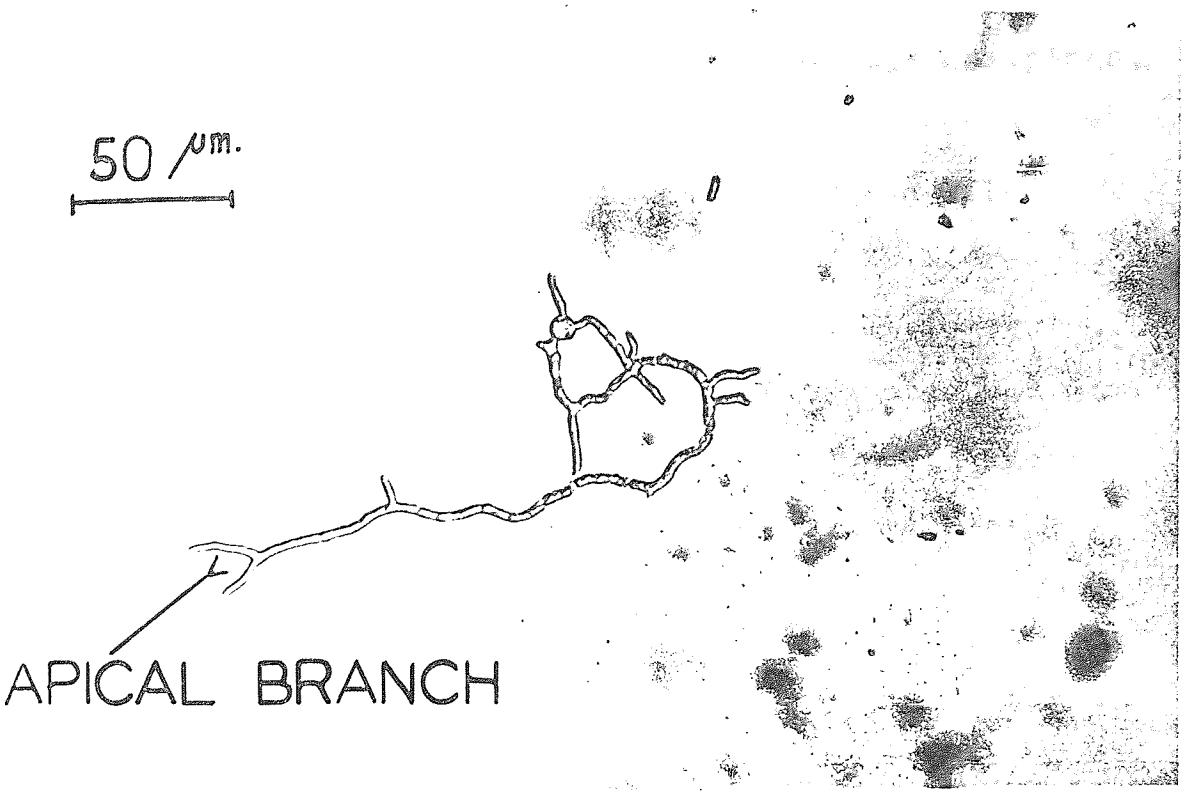
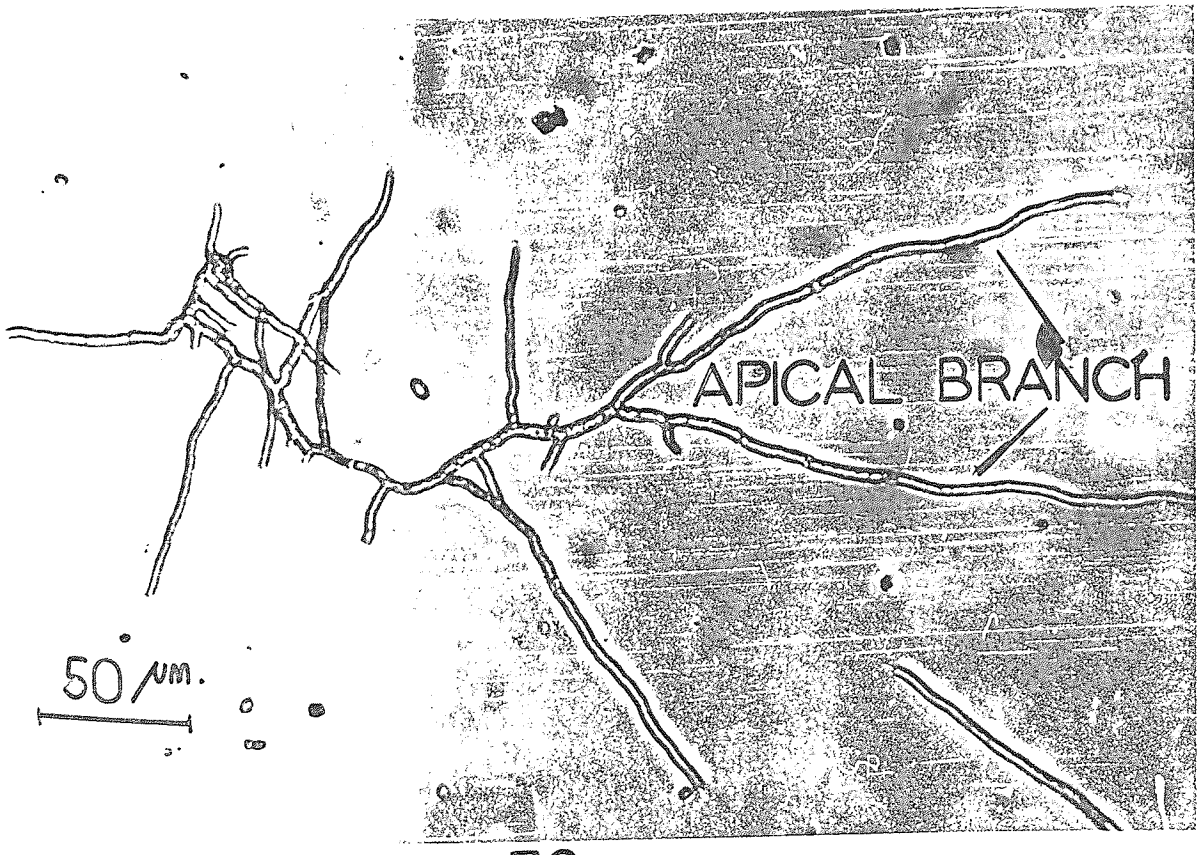


PLATE 6·4 APICAL BRANCHING





Secondary Germ-tube Formation - From the age groupings, a clear pattern concerning the formation of secondary germ-tubes emerged. Sporelings began growth with only one germ-tube (until it was in excess of 25 microns), but by 7 hours after germination all had at least two. The number of second and third germ-tubes observed at each age is shown in Table 6·2 and fig. 6·12. These show that secondary germ-tube formation is directly proportional to the age of the sporeling.

Sporeling age (Hours)	Number in sample	Number with 2 or more Germ-tubes	%	Number with 3 or more Germ-tubes	%
0 - 1	7	1	14	0	0
1 - 2	23	7	30	0	0
2 - 3	26	8	31	1	4
3 - 4	27	17	63	3	11
4 - 5	28	21	75	4	14
5 - 6	26	24	92	4	15
6 - 7	42	42	100	7	17

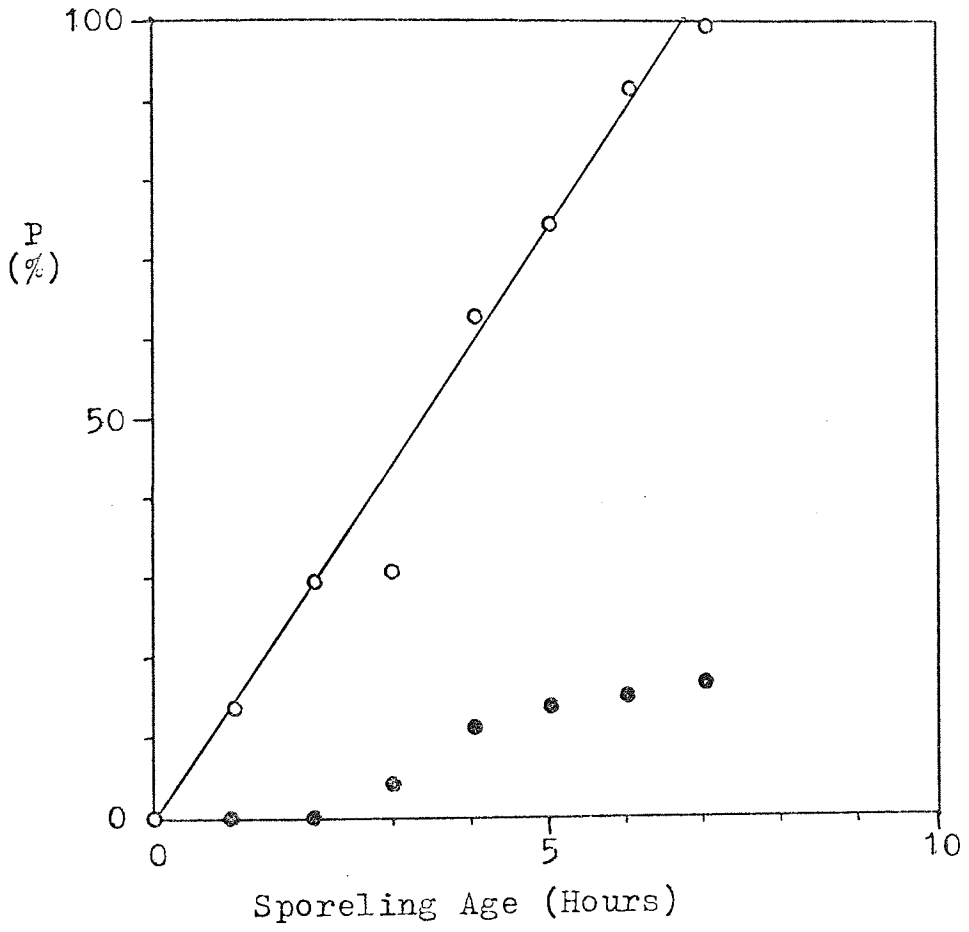
Table 6·2 Incidence of Secondary Germ-tubes in Young Sporelings of A. niger

Fig. 6·12 SECONDARY GERM-TUBE FORMATION - A.niger

Data from experiment BF5

o - 2 or more germ-tubes

● - 3 or more germ-tubes



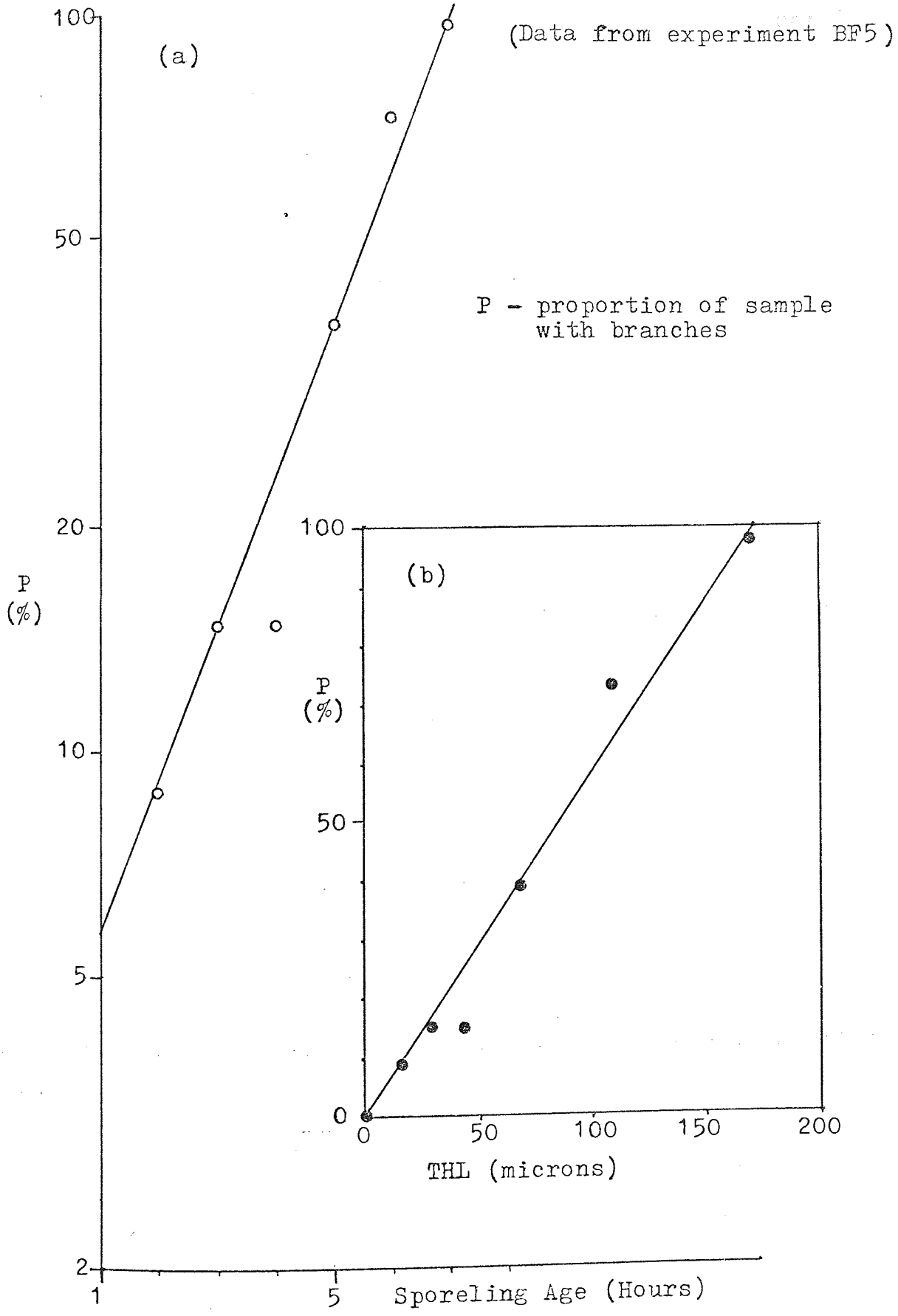
P - proportion of sample with secondary germ-tubes

Incidence of Lateral Branching - Similarly, when the number of sporelings having one or more branches is determined a simple relationship can be discerned (Table 6·3). The formation of branches was found to be an exponential function of time (fig. 6·13a) and approximately proportional to THL (fig. 6·13b).

Sporeling age (Hours)	Number in sample	Number with branches	
			%
0 - 1	7	0	0
1 - 2	23	2	9
2 - 3	26	4	15
3 - 4	27	4	15
4 - 5	28	11	39
5 - 6	26	19	73
6 - 7	42	41	98

Table 6·3 Incidence of Branching in Young Sporelings of A. niger

Fig. 6·13 LATERAL BRANCH FORMATION – A.niger



Lateral Branch Position - Branches are not necessarily formed directly behind the apical tip but (as shown above) can be formed at any point along the parent hypha. To determine the position of a branch along its parent hypha, it was necessary first to determine the time at which the branch formed and then the length of the parent hypha at that time. This involved making an assumption, based on the hypothesis of growth (chapter 3), that branches grow with the same specific growth rate as the total mycelium. A branch will begin growth at a rate based on the contributive length (CL) of parent hypha associated with it. This contributive length is measured along the parent hypha from the newly formed branch, upstream to the next branch or to the spore (see fig. 6·14).

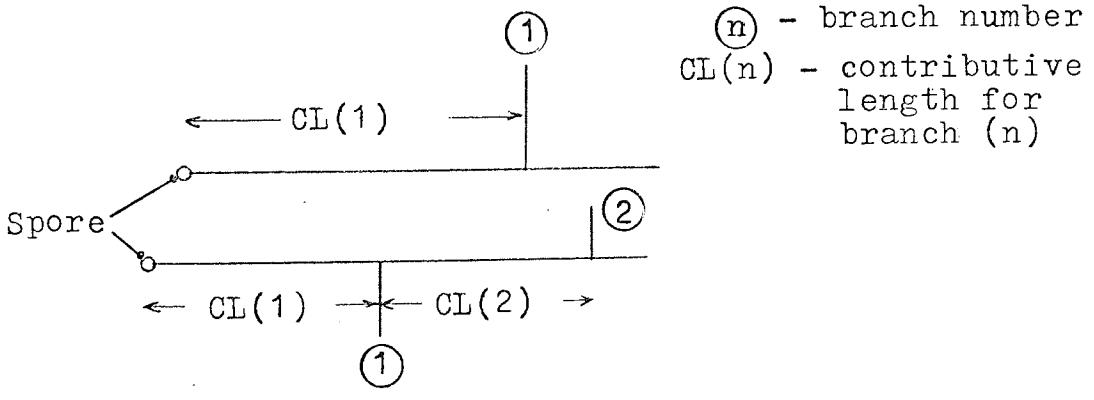
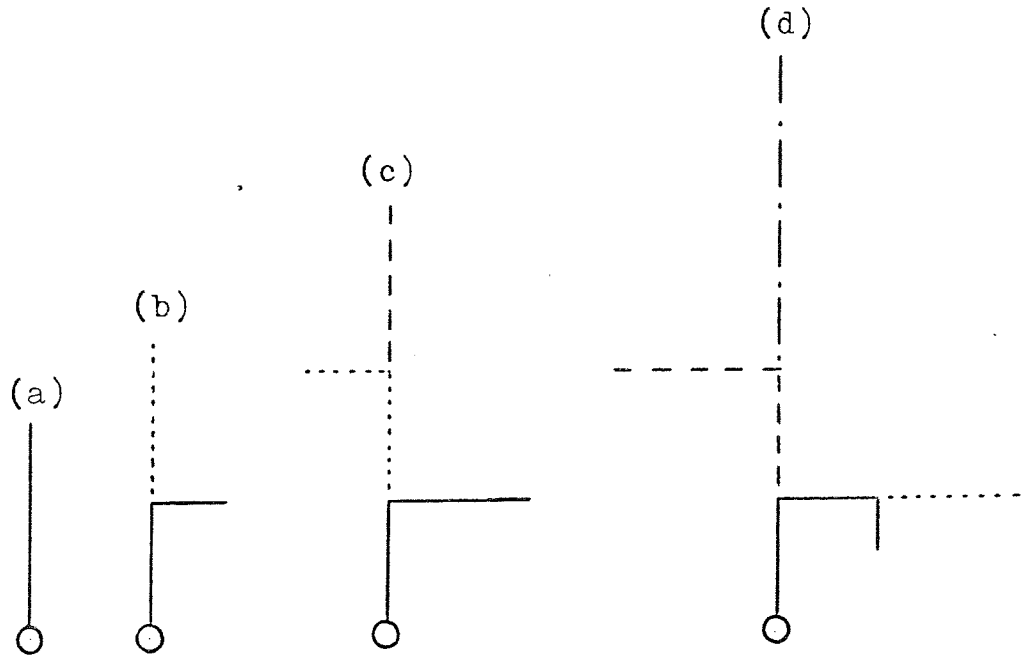


Fig. 6·14 Contributive Length for Lateral Branch Growth

This segmental approach to branching (illustrated by fig. 6·15) conforms to the restrictions imposed by a) septal obstruction of hyphae, and b) growth occuring exclusively at hyphal apices.

FIG. 6.15 THE SEGMENTAL DEVELOPMENT OF A SPORELING STRUCTURE



O - Spore
——— Segment 1
····· Segment 2
- - - - - Segment 3
—·—· Segment 4

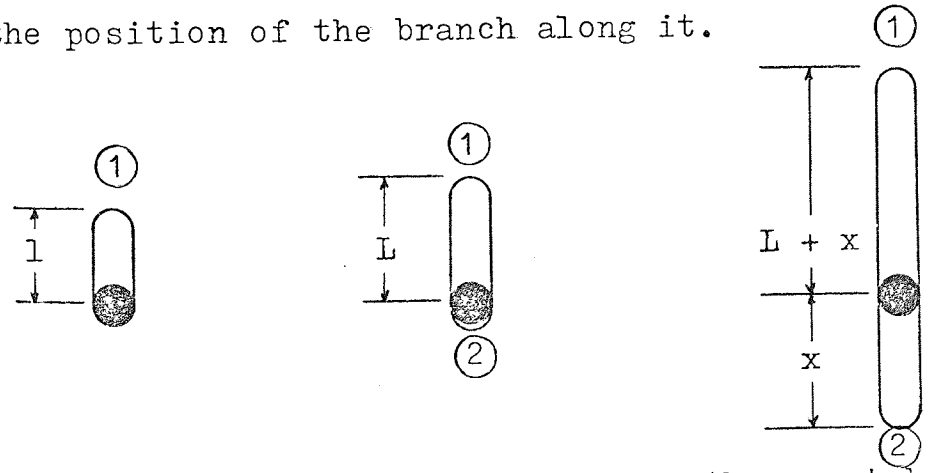
a. Growth confined to one germ-tube

b. First branch formed, about half-way along segment 1. This new branch plus its CL now become segment 1.

c. New branch along segment 2. Segment 1 remains unaltered; the old segment 2 becomes segment 3.

d. New branch along segment 1. This new branch plus its CL now become segment 1 and the remainder of the old segment 1 becomes segment 2; segment 2 becomes segment 3; and so on.

A further assumption concerning secondary germ-tubes had to be made: this was that when a second germ-tube is produced it is supplied with nutrients from the main germ-tube and so behaves in a similar way to an apical branch. Therefore, the difference in length of the two germ-tubes will be constant with time (see fig. 6·16). Third germ-tubes when they are produced, are assumed to be lateral branches formed very close to the spore. On formation of a branch the main and second germ-tubes are considered to become independent. Having made the above assumptions, it was then possible to calculate when a branch was formed and to determine the physical structure of the sporeling at that time. This enabled calculation of the length of the parent segment and the position of the branch along it.



One germ-tube. All growth at apex(1) CL(1) = l

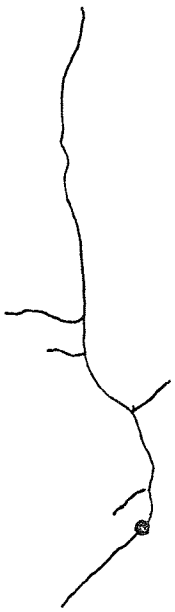
Formation of second germ-tube. Growth now at apex(1) and (2) CL(1) = L/2 CL(2) = L/2

Both germ-tubes have extended by x, the difference in length being still L CL(1) = L/2 + x CL(2) = L/2 + x

Fig. 6·16 Hypothesis for Second Germ-tube Growth

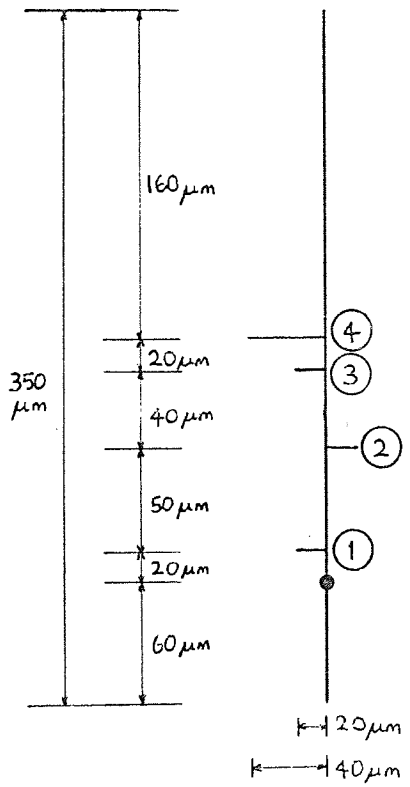
An Example :-

The analysis was, however, often complicated by the uncertainty of the branching order; for purposes of illustration a sample analysis has been included here (see fig. 6·17).



Actual Structure of Sporeling.

Age = 7 Hours
THL = 450 microns
Approx. x250



Graphical Representation.
(n) - Branch Number
Scale = 250 : 1

Fig. 6·17 Sporeling from Sample 7 experiment BF5

Since growth occurs exclusively at hyphal tips then the distance between a branch and the spore will not alter with time. From fig. 6·17 we see that three of the branches are of the same length. Clearly the branch with the smallest contributive length will have been formed first, ie. branch ①. Of the other two branches, branch ③ has the shorter CL and so it will have formed before branch ② : it is also possible to say that branch ③ will have had a CL of 90 microns until branch ② was formed. From the growth equation (6·1) we can determine the time at which branch ② must have formed (based on a CL of 50 microns). For branch ③ we can then determine the length of branch + CL at the time of formation of branch ② , ie. calculate back from 60 (40 + 20) microns. Following this, it is possible to determine at what time branch ③ itself must have been formed (based now on a CL of 90 microns).

Branch ④ will now be considered. It is longer than the rest and has apparently the lowest CL = 20 microns. However if it had been the first branch formed the CL would have been 130 microns and it would have taken little time to grow. Therefore branch ④ was not the first; it was also not the last since to grow from 0 - 40 microns with a CL of 20 microns would take longer than that for the total growth of branch ③.

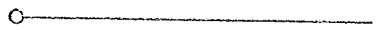
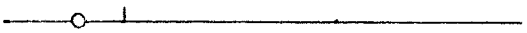
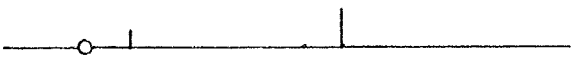
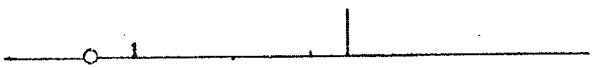
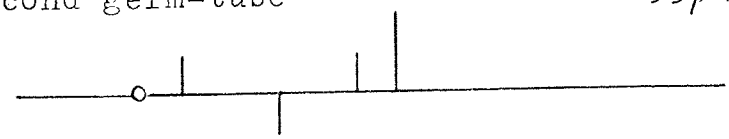
Also, to grow from 0 - 40 microns with a CL of 60 microns would take longer than the growth of branch ② ; so branch ④ must have been the second to form. The order of branching was, therefore, ① , ④ , ③ , ② . Table 6·4 gives details of the times of formation of the branches and the contributive lengths associated with them.

	FT (h)	CL
Time taken for branch ① to grow = 1·55 h	5·45	20
Time taken for branch ② to grow = 0·75 h Length of branch ③ at this time = 3 μm	6·25	50 40
Time for branch ③ to reach 3 μm = 0·1 h Length of branch ④ at this time = 21 μm	6·15	90 20
Time for branch ④ to reach 21 μm = 0·45 h	5·7	110

FT = formation time : CL = contributive length : μm = microns

Table 6·4 Formation Times of Branches

Now, at the time of formation of the first branch the second germ-tube would be of length 30 microns, and the THL would be 240 microns. Therefore, the second germ-tube was formed when the main germ-tube was 180 microns long (ie. 240 - 2 x 30). Table 6·5 gives a summary of the growth of this sample sporeling, and shows the final structure to compare extremely well with that in fig. 6·17. This comparison provides support for the assumptions made in this and other analyses.

Time (h)	Status - Scale = 250 : 1 (μm= microns)
0·0	Start of vegetative growth, no branches Segment 1 (main germ-tube) = 25μm 
4·9	Second germ-tube initiated, no branches Segment 1 = 180μm 
5·45	Branch at 20μm along segment 1 (210μm) Second germ-tube = 30μm 
5·7	Branch at 110μm along segment 2 (210μm) Segment 1 = 22μm Second germ-tube = 33μm 
6·15	Branch at 90μm along segment 2 (133μm) Segment 1 = 26μm Segment 3 = 120μm Second germ-tube = 40μm 
6·25	Branch at 50μm along segment 2 (94μm) Segment 1 = 27μm Segment 3 = 44μm Segment 4 = 125μm Second germ-tube = 41μm 
7·0	Time of sample Segment 1 = 39μm Segment 2 = 70μm Segment 3 = 61μm Segment 4 = 60μm Segment 5 = 170μm Second germ-tube = 59μm 

N.B. Segments are numbered outward from the spore

(···μm) Length of segment prior to branch formation

Table 6·5 Summary of the Growth of an A.niger Sporeling

Using the above method of analysis it was possible to establish parental segment lengths associated with branches and, therefore, the position of branches along their segments. The relationship between branch formation and the size (or age) of the 147 parent segments analysed is shown in fig. 6·18. It is clear that the incidence of branch formation is directly proportional to the length of the parent segment and an exponential function of its age (compare fig. 6·13). Because in practice some branches were formed on parent segments of less than 25 microns length, the theoretical age of the parent segment took a negative value: this is simply due to the form of growth equation used in the calculations (see equation 6·1).

Positions of branches were also calculated as fractions of the parent segment length (measured in the downstream direction). The resultant distribution (see fig. 6·19) is seen to be logarithmic in nature, indicating that the majority of branches are formed along the upper reaches of the segment.

FIG. 6·18 BRANCHING AND PARENT SEGMENT LENGTH (OR AGE)

(Based on data from Experiment BF5)

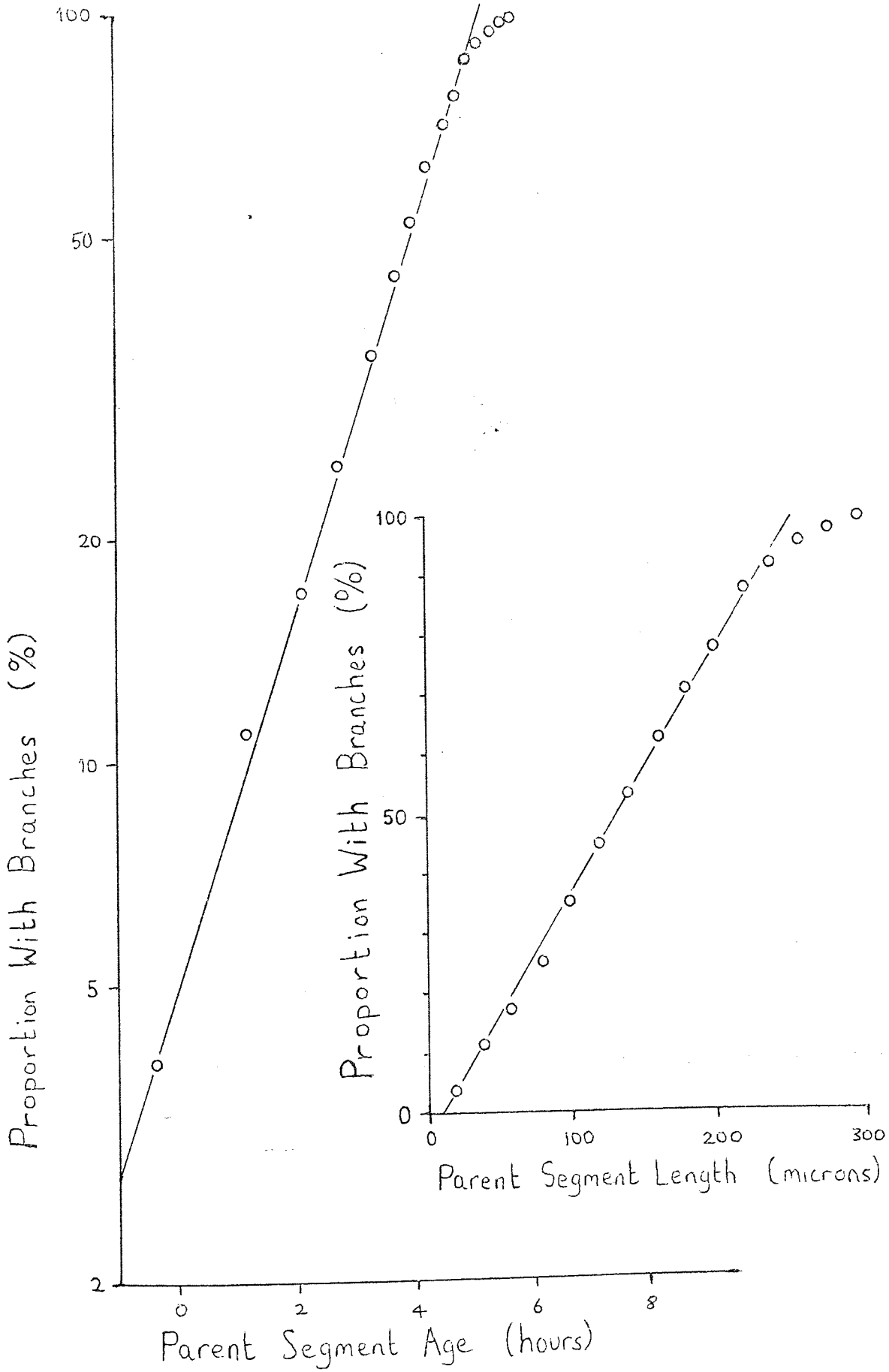
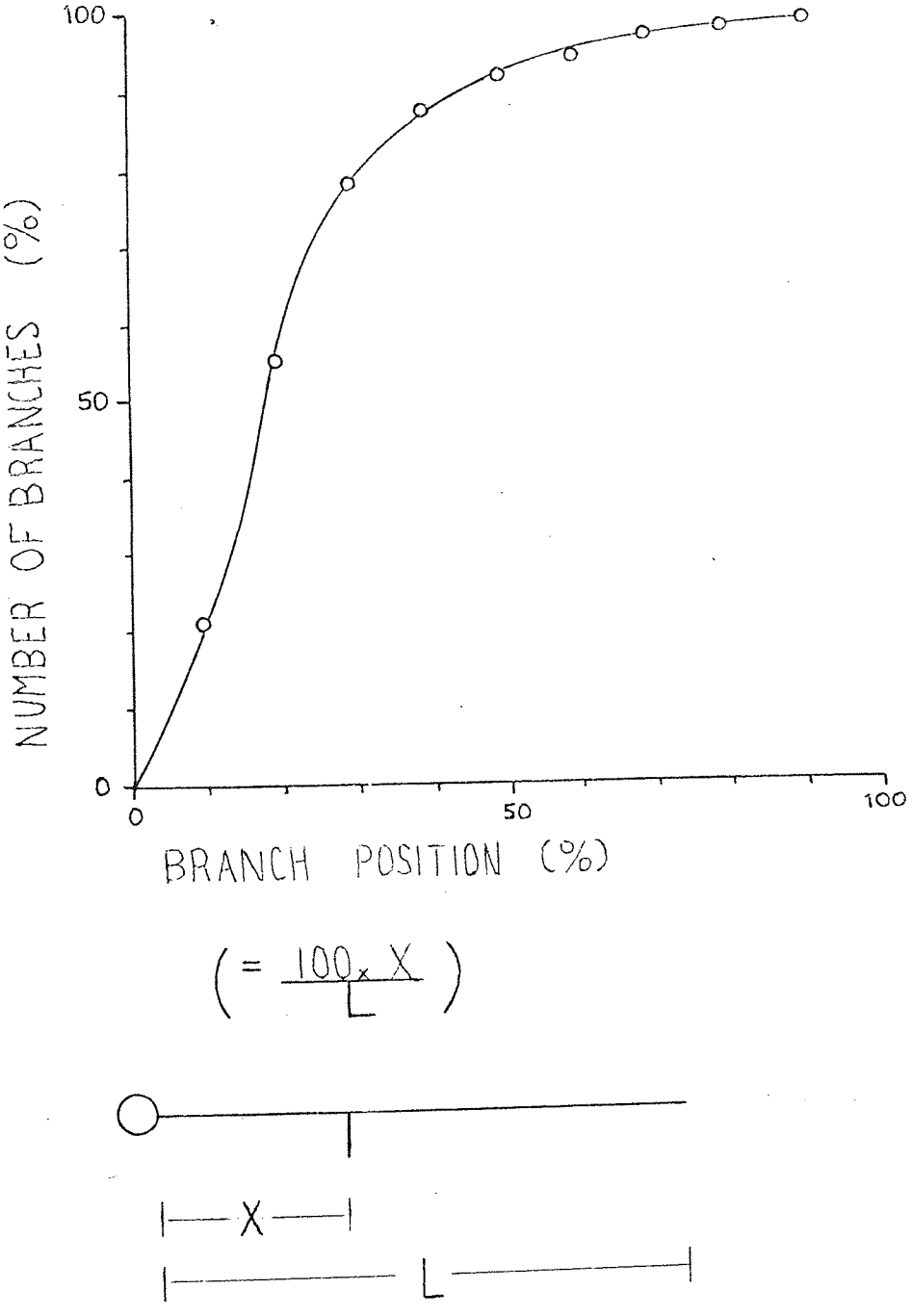


Fig. 6.19 POSITION OF BRANCHES ALONG THEIR PARENT SEGMENTS.

(data from Experiment BF5)



6·3·3 Work with Other Filamentous Fungi

This work was restricted to a study of the early growth of a *Mucor* sp. and a strain of *Penicillium chrysogenum*.

Mucor - The method of analysis described in sections 6·3·1 and 6·3·2 gave meaningful results when applied to data obtained with this fungus, even though the spores are quite different to those of *A.niger*. The spores varied quite widely in size and, unlike those of *A.niger*, appeared to have no tough outer casing. Germination was spread over the larger time range of 5 - 13 h (mean 8·9h), and many sporelings possessed secondary germ-tubes very shortly after germination. Branches were of the lateral type and all sporelings had produced branches by the time a THL of 400 microns had been reached.

Data for the largest THL per sample are represented graphically in fig.6·20. The specific growth rate of 0·46 h^{-1} was similar to those obtained for *A.niger*, though the rapid growth phase preceding exponential growth was much more marked. The line predicted for average growth gave a reasonable fit to the data for the mean THL per sample (see fig. 6·21).

FIG. 6·20 MAXIMUM GROWTH LINE - Mucor

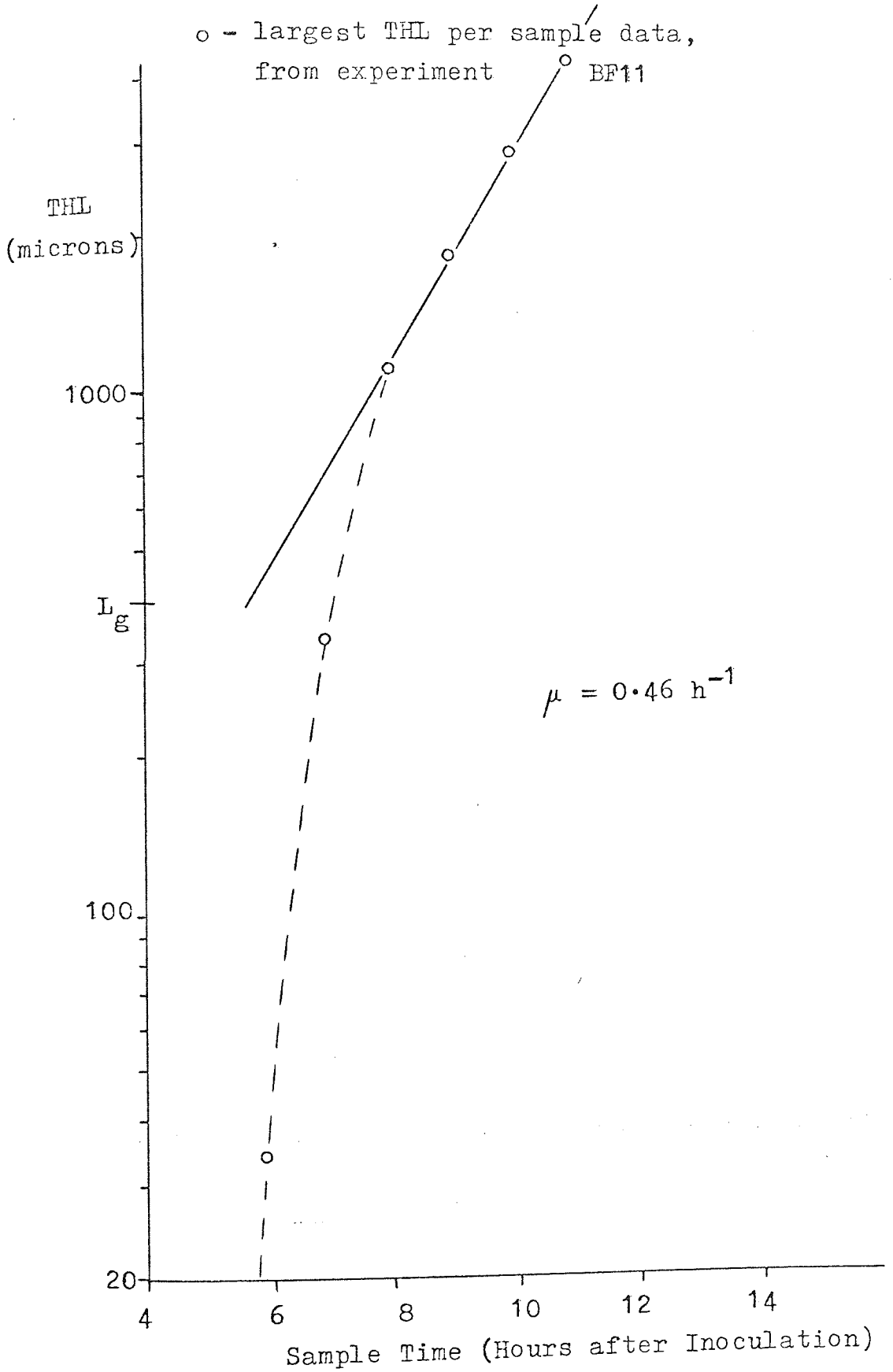
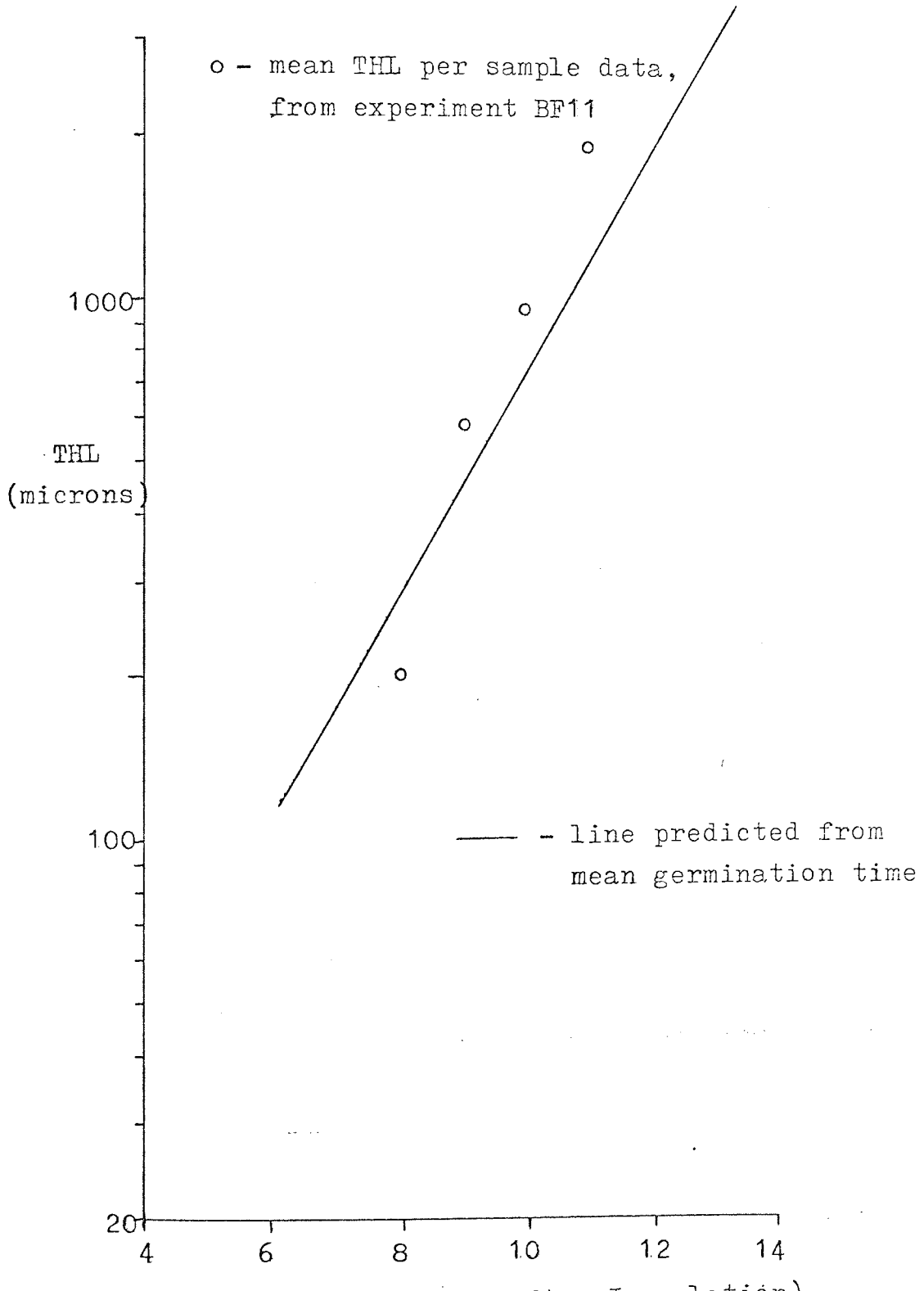
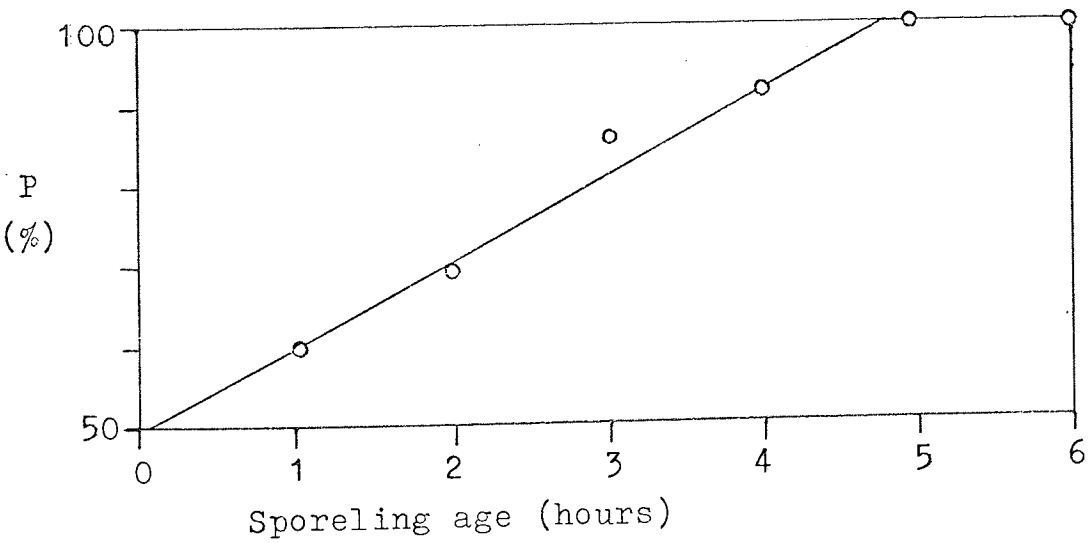


FIG. 6·21 GROWTH LINE BASED ON CALCULATED MEAN GERMINATION TIME - Mucor



The value of L_g = 400 microns (see equation 6·1) for Mucor was much higher than that for A.niger due to the greater initial rapid growth phase.

As with A.niger, secondary germ-tube formation followed a linear relationship with time; note, however, that 50% of the spores produced two germ-tubes actually during germination (see fig. 6·22). Branch formation could not be satisfactorily analysed due to the fact that many were formed during the initial rapid growth phase.



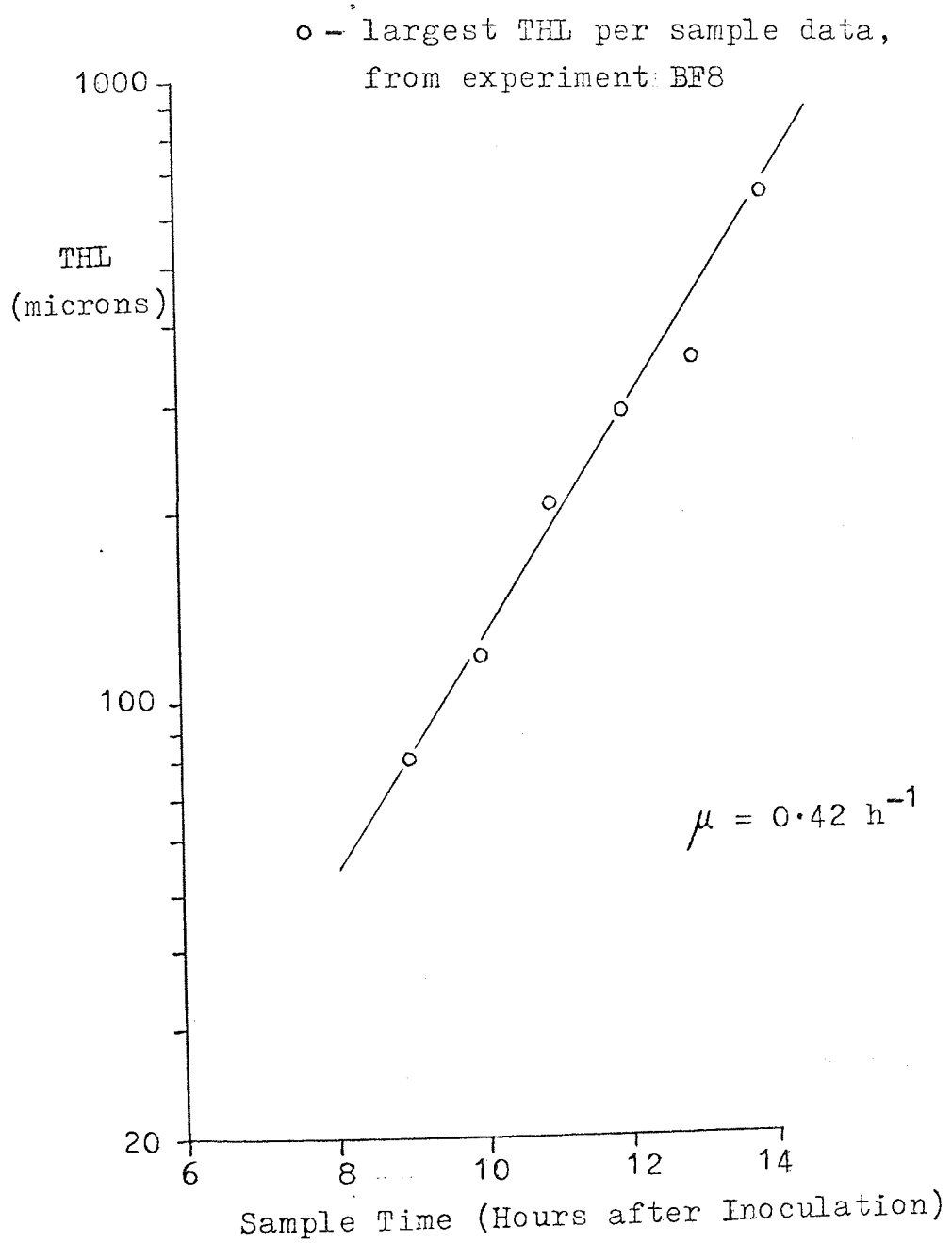
P = Proportion of the sample having at least a second germ-tube.

Fig. 6·22 Secondary germ-tube formation (Mucor)
(Based on data from experiment BF11)

Penicillium chrysogenum - Data for analysis of P.chrysogenum fermentations proved difficult to obtain for two reasons; firstly, aggregation reduced the numbers of single sporelings to such an extent that many microscope slides had to be scanned in order to obtain just a few photo-micrographs; secondly, it was often impossible to detect spores amongst hyphae, as they were of a similar colour and diameter.

However, meaningful data for the largest THL per sample were obtained and are represented graphically in fig. 6·23. The specific growth rate of 0·42 h^{-1} obtained from these data was again similar to those for A.niger and Mucor.

FIG. 6·23 MAXIMUM GROWTH LINE - P. chrysogenum



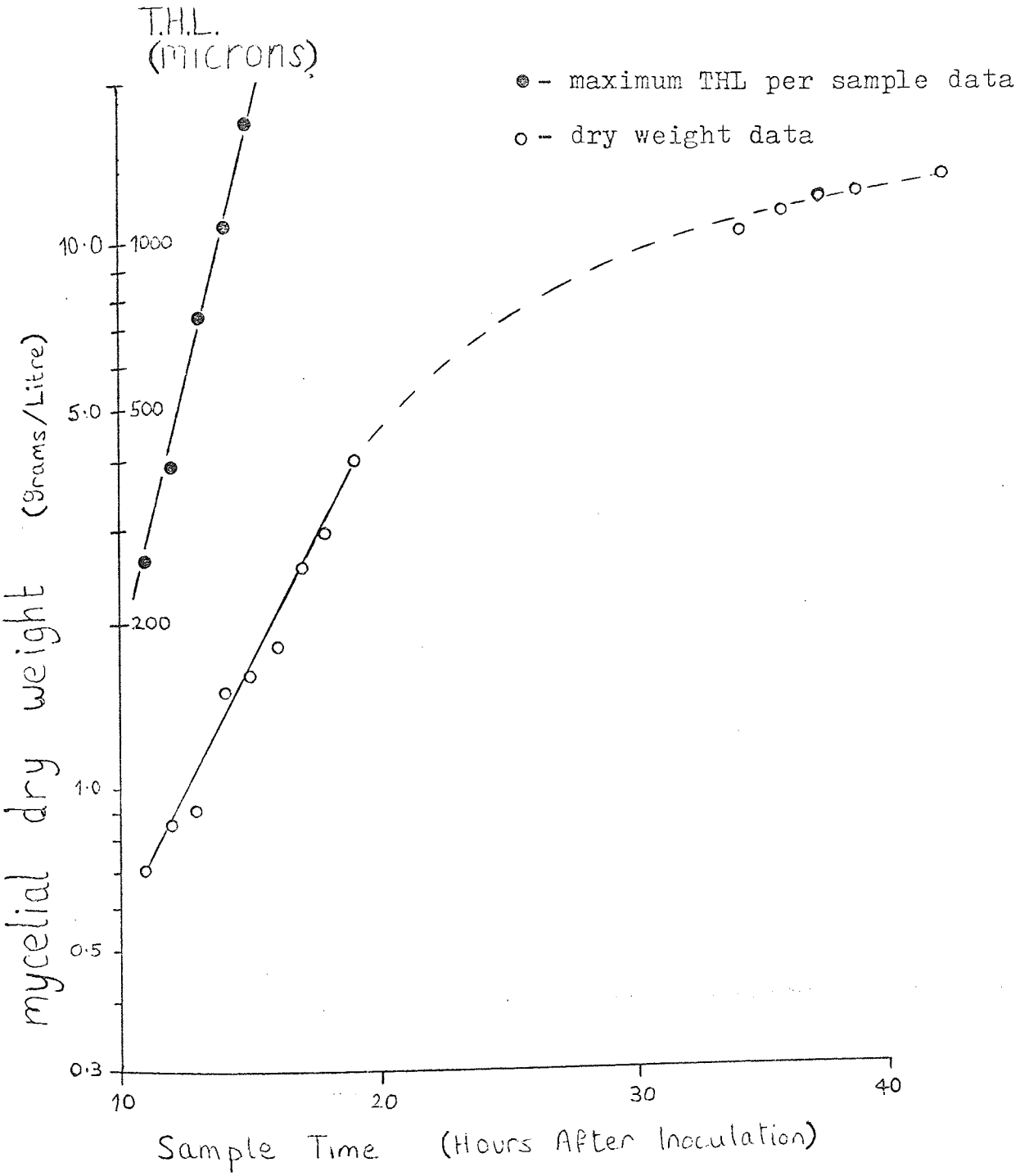
6·3·4 Dry Weight Measurements

Samples taken from the fermenter during the early hours of growth were used to supply both dry weight data and THL data, as shown in fig. 6·24.

It is interesting to note that the two sets of data do not show equivalent relationships with time, even though both represent the same samples. This is in part due to the effects of spore mass on dry weight measurements. Spores were present in high proportion in samples made during the first few hours of growth and must, therefore, be taken into consideration when interpreting the data.

In order to account for spore mass it was first necessary to measure the dry weight of A.niger spores. This was achieved by washing spores from an agar culture with distilled water, accurately pipetting 100 cm^3 of the suspension into a porcelain dish, and then drying to a constant weight. The concentration of spores was determined using a haemocytometer and so the dry weight of a single spore was established. For the $3·775 \times 10^8$ spores which were dried and weighed, an average dry weight of $7·95 \times 10^{-11}$ g was obtained: this compares well with a value of $8·3 \times 10^{-11}$ g based on the mean diameter of an A.niger spore (4·85 microns) and the density of dry mycelial solids (1·39 g/cm^3: from James,

FIG. 6·24 DRY WEIGHT AND THL (data from experiment BF12)



1973). The effect of subtracting the weight of the inoculum spores from the dry weight measurements was to increase the apparent specific growth rate from 0·21 h^{-1} to 0·31 h^{-1}: this latter figure should be compared with 0·47 h^{-1} from THL data.

Another significant feature of the results is that although small amounts of fresh medium were continually added, exponential growth did not continue past about 19 hours into the fermentation. It is suggested that the cause of this is limited oxygen availability throughout the medium. If the rate of oxygen uptake in the organism is limited by the rate at which it is transferred from the gas phase to the liquid phase, then

$$M_O = k_l.a.C_O^* \qquad (6·3)$$

where, M_O = the mass transfer rate of oxygen from air to medium

$k_l.a$ = the mass transfer coefficient from the gas/liquid interface into the bulk liquid times the interfacial area over which the transfer occurs: $k_l.a$ was found to be approximately 0·02 s^{-1} (Dowen, 1979)

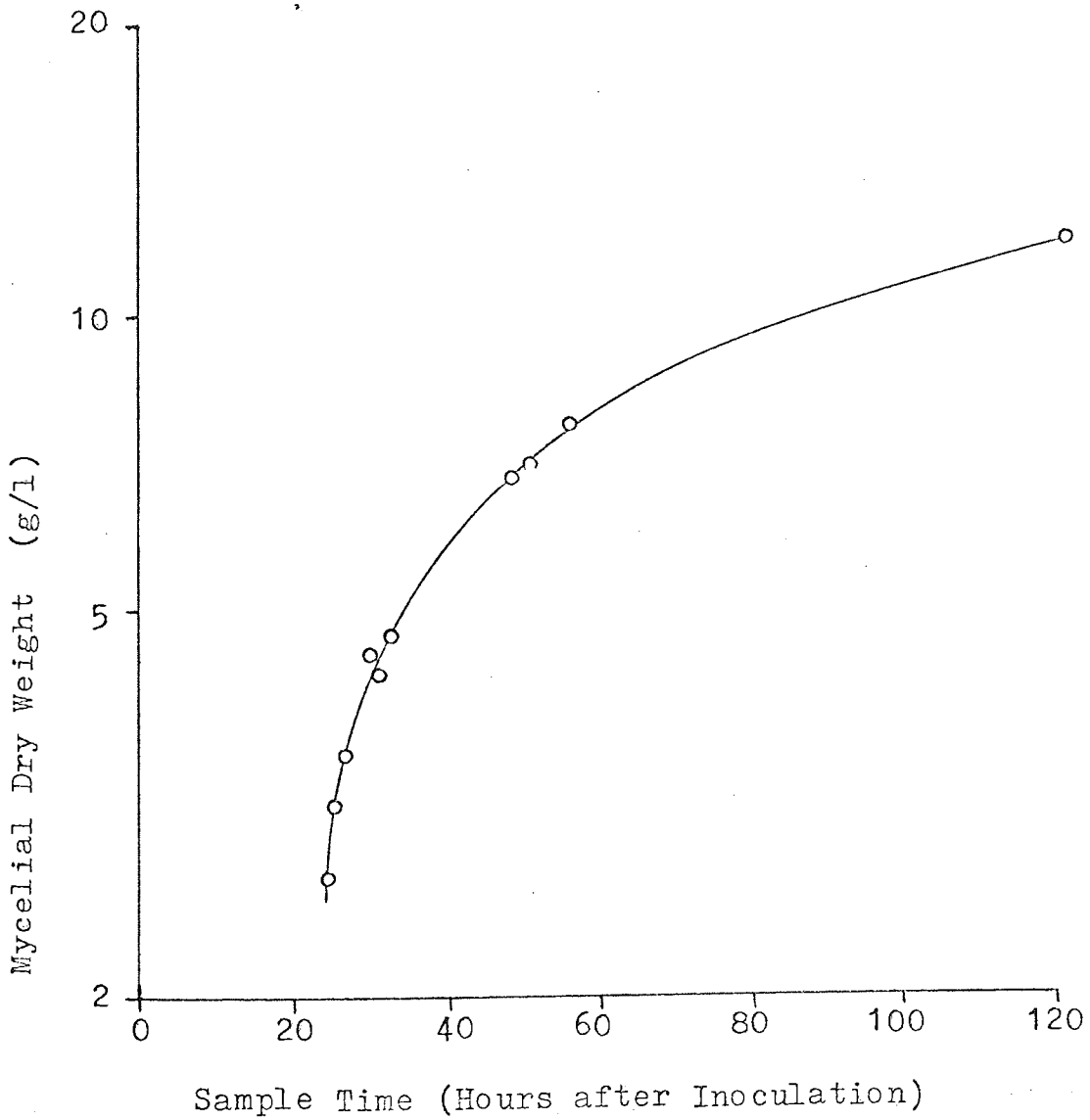
C_O^* = the concentration of a saturated solution of oxygen in the medium and represents the maximum driving force for mass transfer, when all oxygen is being utilised from solution ($\approx$ 7 mg/l).

Hence, the amount of oxygen supplied to the growing fungus is 0·504 g/l.h. which will support the production of only 0·504 g of biomass on a dry weight basis /l.h.

The effects of nutrient limitation can be seen more clearly in fig. 6·25, which presents measurements made over a longer time period. The specific growth rate was approximately 0·17 h^{-1} at 24 hours but had been reduced to 0·006 h^{-1} by 100 hours. Growth was probably restricted by carbohydrate limitation as well as oxygen limitation by this time, since the medium could not support production of more than about 13 g of biomass on a dry weight basis/l.

FIG. 6·25 GROWTH IN TERMS OF DRY WEIGHT DATA

(data from experiment DW2)



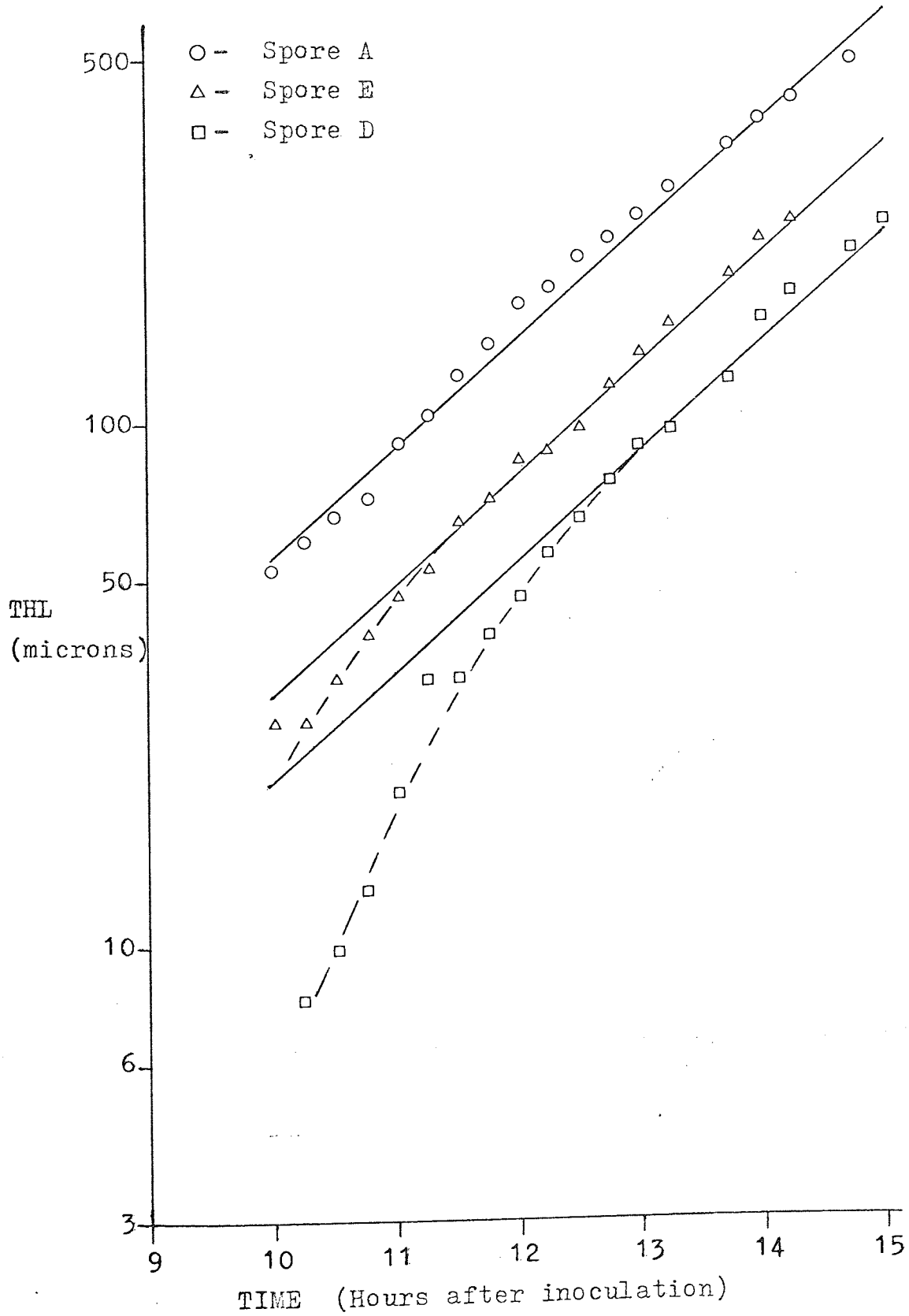
6·3·5 Surface Culture Measurements

When placed on solidified medium, spores of A.niger germinated from 7 to 10 hours after inoculation but proceeded to grow at the same specific growth rate of approximately 0·5 h^{-1}. An initial rapid growth phase was observed but exponential growth of germ-tubes was normal after about 50 microns of growth had occured. Unlike the early growth in submerged culture, no secondary germ-tubes were produced and no branching was observed during at least the first 500 microns of growth.

Fig. 6·26 shows three typical sets of data illustrating the range of germination times for spores of A.niger on nutrient agar. The value for specific growth rate obtained from these data was extremely high when compared with that of 0·27 h^{-1} from the measurements of Trinci (1971b). This may be because spores were kept wet, as described in section 6·2; this technique may have aided diffusion of nutrients from the agar, so simulating to some extent the conditions in submerged culture. However, it must be noted that as far as branching and secondary germ-tube formation were concerned, the sporelings behaved quite differently to those in submerged culture.

Fig. 6·26 SURFACE CULTURE GROWTH (A.niger)

Data from experiment SC1



6·4 COMPARISONS AND CONCLUSIONS

It is now possible to assess the usefulness of the author's method for studying the submerged culture of filamentous fungi and compare it with the more normal methods based on dry weight and surface culture measurements.

A direct comparison between the microscopic study of early growth and dry weight measurements has been presented already (section 6·3·4): this showed that dry weight measurements did not fully reflect the exponential increase in THL and also highlighted the problems of oxygen limitation in aerated fermenters. It is worth noting here that in a study of the growth of Schizosaccharomyces pombe, a fission yeast which grows by elongation rather than by swelling, Mitchison (1963) observed an exponential increase in length but a linear increase in dry weight with time.

With surface culture work. it is the mode of growth and not the method of analysis which causes results to differ from those obtained by the author's method. Surface culture sporelings produced a quite different morphology (much less branching and secondary germ-tube formation), and germinated later than those in submerged

culture. Also, the initial rapid growth phase (germ-tube growth) was much shorter, with the result that growth appeared to be much slower despite the higher specific growth rate. Nevertheless, the hypothesis of equal specific growth rate for sporelings germinating over a range of time (see chapter 3) is clearly supported by the results of the surface culture study, in which the growth of an individual could be monitored continuously.

Although not investigated in depth, successful application of the author's technique to filamentous fungi other than A.niger was achieved. The method's general applicability may, however, be limited to those fungi whose germinated spores can be readily distinguished among a network of hyphae.

Specific growth rates for all the fungi studied were surprisingly similar, ranging only from 0·42 h^{-1} (P.chrysogenum) to 0·49 h^{-1} (A.niger). Consequently, differences in total hyphal length at any time for the different species were due largely to the differing amounts of germ-tube growth and the range of germination times. Mucor spores germinated at much the same time as those of A.niger, but growth at a given time was much more advanced because of the shorter, steeper germ-tube growth phase (compare figs. 6·4 and 6·20). The germ-tube growth

phase is clearly an important stage in overall growth - much more so than anticipated.

In conclusion it can be said that the technique proposed at the beginning of this chapter for studying the early growth of filamentous fungi in submerged culture has proved to be a useful one. The method enabled the collection of quantitative information concerning growth and morphological development. Results obtained were reproducible, and analysis was not restricted to a particular fungus. The specific growth rates obtained in this way are likely to represent the maximum for the organism; such information cannot be determined from dry weight measurements, although surface culture measurements might provide a reasonable estimate of the maximum rate. The author's method is the only one that can be used to obtain detailed information about morphological development (although admittedly the labour involved is considerable).

C H A P T E R 7

INOCULATION AND AGGREGATION

7·1 INTRODUCTION

Inoculation is a most significant stage of a fermentation: it has a bearing on the morphology of the fungus and can greatly influence the quality and productivity of the fermentation (Calam, 1976). Industrial preparation of an inoculum can involve several steps, as illustrated by Blakebrough (1967), and may involve the use of either asexual spores (conidia) or vegetative mycelium. The former is used wherever possible for ease of standardisation. Meyrath and Suchanek (1972) have reviewed inoculation techniques and apparatus, and they describe the effects of inoculum size and type on subsequent growth.

When starting a fermentation from a suspended spore inoculum the major cause of colony formation is aggregation of the spores. Clearly this had to be studied in order to understand and describe colony formation.

A detailed quantitative study of the kinetics of colony growth has not been attempted by the author because of the wide variations in morphology . Rather, the view has been taken that colony kinetics can be predicted by modelling the early growth and formation of colonies.

The objective then, of this part of the research, was to gather information which could later be used to model the formation of filamentous fungal colonies in tower fermenter systems.

Though much of the data presented in this chapter are the result of specifically designed experiments, they have been augmented by data from other experiments within the overall programme.

7·2 EXPERIMENTAL PROGRAMME AND PROCEDURE

To achieve the objective set out above, studies had to be made of :
the aggregation of spores prior to growth,
the aggregation of non-viable spores, and
aggregate size distribution.
The equipment and procedure where different from those specified in chapter 4 are described below.

The Aggregation of Spores Prior to Growth - The fermenter was inoculated with 100 ml of a spore suspension, the concentration of which was determined by haemocytometer. Medium and conditions were as previously described (see page 40).

Small samples of approximately 2 cm^3 were taken from the fermenter at 30-minute intervals and analysed immediately by counting (under the microscope) at least 100 unaggregated spores in a measured volume. Groups of 3 or less spores were also included in the count; groups larger than 3 in number were considered to be aggregates. Because of the drop in spore concentration during experiments due to aggregation, the haemocytometer could not be used in the normal way. Instead, the microscope was tracked across the whole width of the haemocytometer as many times as was necessary to count about 100 spores (see fig. 7·1)

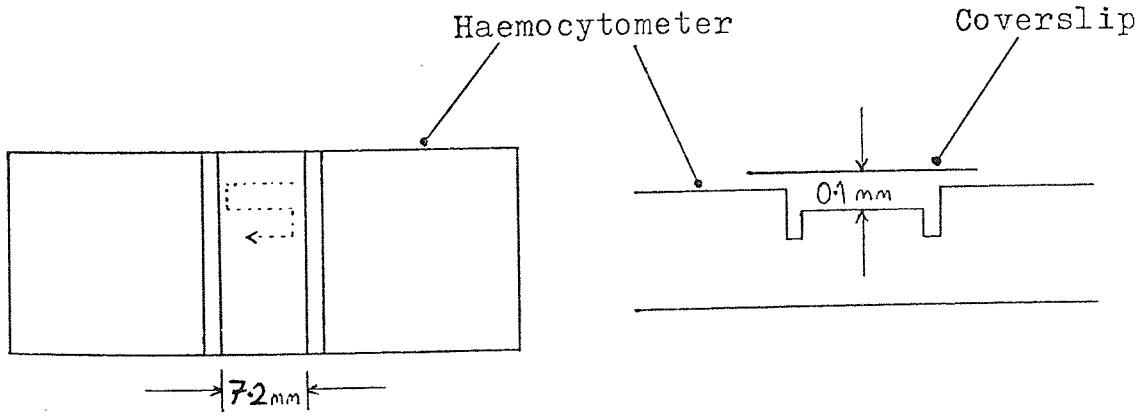


Fig. 7·1 Tracking Method for use with Haemocytometer

The number concentration of spores was then calculated as follows:

$$n_s = \frac{N_s}{V_H \cdot N_w} \qquad (7.1)$$

where, N_s = the total number of unaggregated spores

N_w = the number of widths tracked

V_H = the volume covered by tracking one width.

= 7·2mm x 0·1mm x 0·365mm (microscope field wi

= $2 \cdot 63 \times 10^{-4} cm^3$.

During the course of experiments any spores washed out of the fermenter by the action of air or foam were collected and counted.

A word is necessry about the accuracy of the haemocytometer measurements. Because time did not permit more than one count per sample, the haemocytometer method could only be used to give a guide to spore concentrations in the aggregation experiments. The sample volume used in most cases was only of the order of $10^{-5} cm^3$ and the number of spores counted about 100; consequently, an error of $\pm$ 1 spore in the count affected the concentration figure by as much as $\pm 10^5$ spores/cm^3. In an experiment to check the consistency of measurements from the haemocytometer, ten readings using the same sample varied by as much as 30%.

The results did, however, follow a normal distribution (number of spores: 96 103 108 113 115 116 120 123 129 134 mean: 115·7 standard deviation: 11·5) with a standard deviation which was 10% of the mean.

The level of accuracy achieved when using the haemocytometer must therefore be borne in mind when interpreting the experimental data.

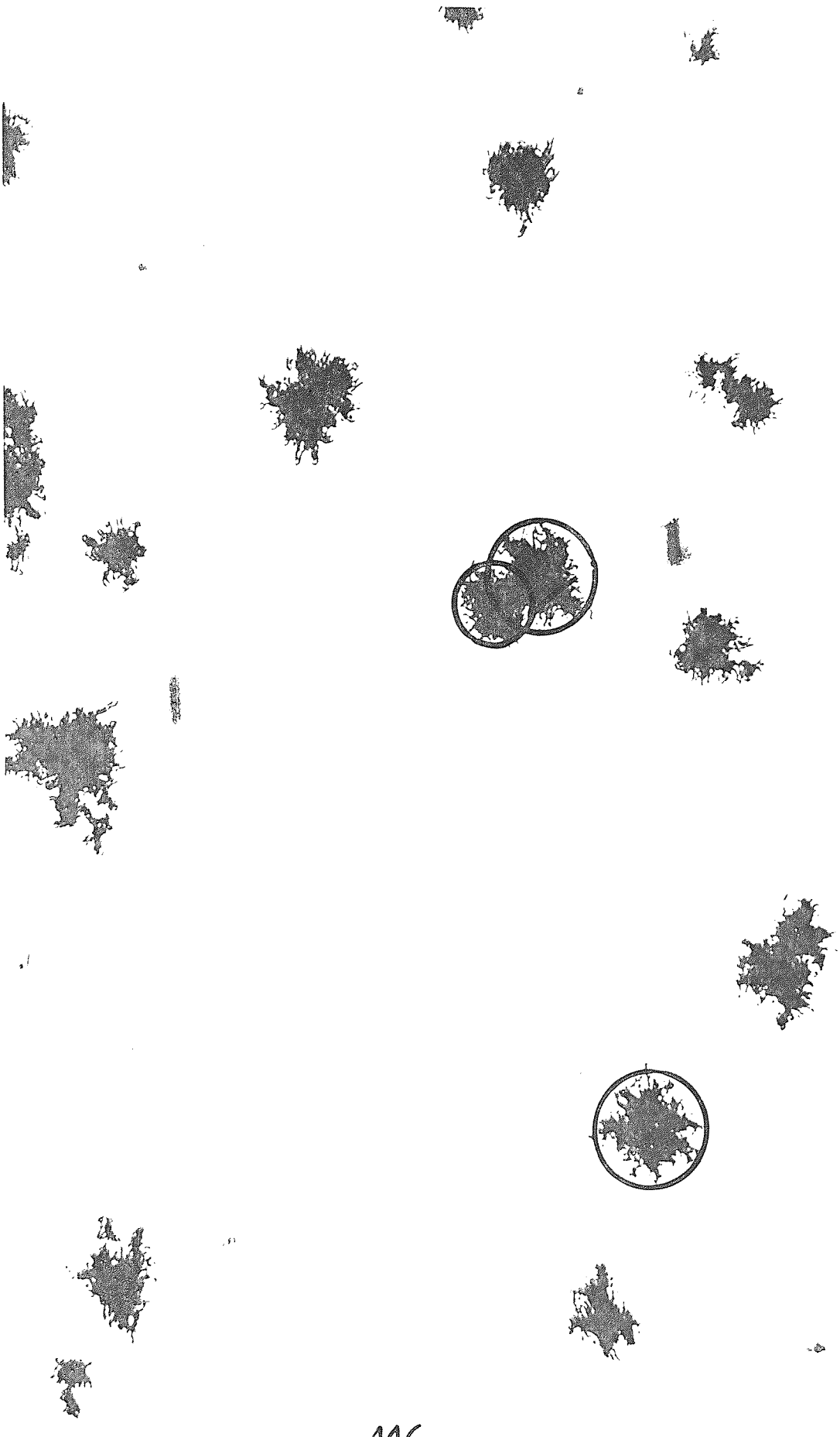
Study of the Aggregation of Non-Viable Spores - The experiments were identical to those described above except that prior to inoculation the spore suspension was either fixed with a 40% w/v formaldehyde solution or sterilized by autoclaving at 120°C for 15 minutes.

Study of Aggregate Size Distribution - Data were collected by taking a large sample from the fermenter during germination and another after 24 hours.

Aggregate concentration in numbers/unit volume were measured by pipetting a drop of the sample onto a weighed cavity slide, reweighing to determine the volume, and then counting the total number of aggregates by microscopic observation. Size distributions were determined by taking thirty photomicrographs of aggregate suspensions and analysing on a Particle Size Analyser (Carl Zeiss 3400, Germany). Calibration was effected by using a photomicrograph of a slide micrometer (Graticules Ltd.).

The Zeiss Particle Size Analyser is operated by placing a photomicrograph over a circle of light, the diameter of which is adjustable. The size of circle is then matched to the particle image size and a trigger records the diameter in an array of size ranges. Because the spore aggregates being analysed in this case were not truly circular (see Plate 7·1) some arbitrary rules had to be used for the sake of consistency. In all analyses, the circle of light was set so as to enclose the whole image regardless of shape, except where the particles clearly comprised two or more individual aggregates in close contact: very irregular shaped aggregates were excluded from the analyses.

PLATE 7·1 AGGREGATE SIZE ANALYSIS



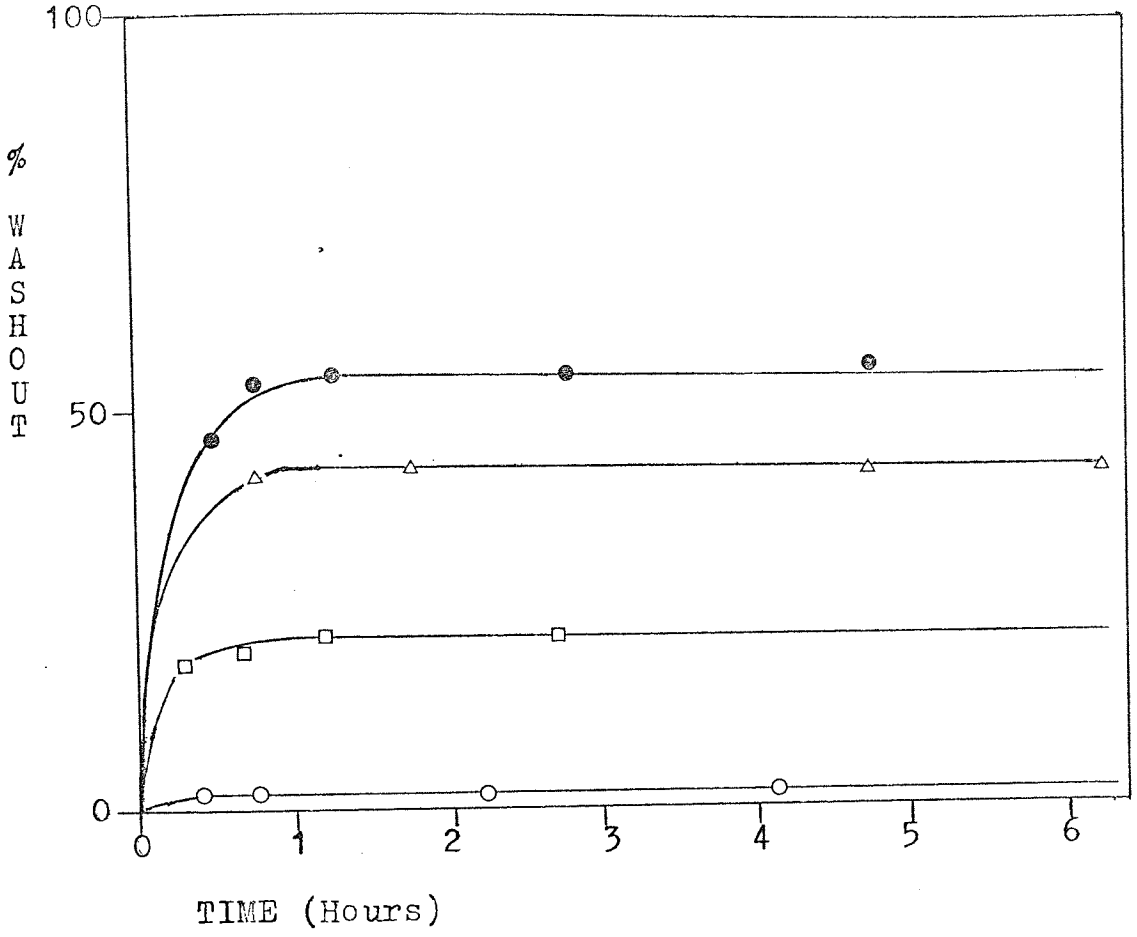
7·3 RESULTS, OBSERVATIONS AND DISCUSSION

7·3·1 Washout of Spores from the Fermenter

One of the first observations to be made during the aggregation experiments was that many of the inoculum spores were washed out of the fermenter by the action of foaming at the top of the tower. Measurements of the quantity of spores washed out of the fermenter from various experiments are represented graphically in fig. 7·2 as percentages of the original inoculum. These results were calculated as follows. The concentration of each sample was determined by counting at least 100 spores on the haemocytometer. This figure multiplied by the total volume collected gave the total number of spores washed out of the fermenter during the time interval between samples. A running total of spores could then be kept and recorded as a percentage of the original inoculum. For detailed results see Appendix 7·1.

Several interesting points of discussion arise from these results.

1. Washout can result in the loss of more than 50% of the inoculum spores under certain conditions.



Data :-

△ - Experiment I6

□ - Experiment I7

● - Experiment I8

○ - Experiment I9

Fig. 7·2 WASHOUT OF SPORES FROM THE TOWER FERMENTER

Although the volumes collected are very small, about 1 or 2% of the fermenter contents, the concentration is almost as high as that of the original inoculum suspension. For example, in the first half hour of experiment I8 the washout volume was 40 cm^3 with a concentration of 91 x 10^6 spores/cm^3; at the same time the concentration in the fermenter was only 2 x 10^6 spores/cm^3. Clearly, the spores concentrate at the top of the fermenter, and are carried over by the foam formed at the air/liquid interface. It is felt that this phenomenon is a result of the non-wetability of the spores. Spores used for inocula cannot be easily dispersed in water without the addition of a surfactant, such as Tween or Triton. This was readily demonstrated by carrying out an experiment in which one spore culture was washed with a 0·01% v/v Triton X-100 solution and another with distilled water. Almost immediately the flask containing Triton X-100 became black with A.niger spores, whereas the one containing the distilled water failed to wet the spores at all. After 1 hour of continuous shaking the concentration of spores in the distilled water had only reached 5 x 10^5 spores/cm^3, while the concentration of spores in the Triton X-100 solution reached 10^7 spores/cm^3 in only a few minutes. In addition the spores in the distilled water did not appear to be homogeneously dispersed, many being

concentrated at the air/liquid interface in the flask.

2. Although over half the inoculated spores may be washed out of the fermenter the bulk of the washout occurs during the first hour. This is thought to be connected with the foam-promoting action of the surfactant in the inoculum. A dense foam is formed shortly after inoculation, but this decreases in volume with time so that no more washout occurs after an hour or so. Thus the agent responsible for ensuring highly concentrated inocula is also in part the cause of losses as a result of washout. However, using the surfactant still provides at least ten times more spores in the fermenter than would be the case if distilled water were used.

3. The medium too may affect the degree of foaming and hence have a marked effect on the washout of spores. This is shown by the results of experiment I7 where, instead of the normal autoclaved yeast extract, a powdered yeast extract was used in making up the medium. Autoclaved yeast extract has a 'soapy' texture and causes a considerable amount of foaming prior to inoculation; powdered yeast extract causes very little with the result that, instead of a loss of 50% of inoculum spores, less than 25% are lost.

4. The lowest washout figure of all in fig. 7·2 is that for experiment I9. Exactly the same conditions were used for this experiment as for the others, autoclaved yeast extract forming part of the medium. The only difference between experiment I9 and the others was that the spores were killed by autoclaving at 120°C for 15 minutes. Shortly after inoculation foaming was as intense as usual and a dense black washout was collected. However, microscopic observation revealed that the very black sample contained comparitively few spores. Therefore, the blackness of the sample must have been due to pigment from the spores (see Yanagita, 1957), leached out during the sterilisation process. It would appear that it is this pigment that provides the hydrophobic coating on viable spores, since samples of washout from experiment I9 were a great deal darker than the fermenter contents. Without the coating of pigment spores wet and remain within the fermenter, while the pigment itself is removed.

The results of experiment I9 are not due simply to the fact that spores have been killed because spores killed with formalin (experiment I6) behaved just as viable spores do.

7·3·2 Spore Aggregation

Figs. 7·3 and 7·4 are sample results from the studies of aggregation before and during germination. They have been chosen to show a) the general trend of aggregation (fig. 7·3 is a typical example) and b) that this trend is not always so obvious (fig.7·4). The remainder of the results are presented graphically in Appendix 7·2.

During the experiments the following observations were made. A proportion of the spores in the inoculum were present as strings of four or more, but by 2·5 hours into the fermentation the population comprised mostly single or pairs of spores. By 4·5 hours the appearance of the fermenter contents changed from an 'inky' blackness to a suspension of discrete particles. At around 7 hours clumps of spores (about 100 microns in diameter) could be seen, and deposits of spores were observed around the top of the fermenter and in sample ports. The same observations were made during all experiments and are summarised in Table 7·1.

The cause of aggregation is unknown but it seems to be connected with the growth process, the stages of which are also summarised in Table 7·1.

FIG. 7·3 SPORE AGGREGATION - A.niger

(data from Experiment I7)

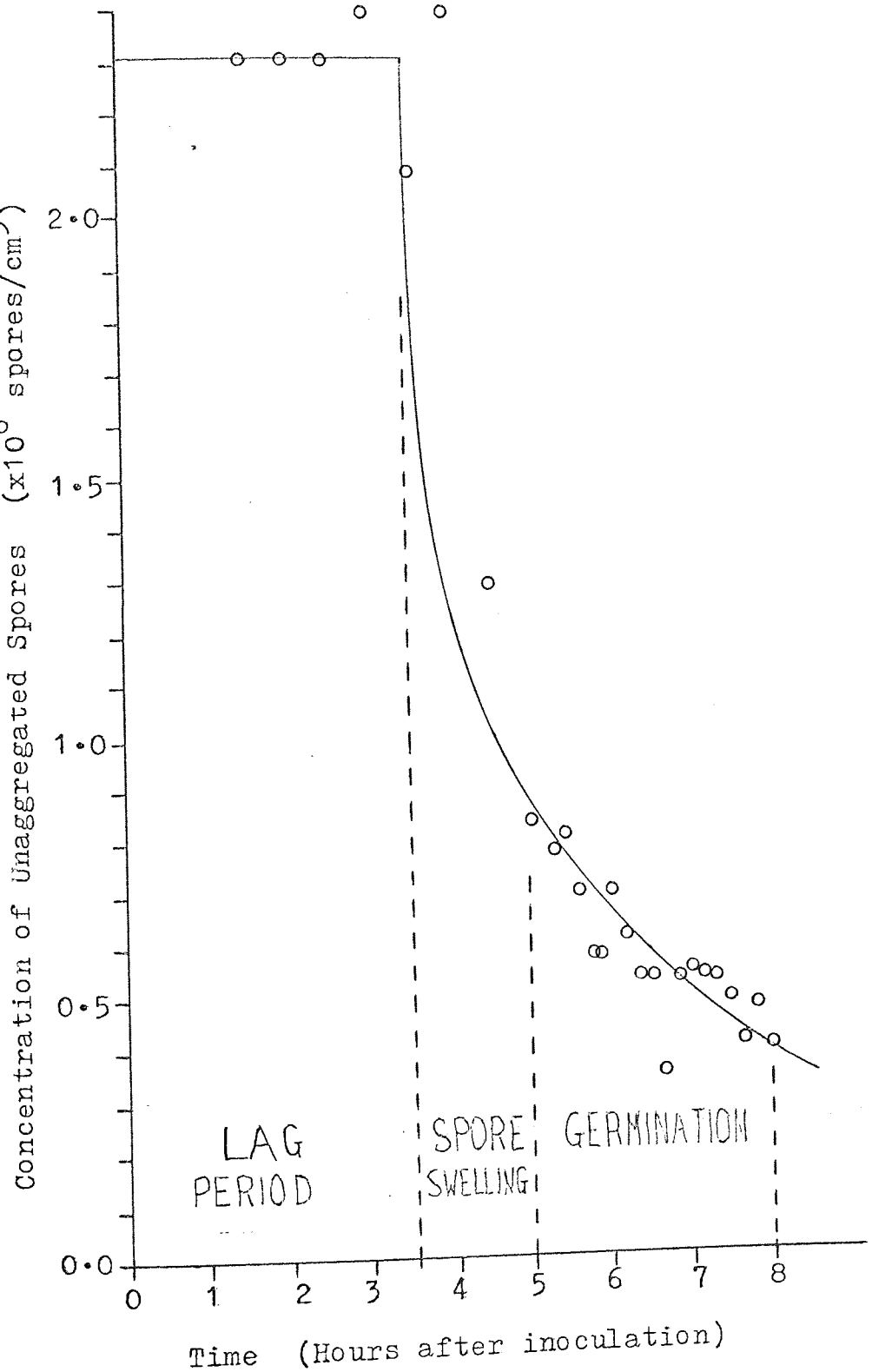
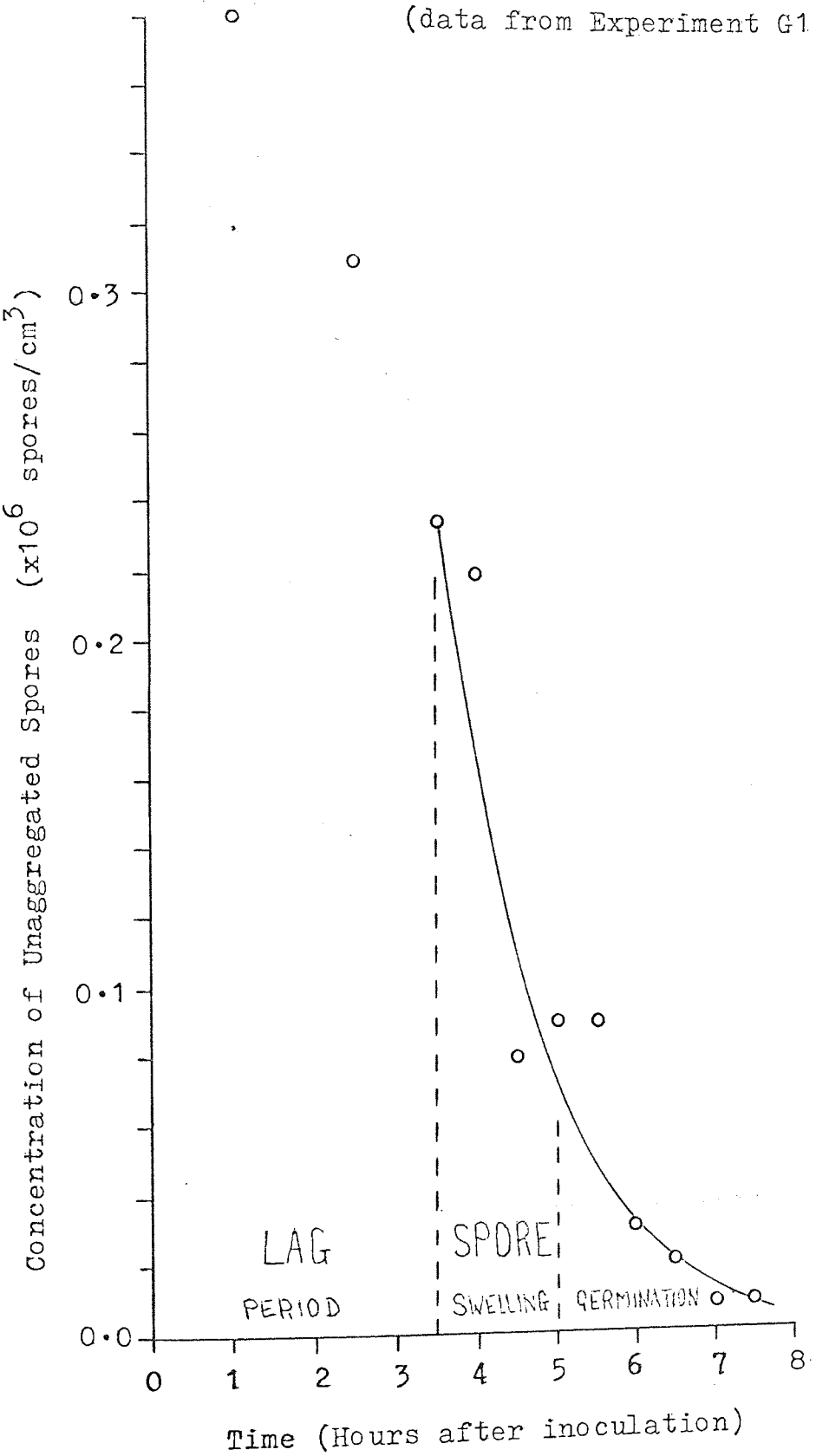


FIG. 7·4 SPORE AGGREGATION A.niger



AGGREGATION	TIME (HOURS)	GROWTH
	0	
little or no aggregation	1 2 3	little or no activity
intense aggregation	4 5	spore swelling
less aggregation	6 7	germination
	8	

Table 7·1 Stages of Aggregation and Growth

The surface properties of spores must change during the swelling phase in order to account for the increased aggregation. It is possible that this change is brought about by the splitting away of the outer skin of the spore (Hawker, 1966), or by the exudation of a sticky substance from the skin of the spore, or by both (see Galbraith and Smith, 1969). It is interesting to note here that in experiments with other organisms, the spores of Penicillium chrysogenum (which are similar to A.niger) were seen to aggregate, whereas those of Mucor sp. (where no hard outer skin is present) were not.

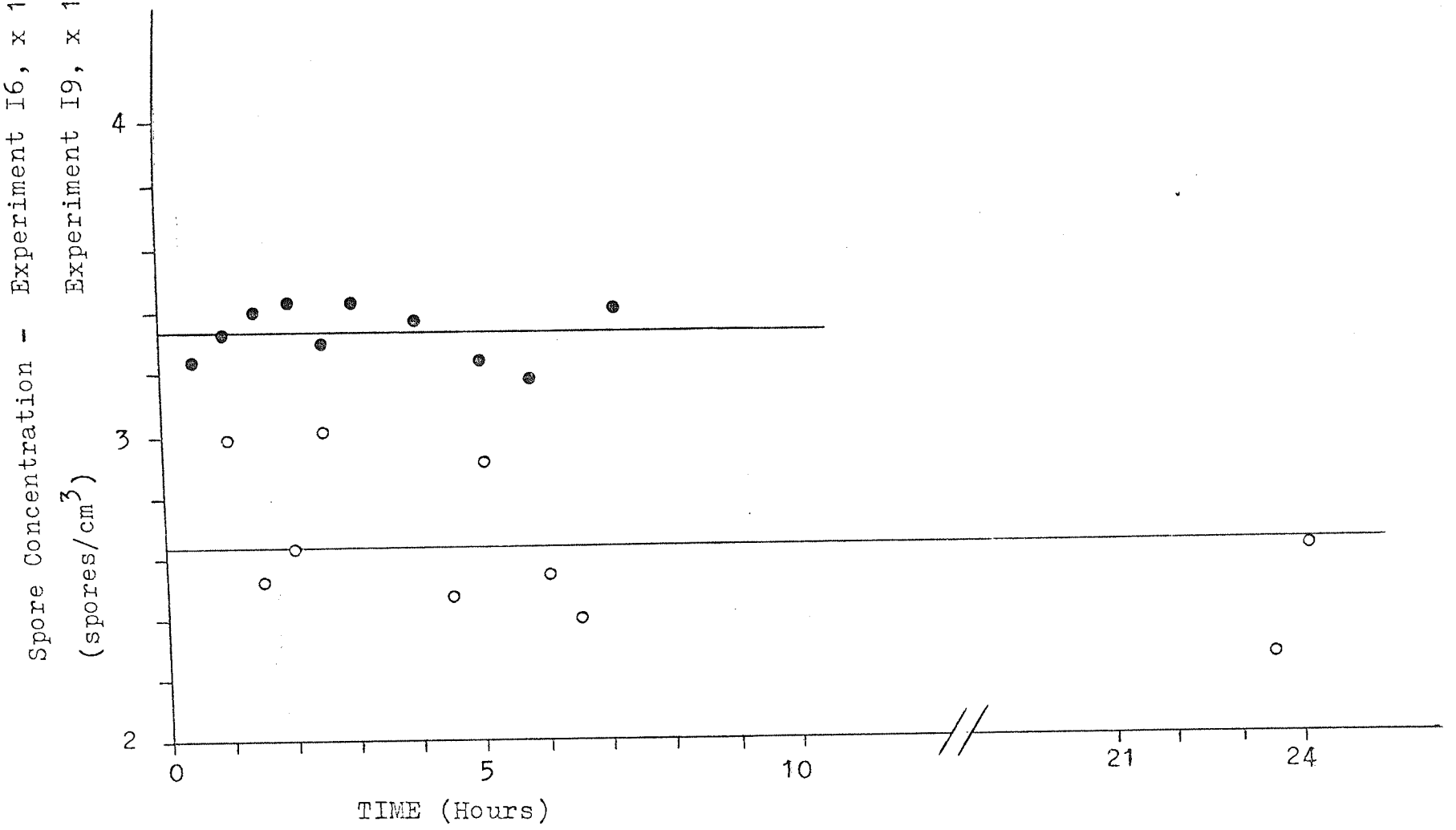
Little has been said so far about the four hour period before aggregation occurs. This is a very difficult period to summarise since it is during this time that washout of spores occurs.

Although there is not a large change in concentration of spores within the fermenter before the 3-4 hour period, there appears from some of the results (I5,G1) to be a certain amount of aggregation prior to spore swelling. To check whether aggregation occurs in spores which do not swell or germinate experiments involving non-viable spores were performed. Fig. 7·5 shows that no aggregation occured in spores rendered non-viable either by fixing with formalin (I6) or by autoclaving (I9).

7·3·3 Aggregate Size Distribution

From a sample taken at 7·5 hours, twelve aggregate concentration measurements were made according to the method described on p.114. The measurements gave a mean concentration figure of $3{\cdot}1 \times 10^3$ aggregates/cm^3, with a standard deviation of $0{\cdot}57 \times 10^3$. A similar sample taken after 26 hours also showed an aggregate concentration of $3{\cdot}1 \times 10^3$ aggregates/cm^3 with a standard deviation of $0{\cdot}7 \times 10^3$. From this information it is possible to calculate the mean number of spores per aggregate. Allowing for a washout of 25% of the inoculum spores (powdered yeast extract was used in the medium), the number available for aggregation was $1{\cdot}36 \times 10^6$ spores/cm^3. At 7·5 hours only $0{\cdot}039 \times 10^6$ spores/cm^3 were unaggregated, so assuming the remainder have formed aggregates there must be an average of 425 spores/aggregate.

FIG. 7·5 THE BEHAVIOUR OF NON-VIABLE SPORES IN SUSPENSION



It was not possible to measure the concentration of unaggregated spores in the 26 hour sample, but even if aggregation had continued to completion the average number of spores/aggregate could only have risen to 438. If no further aggregation occurs after germination (an assumption based on observation and supported by the aggregate concentration data) the diameter of an average spore aggregate can now be calculated from

$$\bar{d}_a = \left(\frac{d_s^3 \left[(1-W) n_{so} - n_s \right]}{(1-\epsilon) n_a} \right)^{1/3} \qquad (7 \cdot 4)$$

where, $\bar{d}_a$ = the diameter of an aggregate containing the mean number of spores

d_s = the mean spore diameter, taken as the swollen diameter which has been measured as 6·9 microns (standard deviation 0·65 microns from 34 measurements)

W = the fractional washout figure, in this case 0·25 since powdered yeast extract was used in the medium (see p.120)

n_{so} = the initial spore concentration, 1.81×10^6

n_s = the unaggregated spore concentration at sample time

n_a = the aggregate concentration at sample time

ϵ = the packed voidage of spores in an aggregate, estimated from empirical results to be 0.4 (Gray, 1968)

This gives a value of 61·5 microns for $\bar{d}_a$.

The results of the size distribution analysis for the 7·5 hour sample are presented as a histogram in fig. 7·6. Of the 766 aggregates analysed, 88% are shown to be normally distributed about a mean diameter of 114·4 microns (fig.7·7). This is not however, the diameter of an aggregate containing the mean number of spores. For this, equation 7·5 is required.

$$\bar{d}_a = \left(\frac{\sum N_i \cdot (d_a)_i^3}{\sum N_i} \right)^{\frac{1}{3}} \qquad (7·5)$$

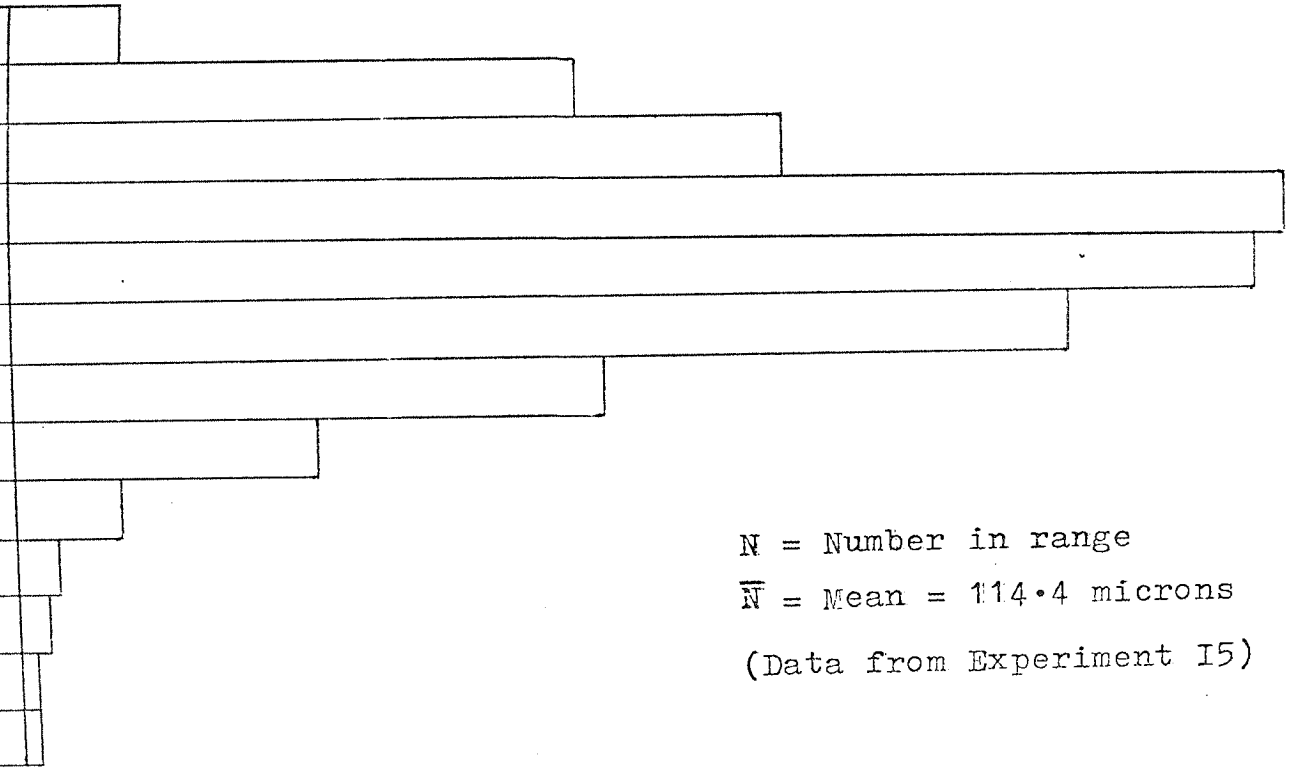
Where, $(d_a)_i$ = aggregate diameter at the mid-point of of range i.

N_i = total number of aggregates counted within diameter range i.

The set of diameters $(d_a)_i$ covering the range of aggregate sizes may be determined from the measurements made in the aggregate size analysis. Since any associated growth was included in the aggregate diameter measurement, allowance for this must be made. By 7·5 hours as much as 58 microns of hyphal growth had occured (see p. 67) so that $(d_a)_i$ can be taken as $(d_a' - 116)_i$, where d_a' is the measured aggregate diameter. Using this in equation 7·5 gives $\bar{d}_a$ = 59 microns. A more detailed presentation of the data appears in Appendix 7·3.

RANGE (μm)	N
13 - 35	14
35 - 57	71
57 - 79	97
79 - 101	159
101 - 124	155
124 - 146	132
146 - 168	74
168 - 190	38
190 - 212	13
212 - 234	5
234 - 256	4
256 - 278	2
278 - 300	2

$\sum N = 766$



N = Number in range

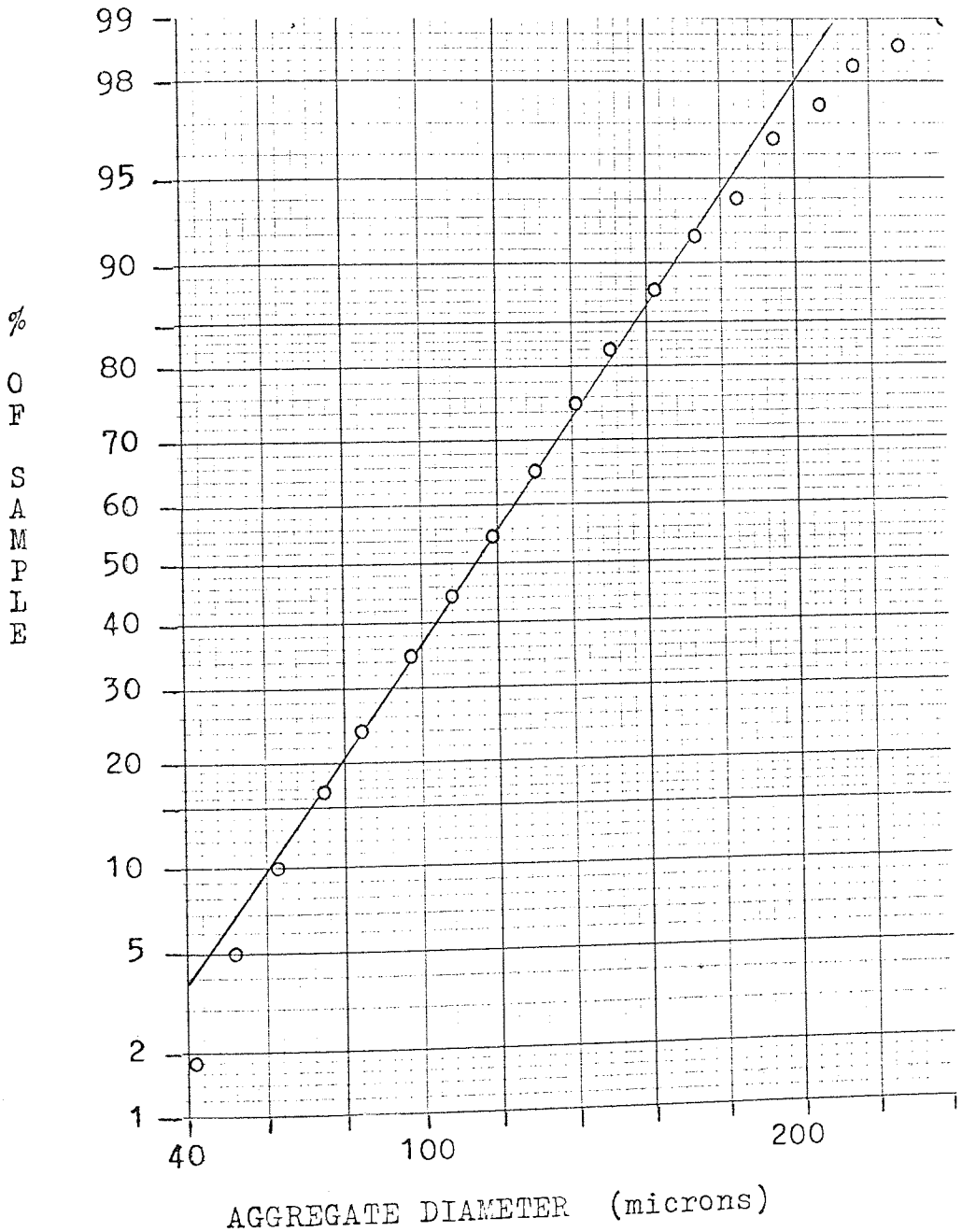
$\overline{N}$ = Mean = 114·4 microns

(Data from Experiment I5)

FIG. 7·6 AGGREGATE SIZE DISTRIBUTION

FIG. 7·7 CUMULATIVE DISTRIBUTION OF AGGREGATE SIZES

(Data from Experiment I5 plotted on probability scale)

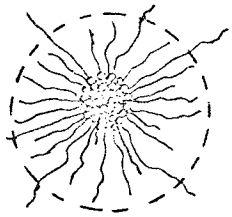


Clearly, the diameters calculated from equations 7·4 and 7·5 are in good agreement, and it would appear that the estimated voidage figure is also a good approximation. However, if the value of $\bar{d}_a$ from equation 7·5 is used in equation 7·4 to calculate the voidage a figure of 32% is obtained. It can be seen, then, that a change in voidage only brings about a cube-root change in aggregate diameter.

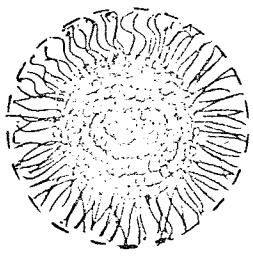
Having to allow for growth in equation 7·5 affects the analysis considerably, since many of the aggregates had diameters of less than 116 microns (the amount allowed for growth). Several observations made during the experiments may help to explain this. The maximum growth lines presented in chapter 6 represent results for only about 9% of the population; the rest of the sporelings are much shorter in varying degrees at any given time. This does not matter with large aggregates which may have thousands of spores at their surfaces, because there will be enough hyphae at the maximum length to verify the need to subtract 116 microns from the overall aggregate diameter. With smaller aggregates, though, there may be much less than a hundred spores at the surface, so it is quite possible that only a few germ-tubes will have reached the maximum length. For aggregates such as these it might be better to subtract

only the average amount of growth, ie. 25 microns (see p. 71). Even making this modification does not account for those aggregates (5% of the population) whose diameters were less than 50 microns, and it may be that human error creeps in here. The boundary of a large aggregate is well defined at the low magnification used for the analysis, but the hyphae growing out from a small aggregate are much more sparsely distributed and, therefore, easily missed. It may well be that for small aggregates the 'circle of light' used to measure diameter is only extended to the periphery of the central spore clump (see fig. 7·8).

with very small aggregates
- tendency to miss hyphae at low magnification



with small aggregates
- tendency to miss large hyphae



with large aggregates
- no problem, though central spore core cannot be easily distinguished

Fig. 7·8 Possible Causes of Error in Aggregate Size Measurement.

The effect of synchronous germination of spores must also be considered. Very often during experiments it was noted that spores within an aggregate had grown to the same extent, suggesting that germination was synchronous throughout the aggregate. Thus, if germination occured later than average, the amount of growth observed for a synchronously growing aggregate would be less than 25 microns. Indeed, at the sample time used, that is to say 7·5 hours, germination was not complete, and so it is quite possible that there were whole clumps of ungerminated spores among the aggregates being measured.

The net effect of these effects on the accuracy of the results is not easily determined: overall, they would probably result in a slightly higher estimate being made of the average aggregate diameter. It can only be said that the data obtained give a guide to aggregate sizes and that the estimate of packing voidage is a reasonable one.

7·4 CONCLUSIONS

The subject of spore aggregation and colony formation is a very large one, and the need for a complete study of the fundamental mechanisms involved has been highlighted by the results of this chapter. Although only a very superficial study, it has produced some interesting information.

Washout of spores from the tower fermenter under constant conditions is dependant primarily on the surface properties of the spores and on the formation of foam at the air/liquid interface. The degree of washout varies with the degree of foaming, which is affected by the type of medium and the use of surfactants. Viability of spores does not affect washout appreciably.

The aggregation of spores is not so easily explained. It is associated strongly with the germination phase of growth and appears to cease on completion of germination. Aggregates may contain more than 12,000 spores or as few as 4 and would appear to be rather tightly packed with a voidage of less than 40%. It is unlikely that the mechanism involved can be explained in terms of a simple collision rate equation. Possible models of aggregation will be discussed in chapter 8.

CHAPTER 8

MATHEMATICAL MODELLING

8·1 INTRODUCTION

Two models have been developed to describe the early growth of filamentous fungi in submerged, aerobic culture. Both are mainly empirical although an attempt has been made to combine experimental data and theory. Throughout the research, many parameters which may affect growth have been held constant, and so the models are only valid for the set of conditions described in chapter 4.

The first model provides a computer simulation of the structural development of the fungus (see section 8·3). The second simulates the build-up of biomass in the fermenter as a function of time (see section 8·4). In section 8·2 the basic ideas underlying the two models are introduced in some detail.

The computations were carried out on the ICL 1904S Computer at Aston University and the CDC 7600 Computer at Manchester University. Graphical output was obtained through use of Gino-F mk 2·5 Routines, and pseudo random numbers were generated by NAG Library Routines. Fortran IV was the programming language used throughout.

8·2 MODEL BASES

During the course of the research experimental results indicated the need to include five phases of the fermentation. These were:-

1. the washout phenomenon;
2. aggregation of spores and aggregate size distribution;
3. germination;
4. vegetative growth;
5. branching and secondary germ-tube formation.

The way in which information about these phases was included in the models is discussed below.

8·2·1 Washout

The loss of inoculum spores due to washout occured very early in the fermentation; for the purposes of modelling it was represented as a simple step function. Although washout was affected by the use of surfactants, the medium, and almost certainly the aeration rate, only one washout rate has been used. Using Triton X-100 to disperse the spores in the inoculum, autoclaved yeast extract in the medium and a superficial air velocity of 1·5 cm/s, about half of the inoculum spores were washed out of the fermenter.

Computation - The effect of washout was introduced into the computation by simply halving the spore concentration within the fermenter one hour after inoculation.

8·2·2 Aggregation

The aggregation of spores is a complex process and not easy to describe in mathematical form. To the author's knowledge the subject is not dealt with in the literature, although equations for describing the flocculation of single-celled organisms and colloids have been proposed. In a treatise entitled "Flocculation Phenomena in Biological Systems" Boyle et.al. (1968) express the rate of increase in number of aggregates containing x organisms with respect to time as

$$\frac{dn_x}{dt} = C_x + D_x + R_x \qquad (8·1)$$

where, C_x = the net rate of formation of aggregates containing x individuals (x - aggregates) resulting from coalescence,

D_x = the net rate of formation of x - aggregates resulting from disruption of larger aggregates,

R_x = the net rate of formation of x - aggregates resulting from cell replication.

For fungal spores R_x is zero, and if disruptive forces are assumed to have a minor effect (ie. the system is adhesion dominant) then D_x tends to zero;

equation 8·1 thus becomes

$$\frac{dn_x}{dt} = C_x \qquad (8\cdot2)$$

This can now be expressed in more detail using the collision frequency principles of Smoluchowski (1916,1917) and the concept of adhesion efficiency; in mathematical form

$$\frac{dn_x}{dt} = \tfrac{1}{2}\sum_{i=1}^{x-1}\left(\beta_{i,x} \cdot A_{i,x-i} \cdot n_1 \cdot n_{x-i}\right) - n_x \sum_{i=1}^{\infty}\left(\beta_{i,x} \cdot A_{i,x} \cdot n_i\right) \qquad (8\cdot3)$$

where, $A_{j,k} \cdot n_j \cdot n_k$ = rate of collision per unit volume between pairs of aggregates containing j and k organisms, respectively ($A_{j,k}$ being the collision constant).

$\beta_{j,k}$ = the probability of a j-k collision being effective.

The first term in equation 8·3 represents the formation of x-aggregates from smaller ones, and the second term describes the disappearance of x-aggregates by coalescence into larger ones.

Unfortunately, there is no theoretical means of determining β, and A in the tower fermenter system is largely dependant on turbulent shear forces which again can only be quantified empirically. An expression

derived by Levich (1962) for turbulent diffusion can be used to replace A in equation 8·3,

ie. $$A_{i,x-i} = 12\pi(r_i + r_{x-i})^3 . K(e_o/\nu)^{\frac{1}{2}} \qquad (8·4)$$

where, r_i = radius of an aggregate containing i individuals

K = a constant

e_o = rate of energy dissipation per unit volume of liquid

ν = kinematic viscosity of the liquid.

A similar model was presented by Gemmell (1975) who also made use of Levich's expression for turbulent diffusion. Vold (1963) has used a random addition method to simulate the growth of an aggregate but considered single particle addition only.

Computation - Attempts were made to simulate spore aggregation using equation 8·3 and the Levich expression (8·4). Because of the nature of the summation an enormous amount of computer time was required, and this made it impossible to include the effect of collisions between aggregates of different sizes on the aggregate size distribution within the fermenter. Since there can be up to 15,000 spores in an aggregate this would have meant 225 million calculations per time interval (and at least 400 time intervals were required for the 4 hour period of aggregation). It was possible, however, to simulate the disappearance of unaggregated spores by

using large time intervals (0·1 hour) and limiting aggregate size (1000 spores). Fig. 8·1 shows a flowcha... of the program, and a listing is given in Appendix 8·1.

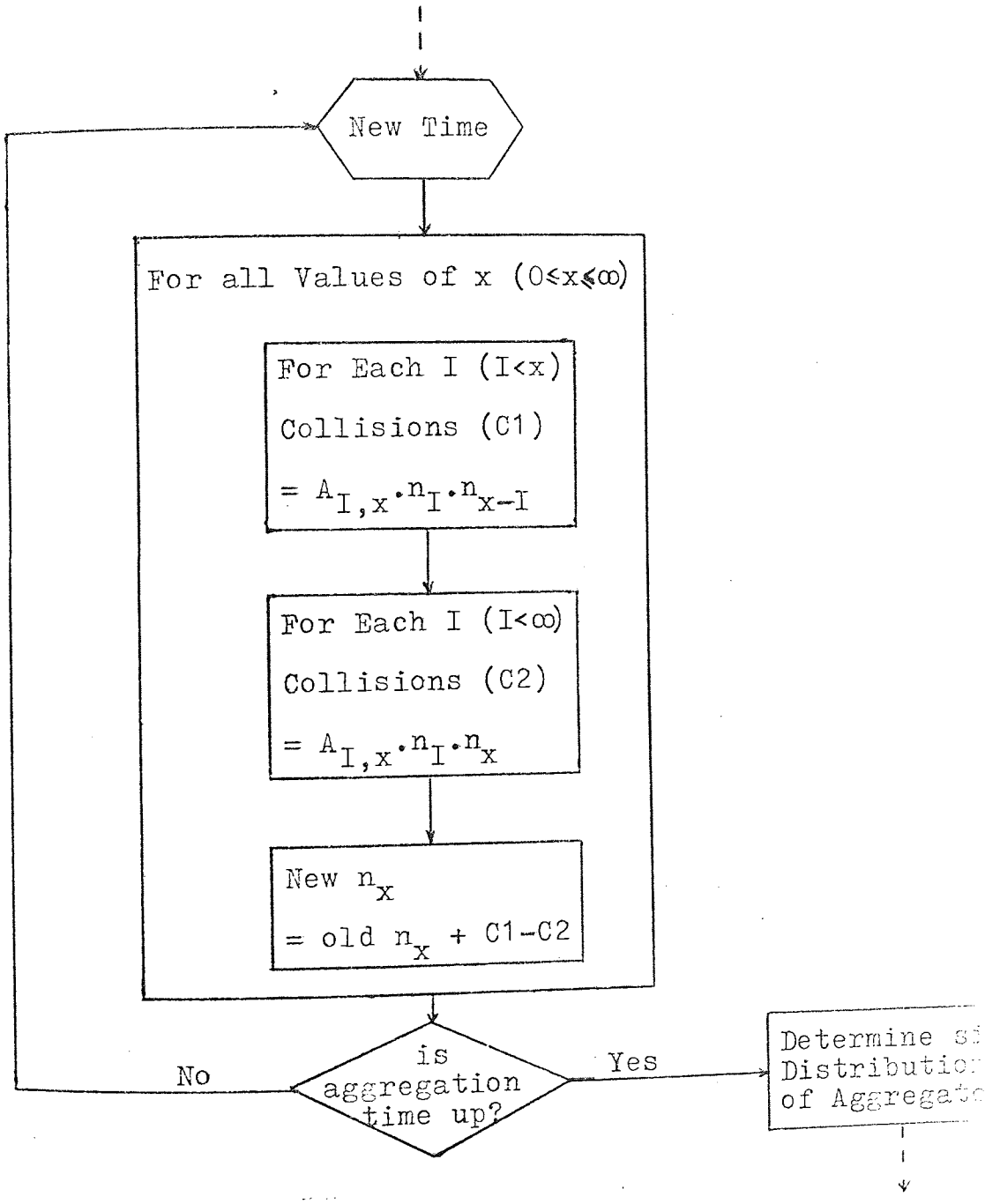


Fig. 8·1 Flowchart for Aggregation Model

Results of the simulation are compared in fig. 8·2 with experimental data. Fig. 8·3 shows that the results are well represented by the relationship

$$1/n_1^{0.4} \propto k_a.t \qquad (8.5)$$

where, k_a is the specific rate constant for aggregation.

When the data for aggregation from other experiments were treated in the same way, fig. 8·4 was the result. The data are described quite well by equation 8·5, k_a being a function of the initial spore concentration (n_{so}). In later modelling, aggregation was represented by equation 8·5, k_a being taken as 0.5×10^{-3} for starting concentrations above 1×10^6 spores/cm^3 (see below).

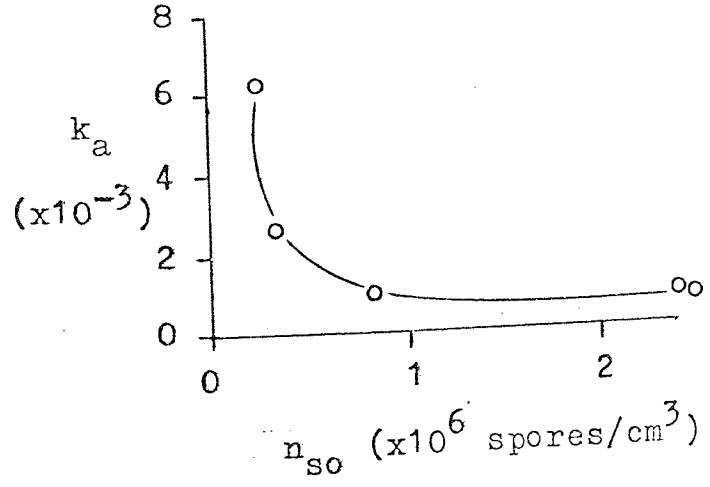


FIG. 8·2 SPORE AGGREGATION

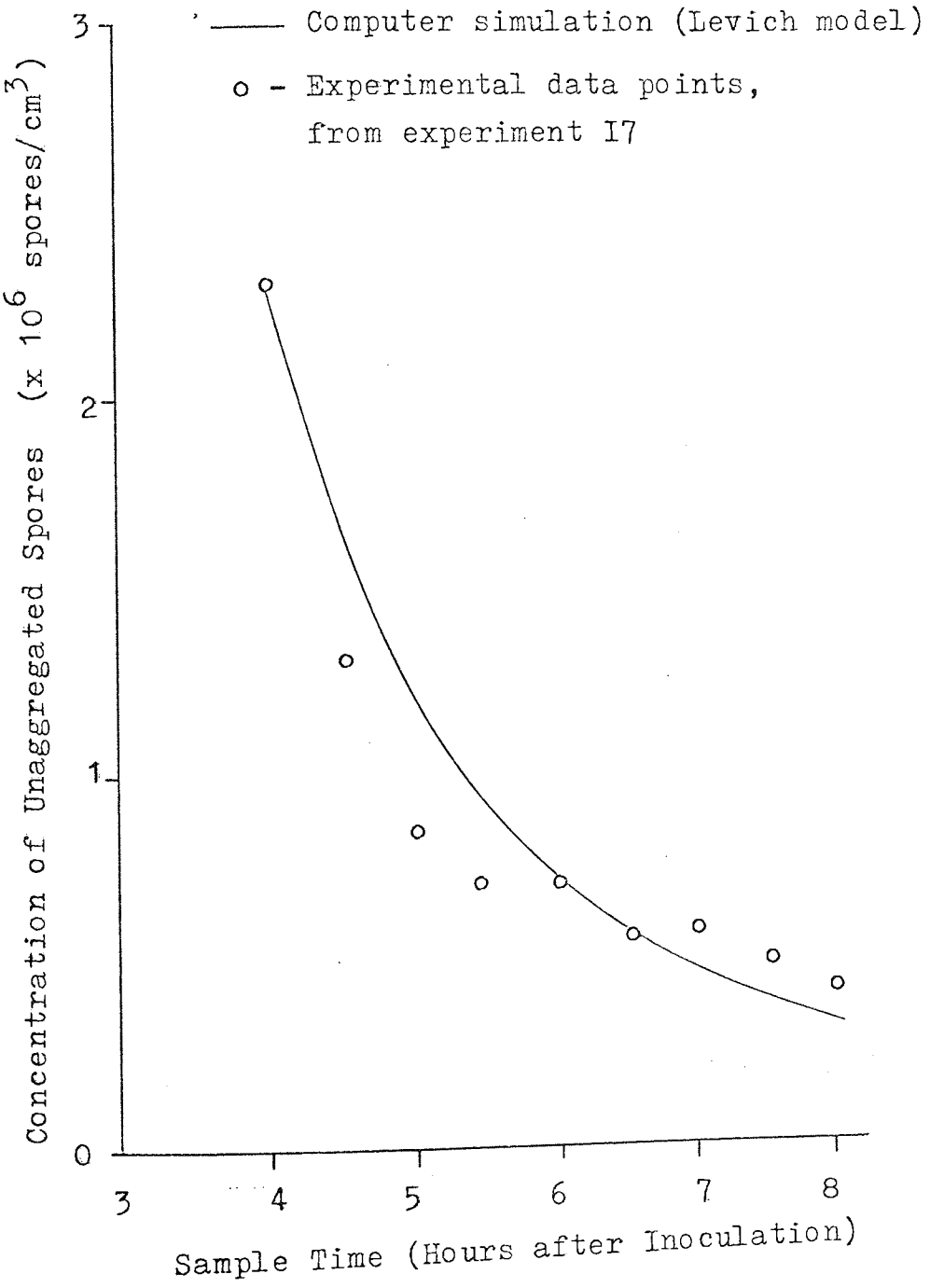


FIG. 8·3 DETERMINING THE ORDER OF THE AGGREGATION "REACTION".

(Experimental data from I7)

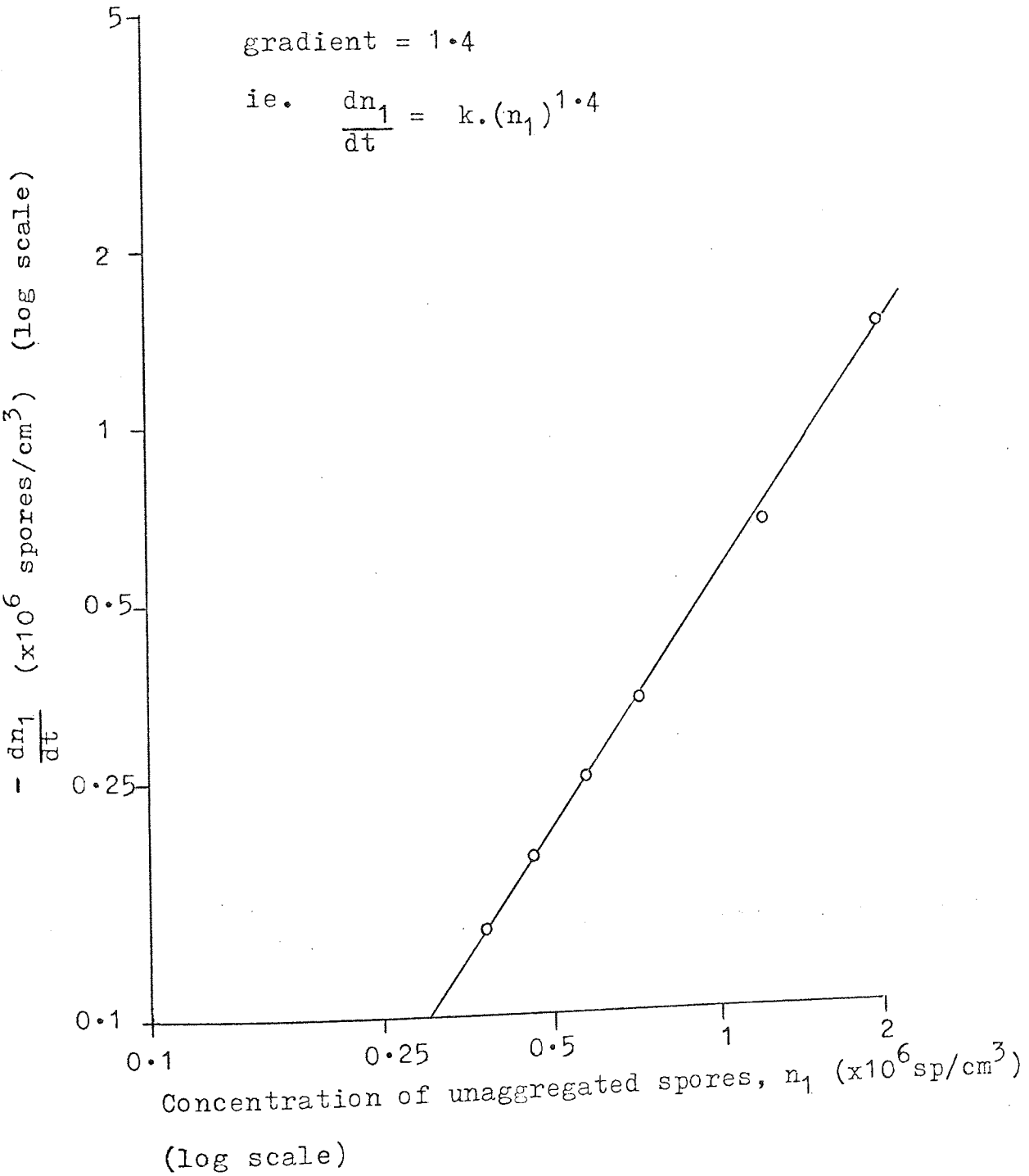
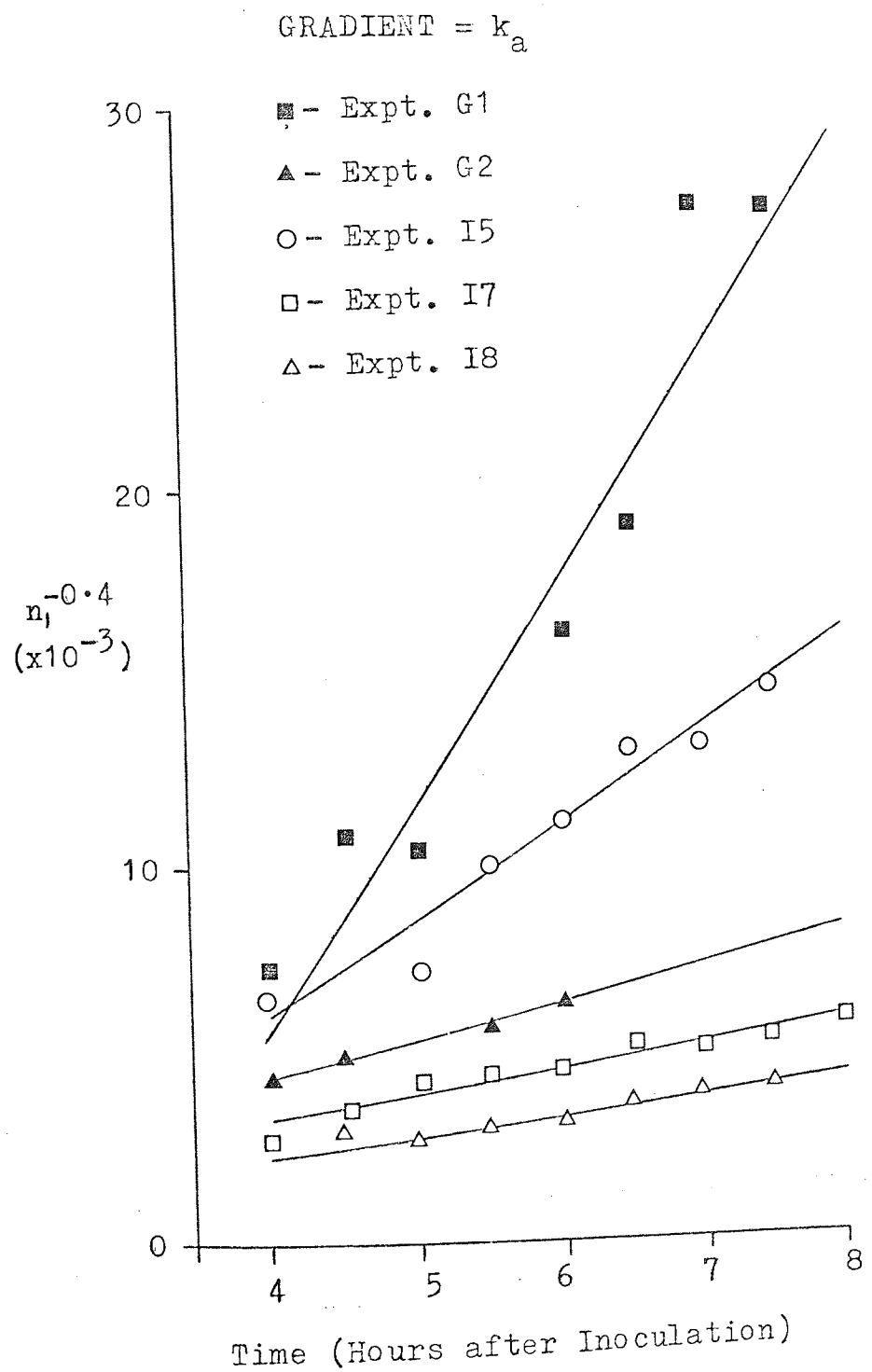


FIG. 8·4 SPORE AGGREGATION AS A FUNCTION OF TIME



8·2·3 Germination

There were two aspects of germination which had to be incorporated into the models - the range of time over which germination occured and the proportion of spores which germinated.

The first involved simulating the germination time distributions obtained experimentally and was thus purely empirical.

The second relied on information about aggregate behaviour. If all individual (unaggregated) spores were considered to germinate along with only those on the surface of aggregates (see chapter 5) a value could be determined for the proportion of spores germinating. The number of spores at the surface of an aggregate is given by equation 5·1:

$$N_{ss} = \frac{4.(d_a - d_s)^2.(1-\epsilon)}{d_s^2}$$

Unfortunately, because of the large amount of computer time and storage capacity required, aggregate size distributions were not obtained. Consequently, the number of spores germinating had to be determined from aggregate size distributions obtained by experiment.

The mean number of surface spores per aggregate, $\overline{N}_{ss}$, is obtained from the relationship

$$\overline{N}_{ss} = \frac{4.(1-\epsilon)}{d_s^2} \cdot \left(\frac{\sum N_i \cdot (d_a)_i^2}{\sum N_i} \right) \qquad (8.6)$$

where, ϵ = voidage

d_s = spore diameter

N_i = number of (i) aggregates

$(d_a)_i$ = diameter of (i) aggregate.

Based on the data of Appendix 7.4 $\overline{N}_{ss}$ has a value of 110.*

Computation - In order to select germination times such that they reproduced the required distribution, pseudo-random numbers had to be weighted according to the distribution. This was achieved by using the method shown in fig. 8.5. The selection procedure was represented in the simulation model by SUBROUTINE DISTRIBUTE. Fig. 8.6 shows some typical results obtained on using the program.

*Note - this is only 25% of the total number of spores in the aggregate (425).

FIG. 8·5 GENERATION OF PSEUDO RANDOM NUMBERS REPRESENTING A SPECIFIED DISTRIBUTION

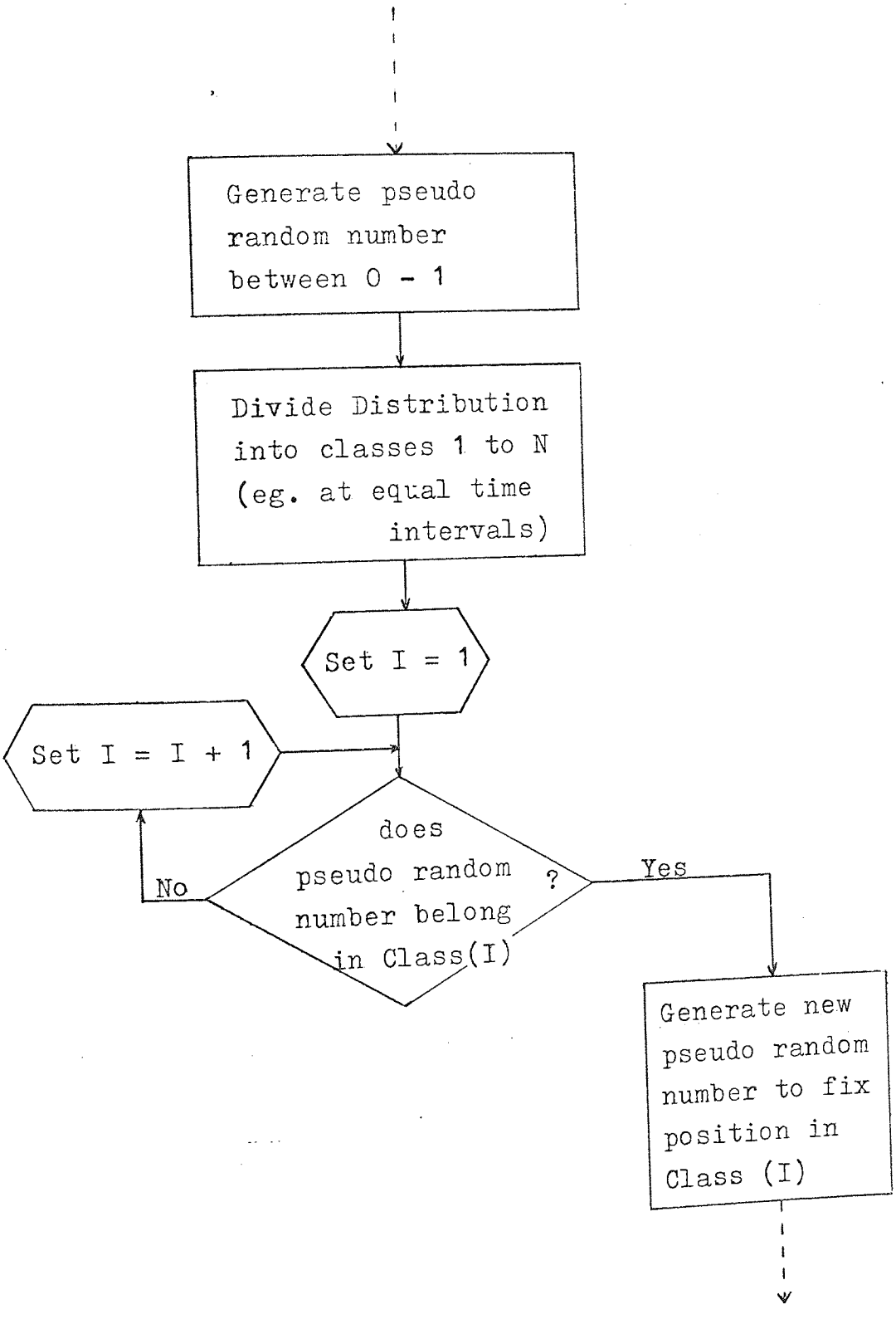
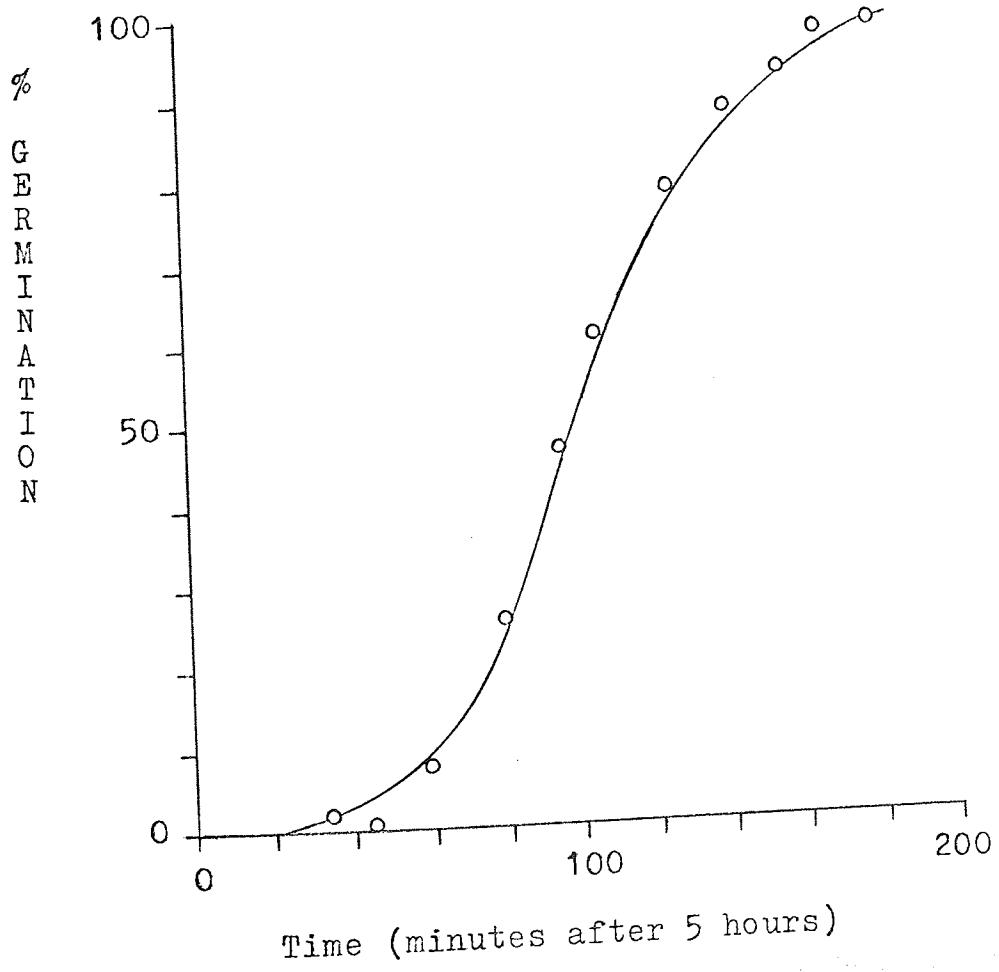


FIG. 8.6 GERMINATION TIME DISTRIBUTION

—— Computer Simulation

o - Data from Experiment G3



8·2·4 Vegetative Growth

Equations describing vegetative growth have been discussed previously (chapter 2). The experimental results presented in chapter 6 show the data to be well represented by the exponential growth equation

$$L = L_g . e^{\mu(t-t_o)} \qquad (8·7)$$

where, μ = empirically determined specific growth rate

t_o = germination time, predicted according to the distributions of chapter 5

L_g = the hypothetical length of hyphae at t_o.

L_g was taken as the value of L obtained by extrapolating the semi-log plot to the time of earliest germination (see fig. 6·4).

Computation - For simulating the growth of A.niger, L_g was always taken as 25 microns and it was found convenient to use a constant time interval $(t-t_o)$. Fig. 8·7 shows the basis of the program used to simulate the increase in total hyphal length (or mass) with time. Results from the computation are compared with experimental data in fig. 8·8.

FIG. 8·7 VEGETATIVE GROWTH MODEL

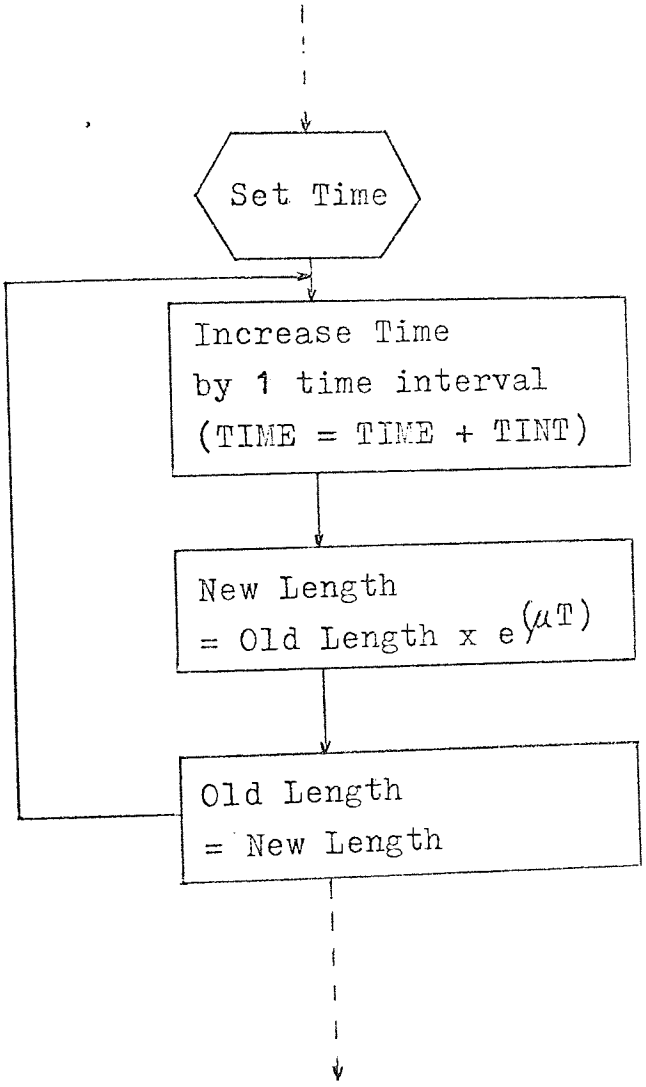
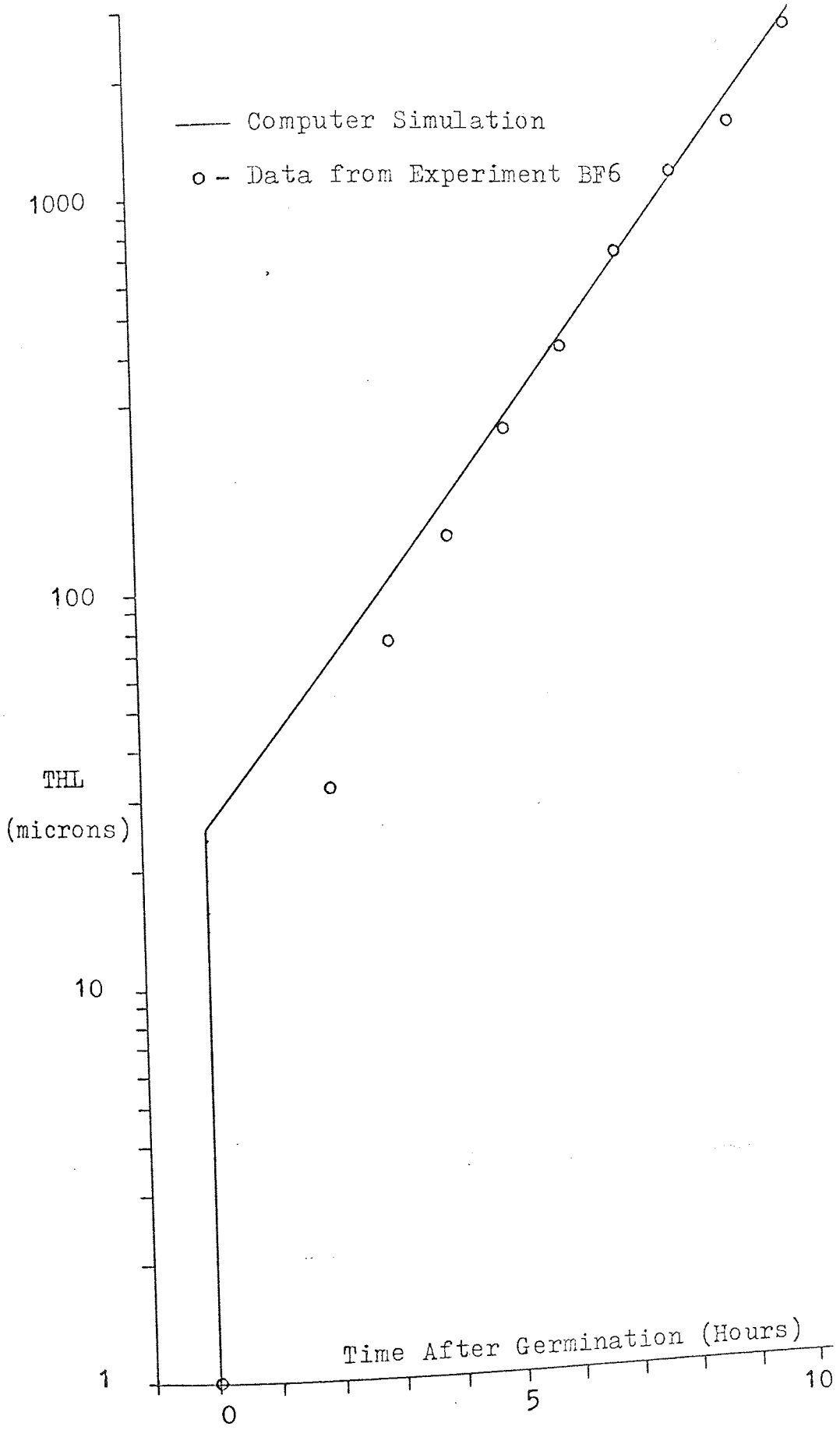


FIG. 8·8 VEGETATIVE GROWTH FUNCTION



8·2·5 Branching and Secondary Germ-tube Formation

It was necessary in the modelling to describe both when and where a new branch was formed. The author's early work was based on an assumption of constant branching interval with linear hyphal apex growth - the 'growth unit' concept (Plomley, 1959). Experimental observation showed, however, that the assumption provided a very poor approximation to the real situation. More realistic branching patterns were suggested by Leopold (1971), who analysed the branching of streams and trees. His ideas were applied to the fungus Thamnidium elegans by Gull (1975), and the computation of such branching systems was illustrated by Honda (1971). Unfortunately, all these studies considered bifurcative (apical) branching only, whereas lateral branching has been far more apparent throughout the author's research. The theory eventually adopted for branch formation was that presented in chapter 3; however, since so few apical branches were observed in practice, only lateral branch formation has been included in the model. Empirically, lateral branching can be represented as a function of either time and specific growth rate or length and specific growth rate. The latter was chosen for convenience, being a linear relationship (see chapter 6, fig. 6·18).

The position of new branches along the parent hypha is of great importance in any structural model and was represented by the distribution shown in fig. 6·19.

The formation of secondary germ-tubes has been observed in all experimental work involving submerged culture growth. All A.niger spores developed a second germ-tube before they were 7 hours old, and the fraction of spores with second germ-tubes was found to be directly proportional to time.

Computation - The linear relationship between the percentage of hyphae having branches and parent hyphal length was simulated on the computer through the generation of pseudo-random numbers. From fig.6·18 it can be seen that by L = 252 microns all hyphal segments had at least one branch. This situation was reproduced by allocating an increasing chance of selection (ie. branch formation) for each equal increment of parent hyphal length; thus

$$\begin{matrix}\text{CHANCE} \\ \text{(of branch} \\ \text{formation)}\end{matrix} = \frac{1}{100-I}$$

where, I = the number of length increments out of a total of 100 (in this case evenly divided along the range 13 - 252 microns).

Computationally this is represented by fig. 8·9.

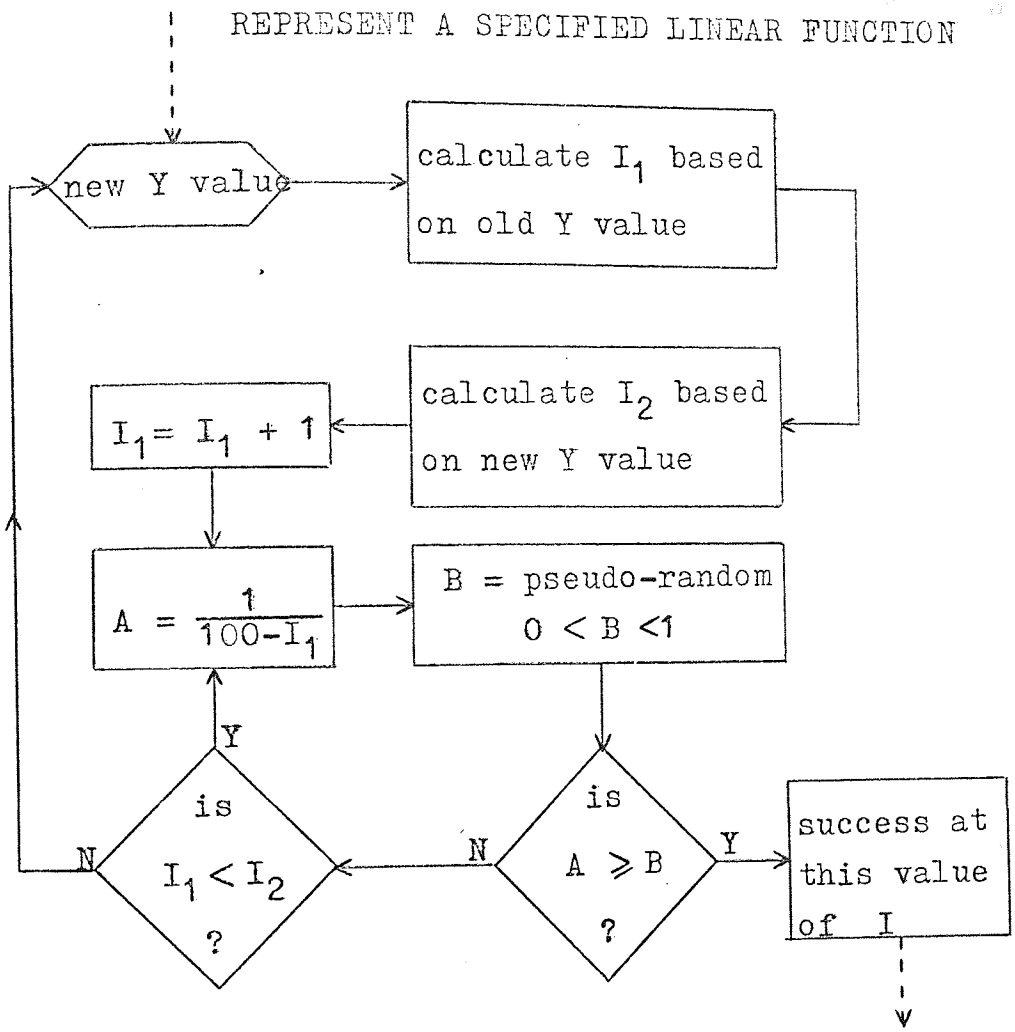
A similar method was used to predict secondary germ-tube formation since this was a linear function of time (ie in this case I = a function of time)

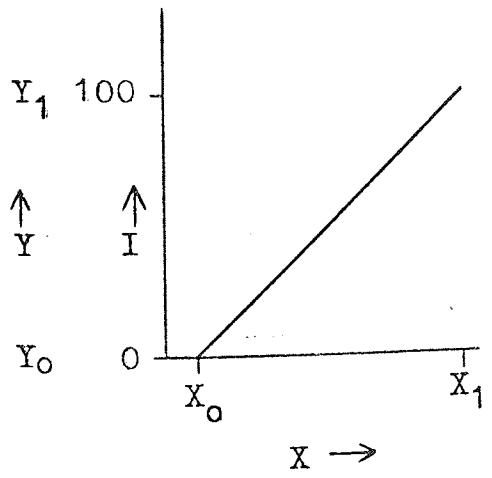
By following the procedure of fig. 8·9 many times the models were tested against the experimental data used in figs 6·18 and 6·12, (see fig. 8·10).

These two selection procedures are represented in the main simulation model by FUNCTION BUD (for branch formation) and by FUNCTION GTPA (for secondary germ-tube production).

Branches were placed along their parent hyphae using the SUBROUTINE DISTRIBUTE described in section 8·2·3. Fig. 8·11 shows the distribution of 1000 computed branch positions compared with experimental data from chapter 6.

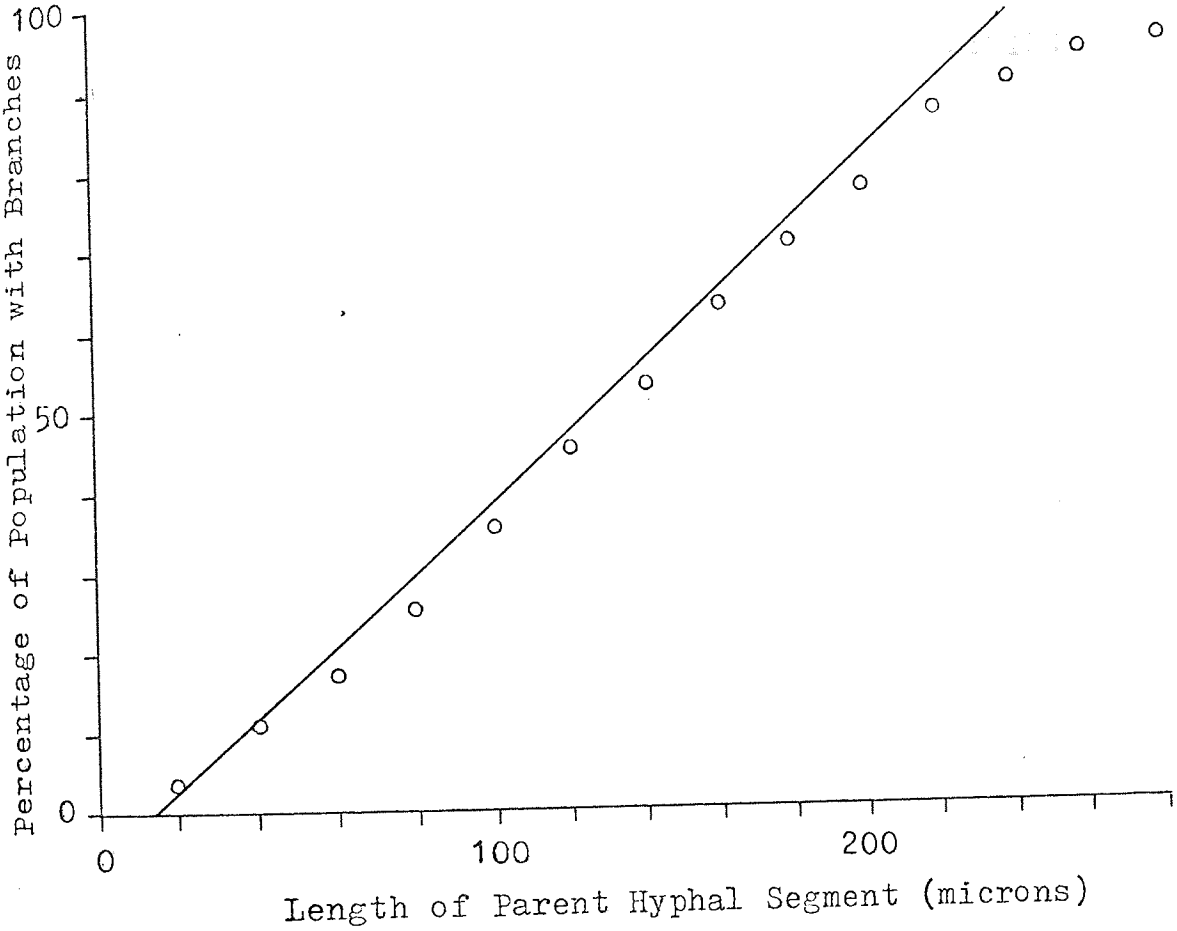
FIG. 8·9 GENERATION OF PSEUDO-RANDOM NUMBERS TO REPRESENT A SPECIFIED LINEAR FUNCTION





$$I = \frac{(X - X_0).100}{X_1 - X_0}$$

FIG. 8·10 BRANCHING AND SECONDARY GERM-TUBE FUNCTIONS



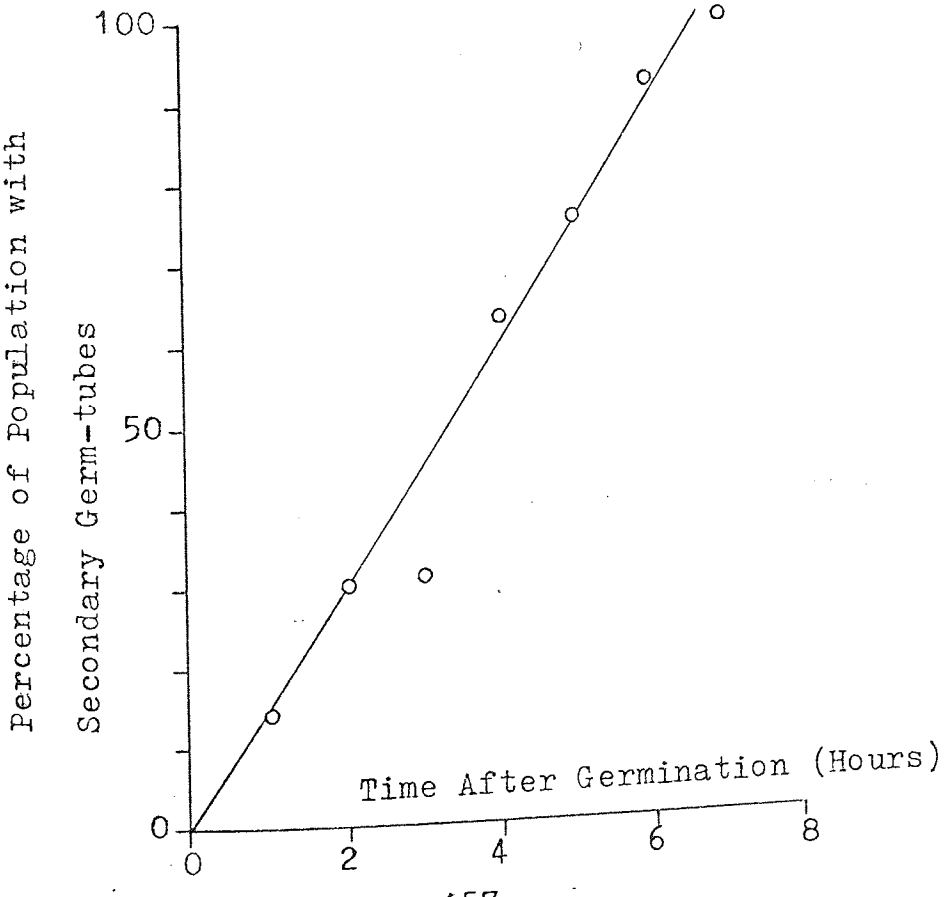
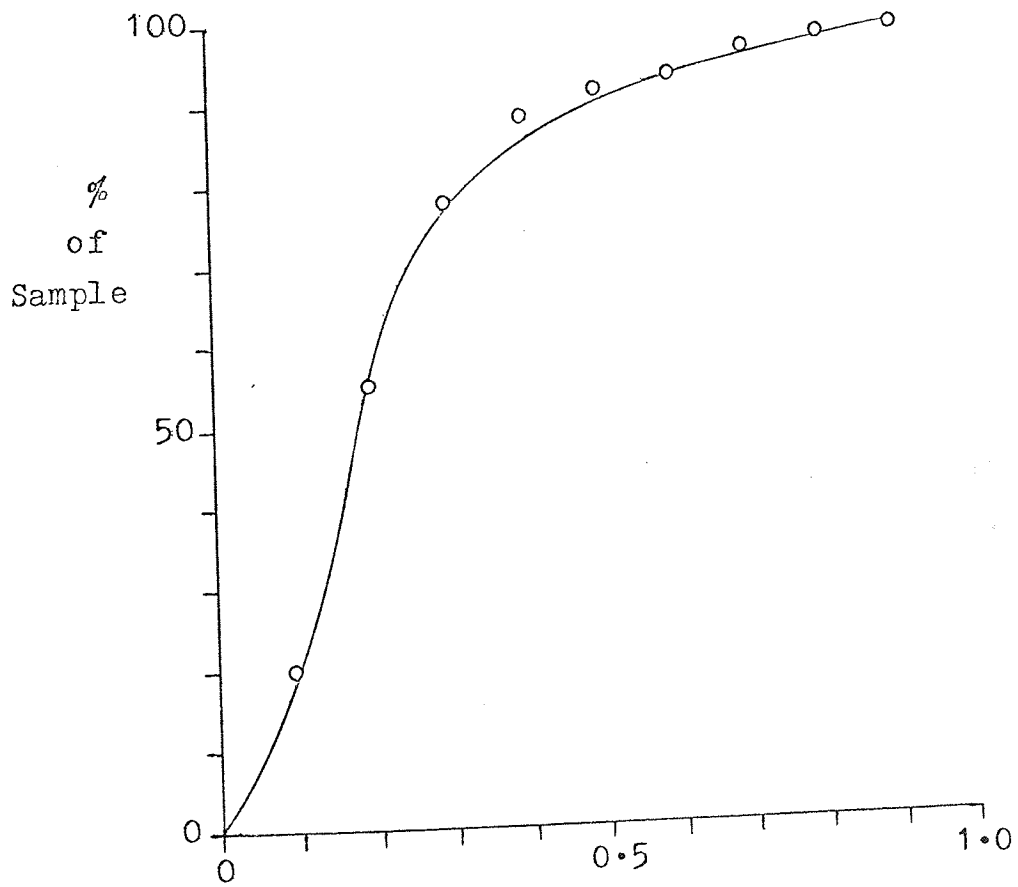


FIG 8·11 CUMULATIVE DISTRIBUTION OF BRANCH POSITION ALONG PARENT HYPHAE

—— Computed Distribution

o - Data from Experiment BF5



8·3 THE STRUCTURAL SIMULATION MODEL

The overall model was used to generate typical sporeling structures, either as individuals or as aggregates. Individual sporeling structures were compared with photomicrographs of sporelings of the same age, while typical aggregate structures were used to provide information regarding their physical properties.

Although based on empirical data the model uses the hypotheses described in chapter 3. It begins by selecting a germination time for the individual, after which growth of a single germ-tube proceeds. Decisions are then made at 0·1 hour intervals regarding the formation of secondary germ-tubes and branches. The development of pellets was modelled by including information regarding inoculum size and aggregation.

All drawings of the structures are two-dimensional and comprise straight lines with branches at right-angles to parent hyphae. Aggregates were assumed to be spherical and were represented by a section through their centre. Only the spores at the surface of aggregates were assumed to germinate.

Fig. 8·12 shows a flowchart of the computer program for the structural simulation of both individual and aggregated sporelings. More detail of the growth and branching section of the program is given in fig. 8·13. and a program listing is given in Appendix 8·2.

Fig. 8·12 Program for Simulating Sporeling Structures

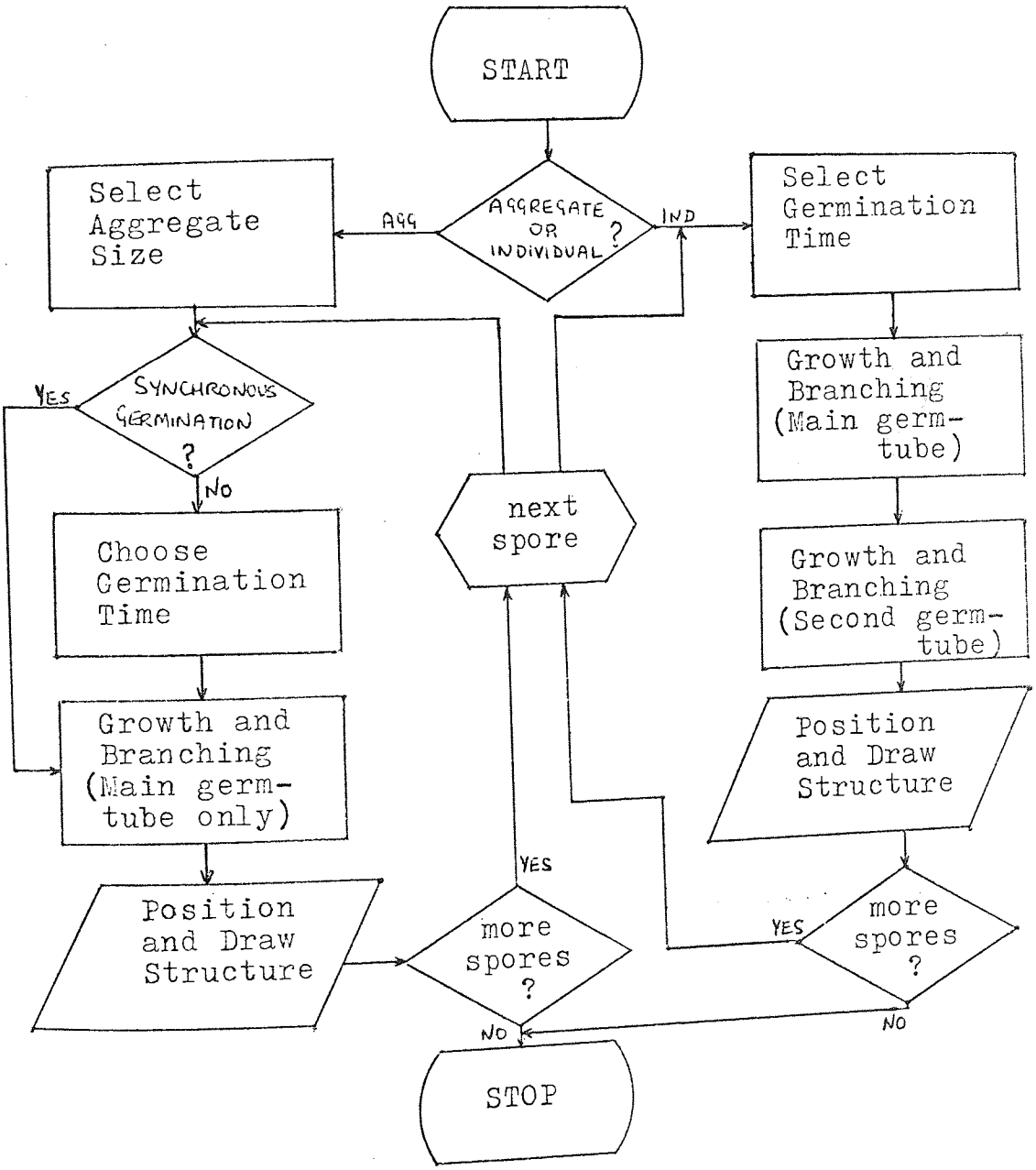
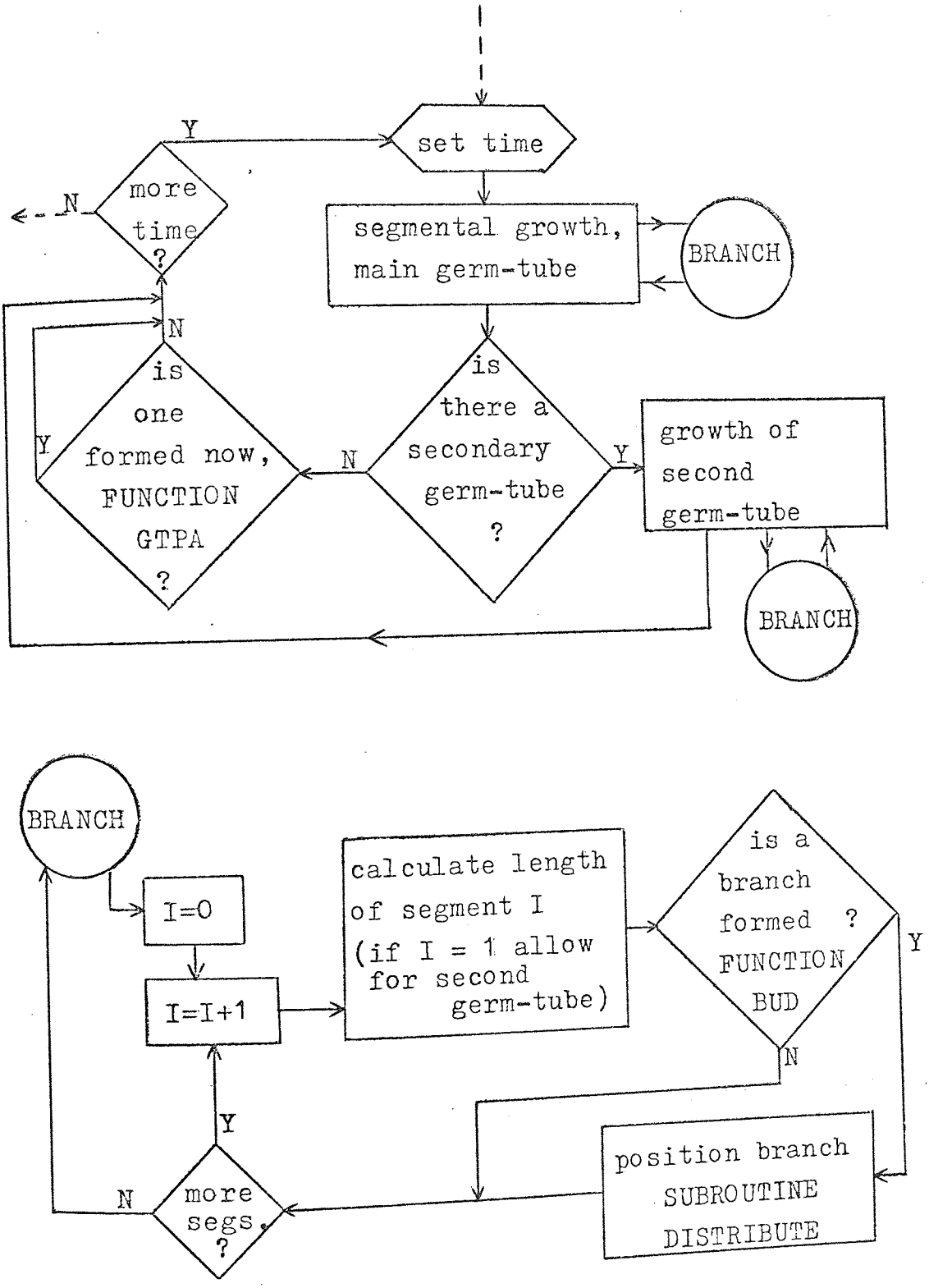


FIG. 8·13 GROWTH AND BRANCHING



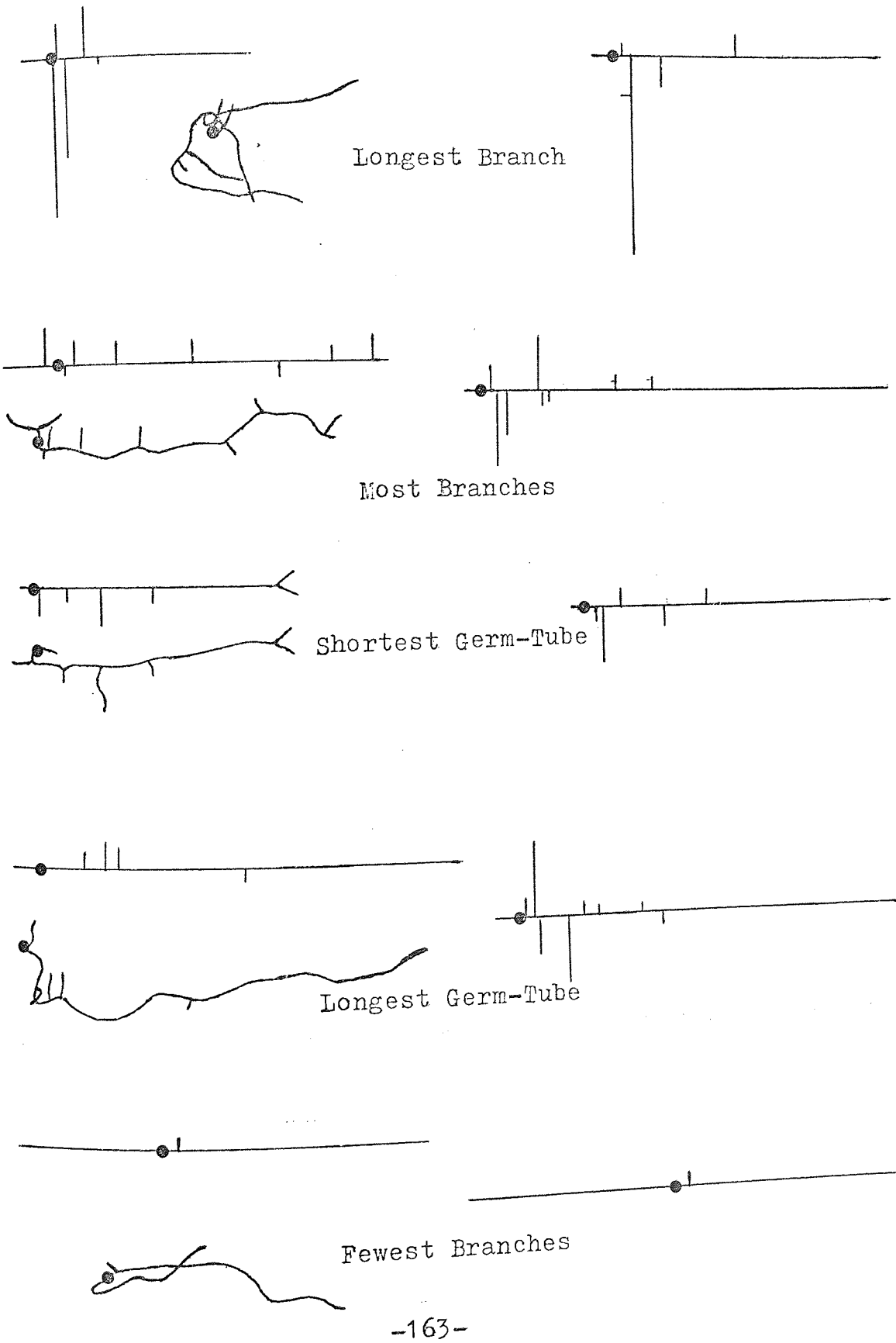
The simulated structures compared favourably with those in actual photomicrographs, when many structures of the same age were generated. Table 8·1 shows a comparison between sporelings from two experiments and the simulated structures. The only change in the model for the two sets of data was the value of the specific growth rate. It was found that, for the purpose of this model, branching was best represented as a function of length/specific growth rate squared, although this approach has not been fully tested. From the set of 42 structures generated to compare with the 42 photomicrographs of experiment BF5, various extremes (eg. most branches) are shown in fig. 8·14. Accompanying each photomicrograph trace is a straight line representation of the sporeling. It is clear from this figure that the simulated structures cover a similar range to that of actual structures.

Branches		0	1	2	3	4	5	6	7	8	9	10	11	12	13	Mean(s.d)
BF5	A	0		2	3	9	8	11	4	3	1					4·3(1·6)
	S	1	0	6	14	8	9	2	2	0						4·4(1·6)
BF6	A					1	4	12	7	7	4	3	2	1		6·3(1·9)
	S			1	1	5	7	9	11	1	3	2	0	0	1	6·3(2·1)

A - Actual sporeling structures S - Simulated structures

Table 8·1 Numbers of Branches on 6-7 hour Sporelings

Fig.8.14 COMPARISON BETWEEN COMPUTER SIMULATED SPORELING STRUCTURES (RIGHT) AND PHOTOMICROGRAPHS (LEFT) AT 6 - 7 HOURS AFTER GERMINATION



The simulation of a synchronously germinated aggregate (fig. 8·15) shows some interesting features, such as the denser inner core often observed with pellet growth and the very open nature of the structure. A series of computer drawings simulating the structural development of such an aggregate is included in Appendix 8.4. Fig. 8·16 shows a simulated aggregate structure in which spores were allowed to germinate according to the distribution shown in section 8·2·3.

Even with the limitations of straight lines and right-angles, colonies originating from a single spore eventually become circular (fig. 8·17). This supports the observation that pellets can form from single spores.

FIG. 8·15 COMPUTER SIMULATION OF SYNCHRONOUS GROWTH

Aggregate age - 6·5 hours (Scale 100 : 1)

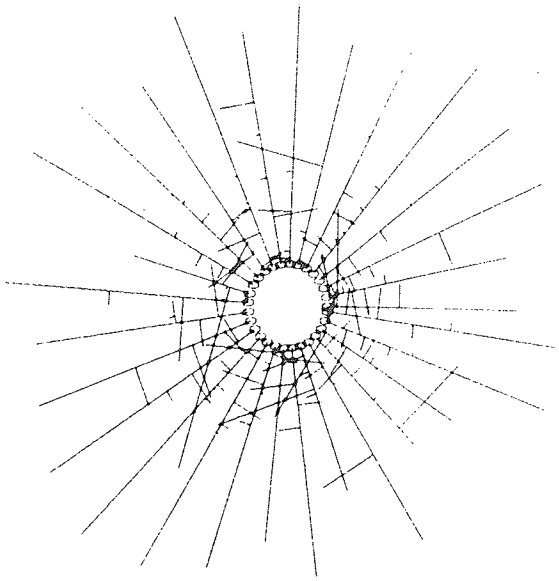


FIG. 8·16 COMPUTER SIMULATION OF ASYNCHRONOUS GROWTH

Average sporeling age - 6·5 hours (Scale 100 : 1)

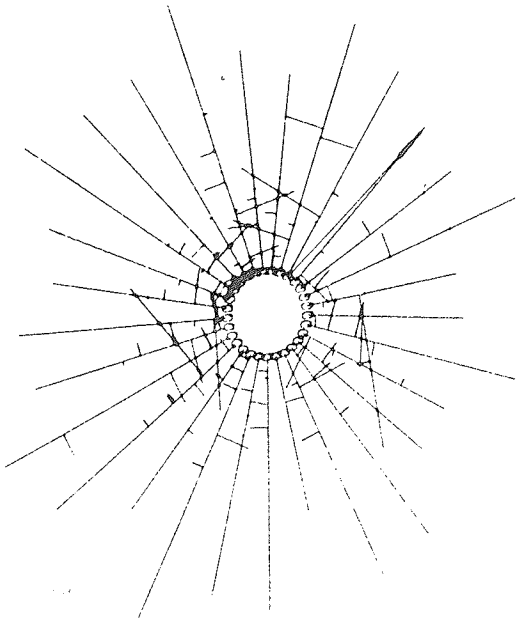
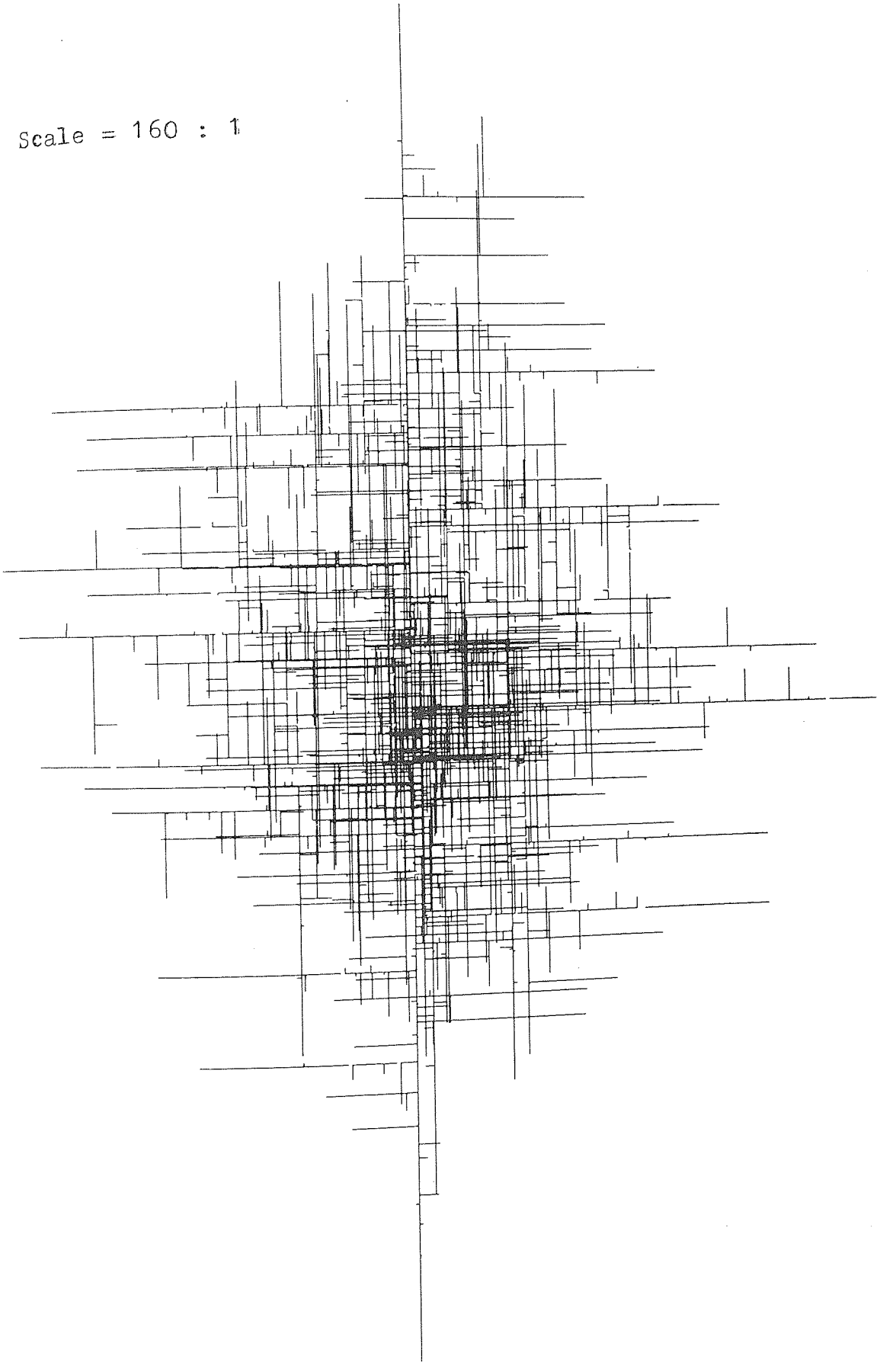


FIG. 8·17 COLONY GROWTH FROM A SINGLE SPORE (AGE ≈ 19 h)

Scale = 160 : 1



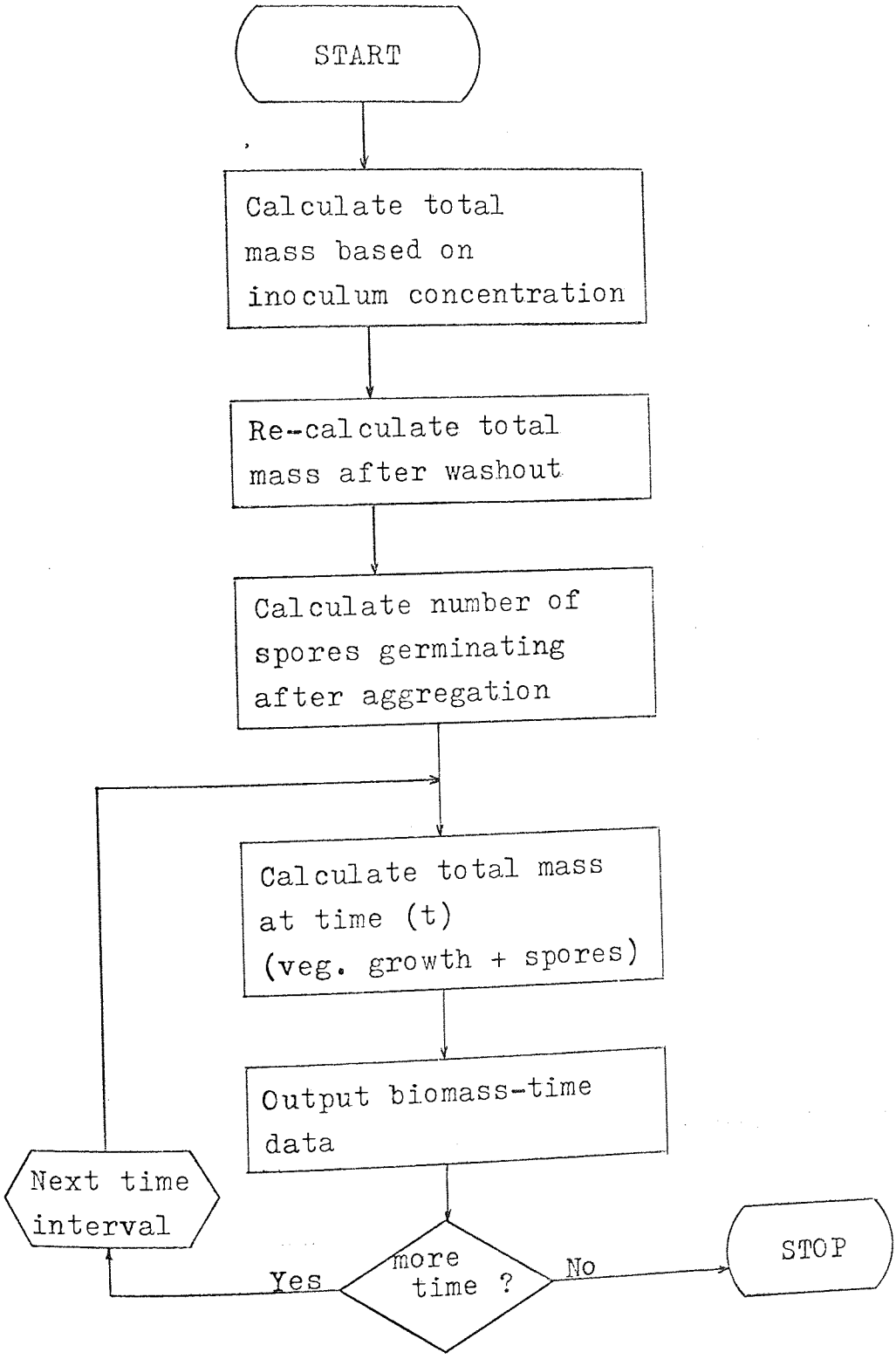
8·4 THE BIOMASS SIMULATION MODEL

Based on the inoculum concentration and specific growth rate alone, the biomass simulation model predicts the dry weight concentration of fungus within the fermenter as a function of time. The model is based upon average values for aggregate diameter, number of spores per aggregate, and germination time, taken from experimental data: growth of individual sporelings or aggregates is not considered. Fig. 8·18 shows a flowchart of the computer program, and appendix 8·3 gives a program listing.

To account for oxygen limitation, exponential growth is allowed to continue only to the point where production of biomass reaches the maximum that can be supported, ie. 0·504 grams/l/h (dry weight basis), (see chapter 6). After this a limited amount of biomass (eg. 1·12g/l at μ=0·45 h^{-1}) is considered to be actively growing at any time; this results in a constant increase in biomass concentration per unit time.

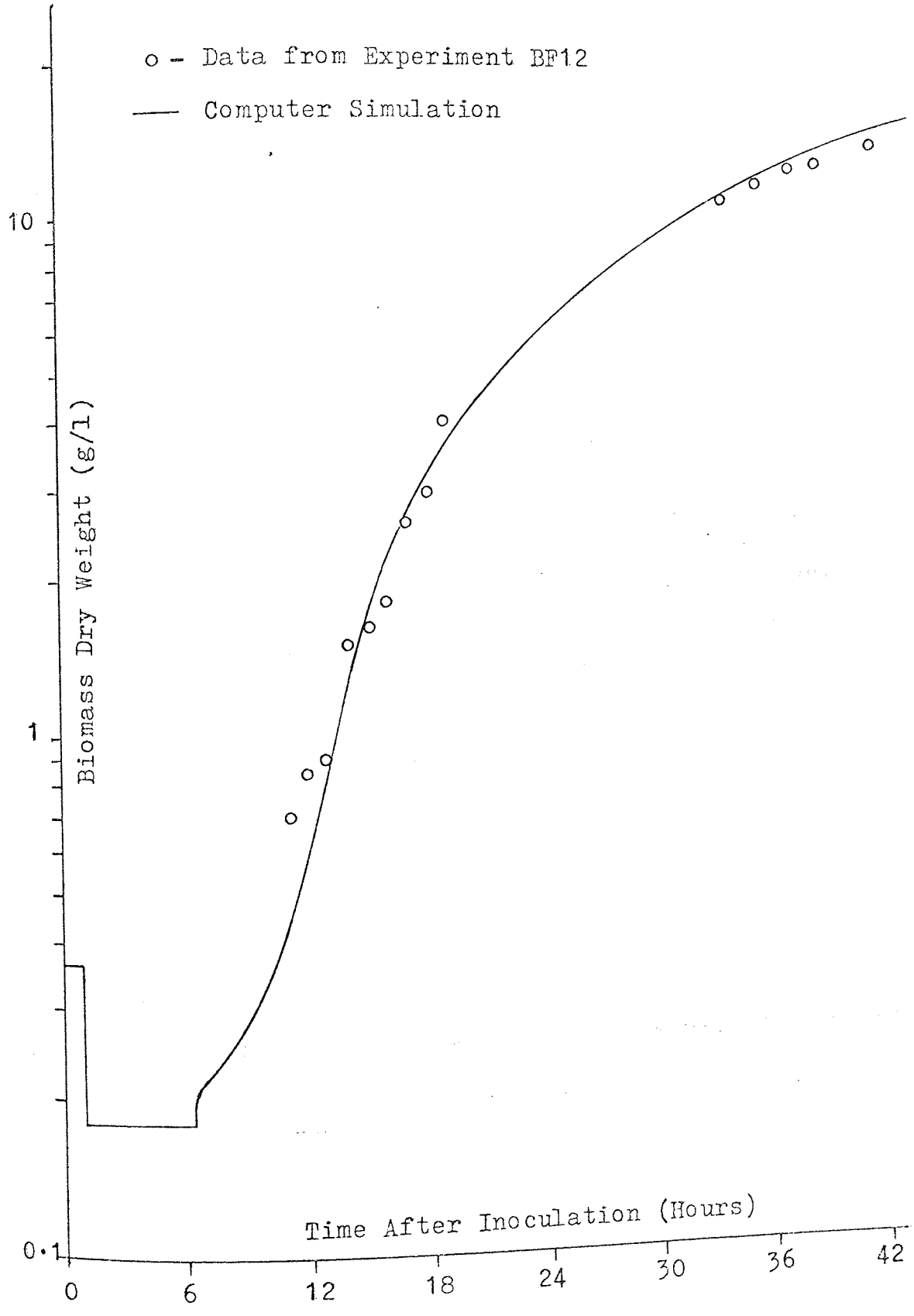
The value used for the mass of an A.niger spore was determined experimentally, and the dry weight per unit length of hyphae was based on the measurements of James (1973), (see also chapter 6).

FIG. 8·18 THE BIOMASS SIMULATION PROGRAM



A set of results from the biomass simulation model is compared with experimental data in fig. 8·19. The fit is surprisingly good and shows that oxygen limitation plays a large part in slowing down the growth-rate. The specific growth rate for the data presented in fig 8·19 was determined experimentally (see chapter 6).

FIG. 8·19 BIOMASS SIMULATION - A.niger



8·5 DISCUSSION

Both the Structural Simulation Model and the Biomass Simulation Model have been shown to reproduce experimental results with reasonable accuracy, and both provide additional information worthy of discussion.

The Structural Simulation Model - It is extremely difficult to make microscopic or other observations of the structure of spore aggregates and the fungal pellets that subsequently develop. However, by assuming that spores within an aggregate grow in much the same way as single spores do, the structural simulation model provides an indication of likely pellet structures (appendix 8·4).

Perhaps one of the most interesting features of pellets modelled in this way is the 'filling-in' phenomenon (observed by Robertson (1965) amongst others in surface cultures). As the pellet becomes larger so its centre becomes denser and the very open structure associated with the leading hyphae becomes filled in by branch growth. It would appear then that this phenomenon is simply a consequence of the positioning of lateral branches along hyphae.

As a result of the 'filling-in' process, pellet density can be seen to increase with pellet diameter. Table 8·2 shows pellet densities calculated from results of the structural simulation of growth from a typical spore aggregate. Once the effect of the original spore aggregate has become negligible, pellet density is seen to increase with pellet diameter. Fig. 8·20 shows the data of table 8·2 along with the results of Van Suijdam. Van Suijdam(1979) has observed that pellet density (defined as the mass of dry mycelial solids per unit volume of pellet) increases with diameter in small (less than 1mm) pellets of Penicillium chrysogenum.

As pellets become larger, however, the effects of diffusion limitation become apparent and the more normally observed decrease in density with pellet diameter results.

A further consequence of 'filling-in' is that pellet radius may increase linearly with time while the increase in total hyphal length remains exponential. This is shown in fig. 8·21. Initially pellet radius is proportional to THL, but as branches are formed it slows to a constant rate of increase. Observations of such 'linear' growth of pellets has often been taken as

an indication that cube-root kinetics apply (Emerson, 1940; Robertson, 1968), but since the pellet is not necessarily a sphere of uniform density this evidently need not be the case.

Details for Table 8·2 :-

Time - measured in hours, from germination

THL - based on $\mu = 0{\cdot}44\ h^{-1}$, starting at t=0, L_o=25 microns

THV - (Total Hyphal Volume) based on a constant hyphal diameter of 3 microns, and germination of surface spores only.

W_{ds} - (Weight of dry solids) based on 25% of THV remaining as solids after drying, with a density of 1·39 g/cm^3, (James, 1973). W_{ds} also includes the weight of spores associated with the aggregate.

r_p - (Pellet radius) measured directly from computer simulations (see Appendix 8·4)

V_p - (Pellet volume) calculated from the radius measurements.

ρ - (Pellet density) calculated as W_{ds}/V_p (Van Suijdam, 1979)

Diameter of spore aggregate = 114·5 microns

Total number of spores = 2734 (Mass = 2·3 x10^{-7}g)

Number of surface spores = 659

TABLE 8·2 DATA FROM COMPUTER SIMULATED PELLET STRUCTURES

Time	THL	THV	W_{ds}	r_p	V_p	ϱ
(h)	(cm) $(x10^{-4})$	(cm^3) $(x10^{-7})$	(g) $(x10^{-6})$	(cm) $(x10^{-4})$	(cm^3) $(x10^{-6})$	(kg/m^3)
1	39	1·8	0·29	90	3·1	93·5
2	60	2·8	0·33	110	5·6	58·9
3	94	4·4	0·38	140	11·5	33·0
4	145	6·8	0·47	190	28·8	16·3
5	226	10·6	0·6	260	73·8	8·1
6	350	16·4	0·8	340	165	4·8
7	544	25·5	1·1	370	213	5·2
8	845	39·5	1·6	480	462	3·5
9	1311	61·3	2·4	550	695	3·5
10	2036	95·3	3·5	590	858	4·1
11	3162	148	5·4	630	1045	5·2
12	4909	230	8·2	670	1257	6·5
13	7623	357	12·6	700	1434	8·8

Note - from 9 hours on, the spore mass constitutes less than 10% of W_{ds}.

FIG. 8·20 PELLET DENSITY AS A FUNCTION OF DIAMETER

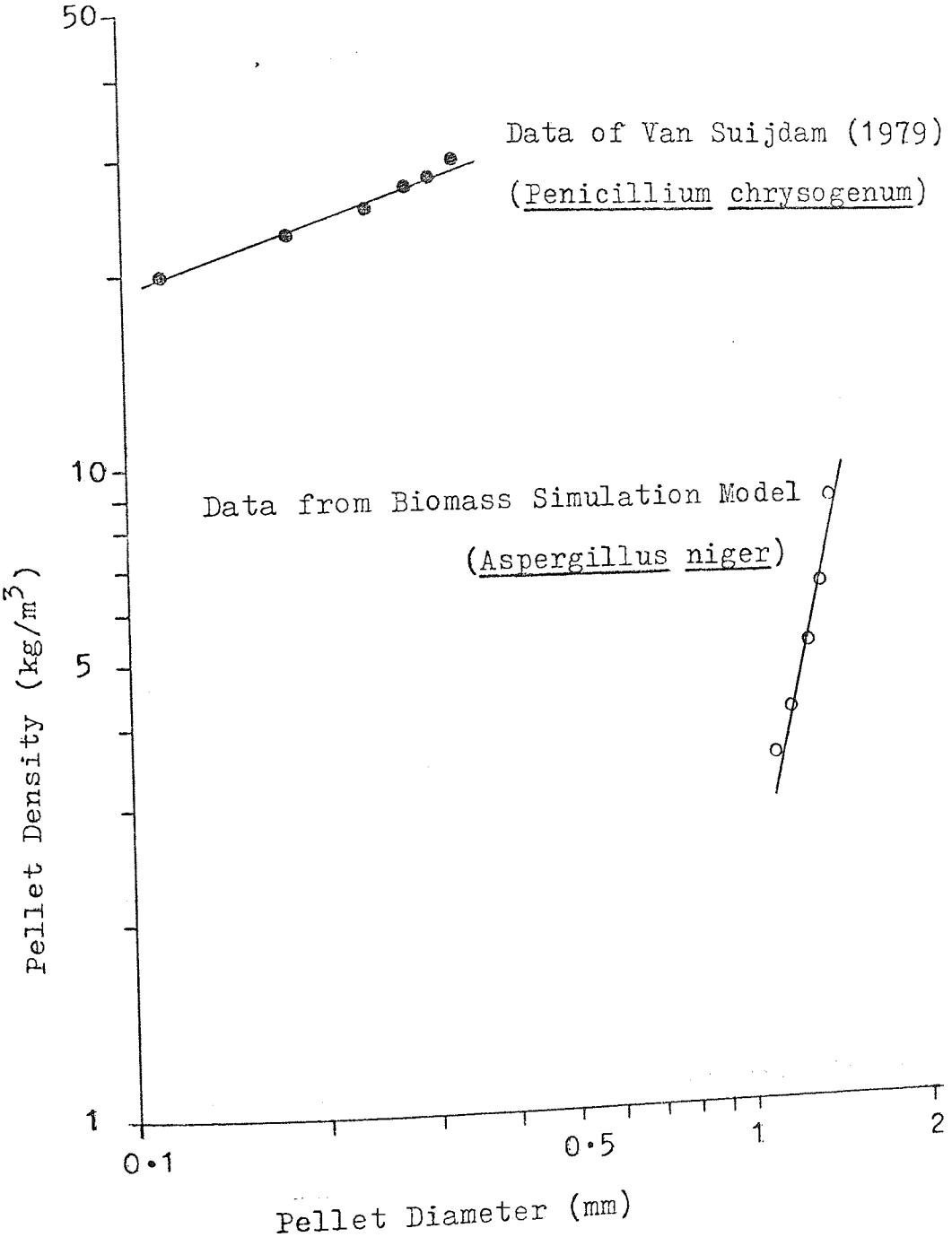
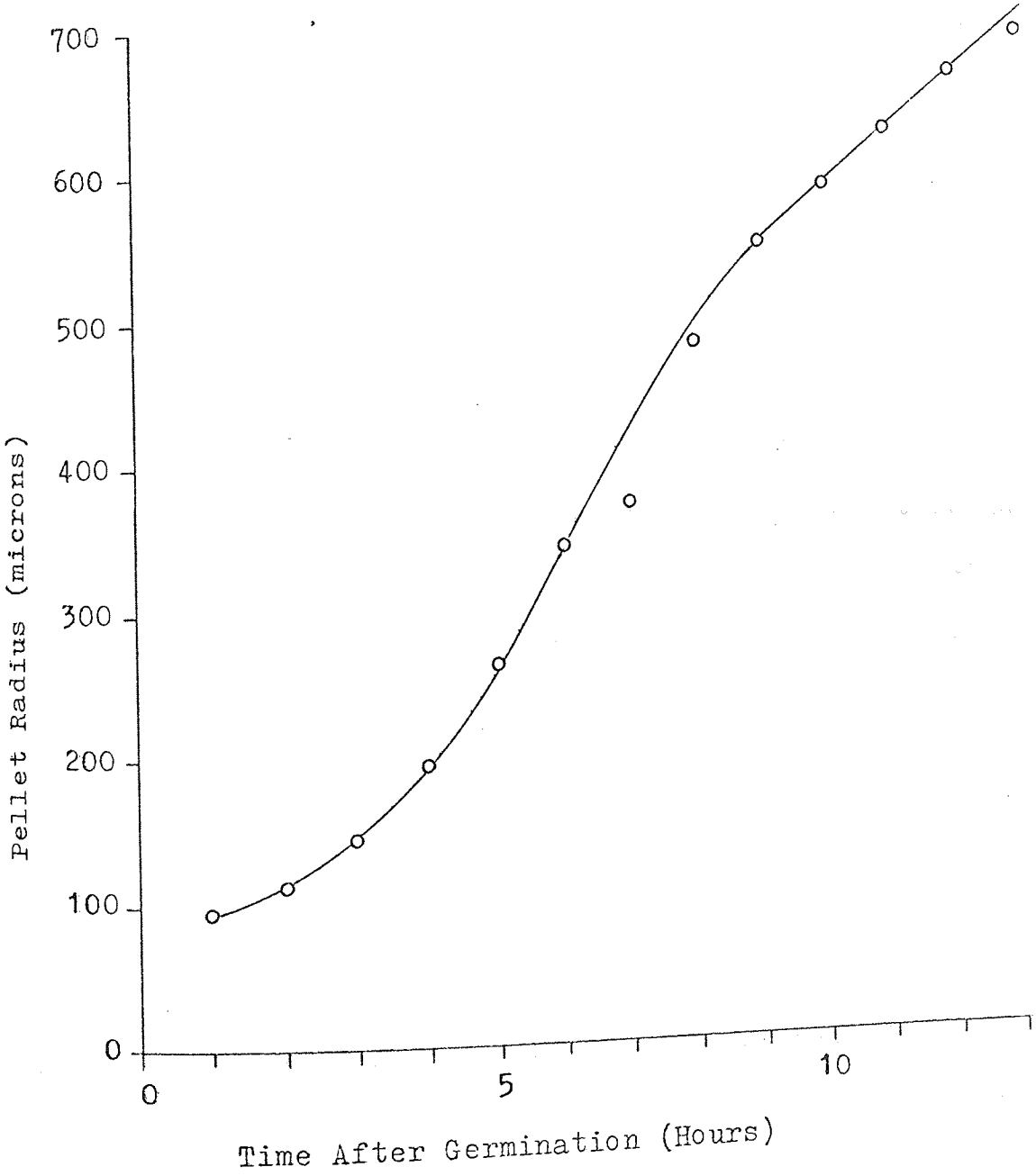


FIG 8·21 RADIAL GROWTH OF SIMULATED PELLET STRUCTURE



The Biomass Simulation Model - A generalised growth curve such as that presented in chapter 6 (section 6·3·1, fig 6·9) shows an acceleration phase immediately preceding the exponential phase. The author's length measurements showed that such an acceleration phase did not occur: indeed just after germination growth was even more rapid than in the exponential phase. However, when these data were used in the model and the results plotted an acceleration phase could be seen (fig. 8·19). This is due to the effect of spore mass on dry weight during the early stages of growth.

Fig. 8·19 also shows a deceleration phase following exponential growth. In this case the deceleration is caused by oxygen limitation and does not necessarily indicate an imminent cessation of growth due to depletion of other nutrients. The available oxygen in the system can only support a certain amount of growth per unit time and the change in kinetics shown in fig.8·19 merely demonstrates that this level has been reached. Because the oxygen in the system is continually being replenished the amount of biomass in the fermenter will continue to increase at a constant rate until one of the nutrients other than oxygen becomes scarce. It is interesting to note that the effects of oxygen limitation become apparent at such an early stage in the fermentation (ca. 18 hours after inoculation).

An important feature of the biomass simulation is that it integrates results obtained using the microscopic method and the dry weight method. The simulation was based on results from microscopic analysis (specific growth-rate - 0.47 h^{-1}) but provides a good fit to dry weight data from the same experiment.

CHAPTER 9

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

9·1 GENERAL DISCUSSION

- The Hypothesis for Growth
- The Growth Simulation Models

9·2 CONCLUSIONS

- Germination and Spore Aggregation
- Initial Growth of Sporelings
- Secondary Germ-tube and Branch Formation
- Growth Rates Based on THL and Dry Weight Measurements
- Experimental Technique

9·3 RECOMMENDATIONS FOR FURTHER WORK

9.1 GENERAL DISCUSSION

Although results on specific topics have been discussed in earlier chapters of the thesis, it is also useful to take an integrated view of the overall research programme and achievements.

The two major functions of the experimental programme were to test the hypothesis for growth (chapter 3) and to provide data on which to base the simulation models. It is with respect to these two areas that results will now be discussed.

The Hypothesis For Growth - To summarise, the hypothesis for growth contained the following proposals. When a fermenter is inoculated with a population of viable spores and operated under conditions such that growth is not limited

1. germination occurs over a range of times;
2. growth is autocatalytic in nature with all individuals being subject to the same specific growth rate;
3. apical branches are formed as a result of hyphal apices reaching a maximum extension rate, although the formation of septa and subsequently, lateral branches reduce the chance of apices ever attaining this maximum rate;

4. aggregation of spores occurs during the swelling phase and only those spores on the outside of an aggregate germinate.

The first of these assumptions was well supported by direct experimental evidence.

The idea that growth can be autocatalytic was also supported by experimentation. The nature of the technique used to obtain growth data did not allow direct observation of autocatalytic growth, although it was strongly suggested by the results obtained from the analysis. By making the assumption that all sporelings were growing with the same specific growth rate, extrapolation of the data yielded a likely germination time for each individual sporeling. The "theoretical" germination-time distribution thus obtained compared well enough with experimental data to provide reasonable confidence in the assumption. Consideration of the mean spore growth further strengthened support for the proposed hypothesis and also served to confirm the usefulness of the experimental and analytical techniques developed during the studies. Finally, direct observations of surface cultures showed that autocatalytic growth, at a single specific growth rate, certainly occurs in this mode.

The third proposal concerning branching was not tested effectively by the studies of this research, since apical branches were too few in number for meaningful conclusions to be drawn. The results of the analysis of lateral branching did, however, lend support to the idea of septation and segmental growth (see chapter 6). Recent work by Prosser and Trinci (1979) relied on the connection between septation and branching, and it is interesting to note the similarity between the results of their simulations and those presented by the author in chapter 8.

Finally, the fourth proposal would appear to be supported directly from experimental observation, although only in a qualitative way.

Although further testing of the hypothesis was (and is still) needed, it has been developed and examined to such a degree that modelling of growth can be undertaken with some confidence.

The Growth Simulation Models - Although simple in structure, the models served to strengthen the connection between the experimental results and the hypothesis. By using the growth hypothesis and empirical data, simulated structures were produced which matched those obtained by experiment.

Indeed, it was even possible to predict dry weight data for an experiment using only the results of microscopic analysis.

The success of the models in fitting and simulating data gave considerable confidence when predictions were made of growth beyond the range covered by the experiments. Some interesting results were provided by these extended simulations, and it is quite probable that further analysis would yield information useful, for example, in mass transfer and sedimentation studies.

9·2 CONCLUSIONS

The primary objectives of the research have been met, and it is felt that the results provide the basis for a better understanding of the behaviour of microbial aggregates in tower fermenter systems. Specific conclusions are listed below.

Germination and Spore Aggregation - The germination of fresh, mature spores of Aspergillus niger occurs over a range of time, following a period of spore swelling. The spores begin to swell approximately three hours after inoculation of a suitable medium, and germination times

are distributed normally between the fifth and eighth hours. The age of spores used for an inoculum affects their germination.

During the swelling phase spores tend to aggregate into clumps often containing many thousands of spores, and germination at the centres of these aggregates is suppressed even though the spores retain their viability. The aggregation process itself is strongly associated with that of spore swelling and only occurs amongst viable spores. Growth within a spore aggregate tends to be synchronous even though there are wide variations between aggregates.

Initial Growth of Sporelings - Growth, both in submerged and surface culture, is initially extremely rapid ('germ-tube growth') but becomes exponential in nature as vegetative growth takes over. All sporelings grow at the same specific growth rate for given environmental conditions, although the morphological development in the two modes of growth is quite different.

Secondary Germ-tube And Branch Formation - As growth proceeds to the vegetative phase septa are laid down, resulting in the formation of lateral branches. In submerged culture, these branches are usually formed

well upstream of the hyphal apex, and their numbers appear to be directly proportional to total hyphal length (THL). As well as branches, secondary germ-tubes are always formed during submerged culture growth. Stochastic models based on observations of branching behaviour can be successfully used to simulate the morphological development of individual and aggregated sporelings.

Growth Rates Based on THL and Dry Weight Measurements - Increases in THL do not necessarily result in proportional increases in dry weight of the fungus. Hence specific growth rates measured by the two methods can differ: nevertheless, models based on THL data can be used to successfully predict dry weight measurements.

Experimental Technique - Several of the conclusions outlined above are applicable also to fungi other than A.niger, as is the technique employed to obtain the necessary data. It is felt that the development of the technique constitutes one of the major contributions of this work and so it is summarised briefly below.

1. Fungi are grown from a suspended spore inoculum in an aerated, submerged culture, samples being taken at hourly intervals.

2. Photomicrographs of individual sporelings are taken from each sample, and THL, secondary germ-tube lengths, and branch lengths and positions from spores are made.

3. A plot of the largest THL from each sample is made against time to establish the specific growth rate (SGR).

4. Germination times for all individual sporelings from each sample are determined by assuming they grow at the same SGR, and then a mean germination time is calculated.

5. A line, representing the SGR, starting at the mean germination time is drawn and compared with the data for mean THL per sample.

6. All individuals are regrouped into age ranges making possible assessment of branching and secondary germ-tube characteristics.

The first step is straightforward with the fermenter being run for about 20 hours after inoculation; however, preparation of equipment, media, and the inoculum does take a day or so. Careful consideration must be given to the inoculum size: too few spores will result in excessive time demands at the sample scanning and photography stage; too large an inoculum may result in growth

becoming oxygen-limited before the end of the experiment. An inoculum size of around 1×10^6 spores/cm^3 of fermenter contents has been found to be the optimum figure.

The next phase of the procedure can be time consuming, several hours normally being required for each sample to be scanned and photographs prepared for later analysis. Only individual sporelings are considered for analysis, as groups of sporelings have been observed to germinate synchronously. Photographing is stopped when it becomes difficult to ascertain whether or not the mass of hyphae under the microscope lens has originated from a single spore.

Data are now collected from projections of the photomicrographs - approximately one hour per sample is required for this. Once data have been collected, the tedious task of analysing them (which can take several days per sample by hand) can be shortened by using a computer: the overall procedure from preparation of the fermenter to presentation of the results can then be performed in 7 or 8 days.

9.3 RECOMMENDATIONS FOR FURTHER WORK

Probably the most unexpected observation to be made during the course of this research was that concerning germ-tube growth. When the initial, rapid, germ-tube growth was first observed, it was felt that it would have only a small effect on the overall development of a spore-ling, but later results showed it to be a most significant phase of growth. It is for this reason that further study of the germ-tube growth of filamentous fungi is strongly recommended.

Germination and the onset of growth are important and crucial stages in a fermentation where the inoculum is a suspension of spores, and knowledge of the numbers of spores germinating is essential. As a result of the combined effects of spore washout, aggregation and inoculum spore age, germination can be as low as one spore in ten, and so further study of each of these factors is desirable. A relationship between spore age and germination would also provide a useful extension to the simulation models.

The mechanism of aggregation is clearly little understood, and although an attempt has been made here to study it, a more detailed investigation is required.

In view of the success of the author's technique for studying submerged culture growth of A.niger, further studies with other filamentous fungi would prove useful in assessing its general applicability. As a result of such work, it should be possible to extend the simulation models developed during this research to other fungi.

Finally, it is felt that much interesting and useful information has yet to be gained from the use of the computer models, in particular aspects of mass transfer and fluid mechanical behaviour of microbial aggregates.

APPENDICES

APPENDIX 7·1

DATA FOR WASHOUT OF SPORES FROM THE TOWER FERMENTER

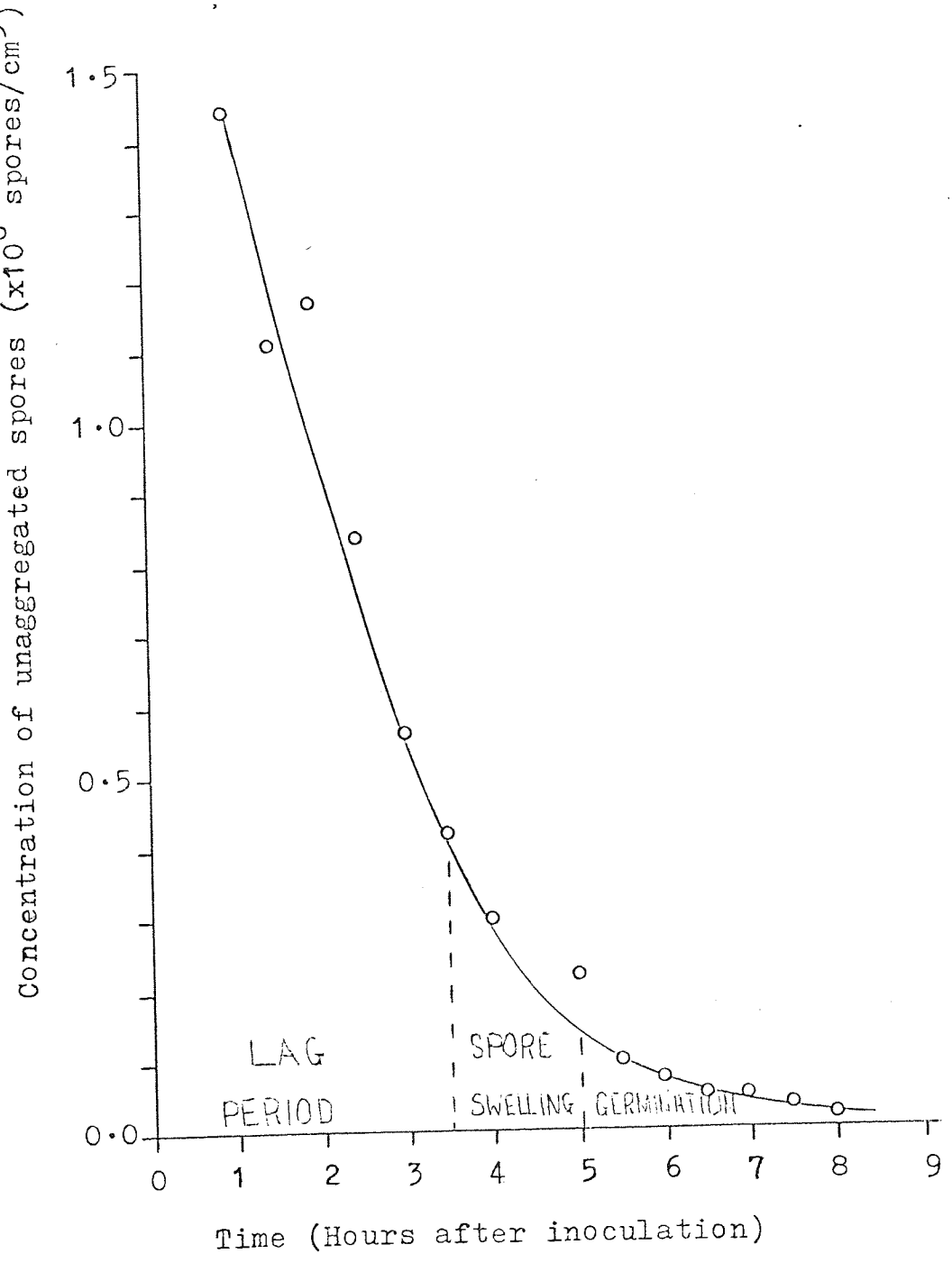
	V_s (cm^3)	t_s (h)	N_s	$\sum N_s$	W (%)
Experiment I9 $N_{so} = 1·0 \times 10^{10}$	43	0·4	$2·82 \times 10^8$	$2·82 \times 10^8$	2·8
	20	0·75	$2·24 \times 10^6$	$2·84 \times 10^8$	2·8
	4·5	2·2	$3·61 \times 10^6$	$2·88 \times 10^8$	2·9
	5	4·1	$1·24 \times 10^6$	$2·89 \times 10^8$	2·9
	7	7·3	0·0	$2·89 \times 10^8$	2·9
Experiment I8 $N_{so} = 7·7 \times 10^9$	41	0·5	$3·65 \times 10^9$	$3·65 \times 10^9$	47
	22	0·75	$5·43 \times 10^8$	$4·20 \times 10^9$	54
	2·5	1·25	$7·7 \times 10^7$	$4·27 \times 10^9$	55
	5	2·75	$1·8 \times 10^7$	$4·29 \times 10^9$	55
	4·5	4·75	$1·5 \times 10^7$	$4·31 \times 10^9$	56
Experiment I7 $N_{so} = 2·2 \times 10^{10}$	65	0·33	$4·2 \times 10^9$	$4·20 \times 10^9$	19
	30	0·67	$3·4 \times 10^8$	$4·54 \times 10^9$	20
	22	1·17	$3·2 \times 10^8$	$4·86 \times 10^9$	22
	2	2·67	$2·9 \times 10^6$	$4·86 \times 10^9$	22
Experiment I6 $N_{so} = 1·2 \times 10^9$	10	0·75	$5·07 \times 10^8$	$5·07 \times 10^8$	42
	2	1·75	$1·33 \times 10^7$	$5·20 \times 10^8$	43
	3	4·75	$1·13 \times 10^6$	$5·21 \times 10^8$	43
	1·5	6·25	$3·59 \times 10^5$	$5·22 \times 10^8$	43
	16·5	23·75	$6·52 \times 10^6$	$5·28 \times 10^8$	43

V_s = sample volume : t_s = sample time
N_s = total number of spores in sample (N_{so} at t_o)
W = fractional washout ie. N_s/N_{so}

APPENDIX 7·2

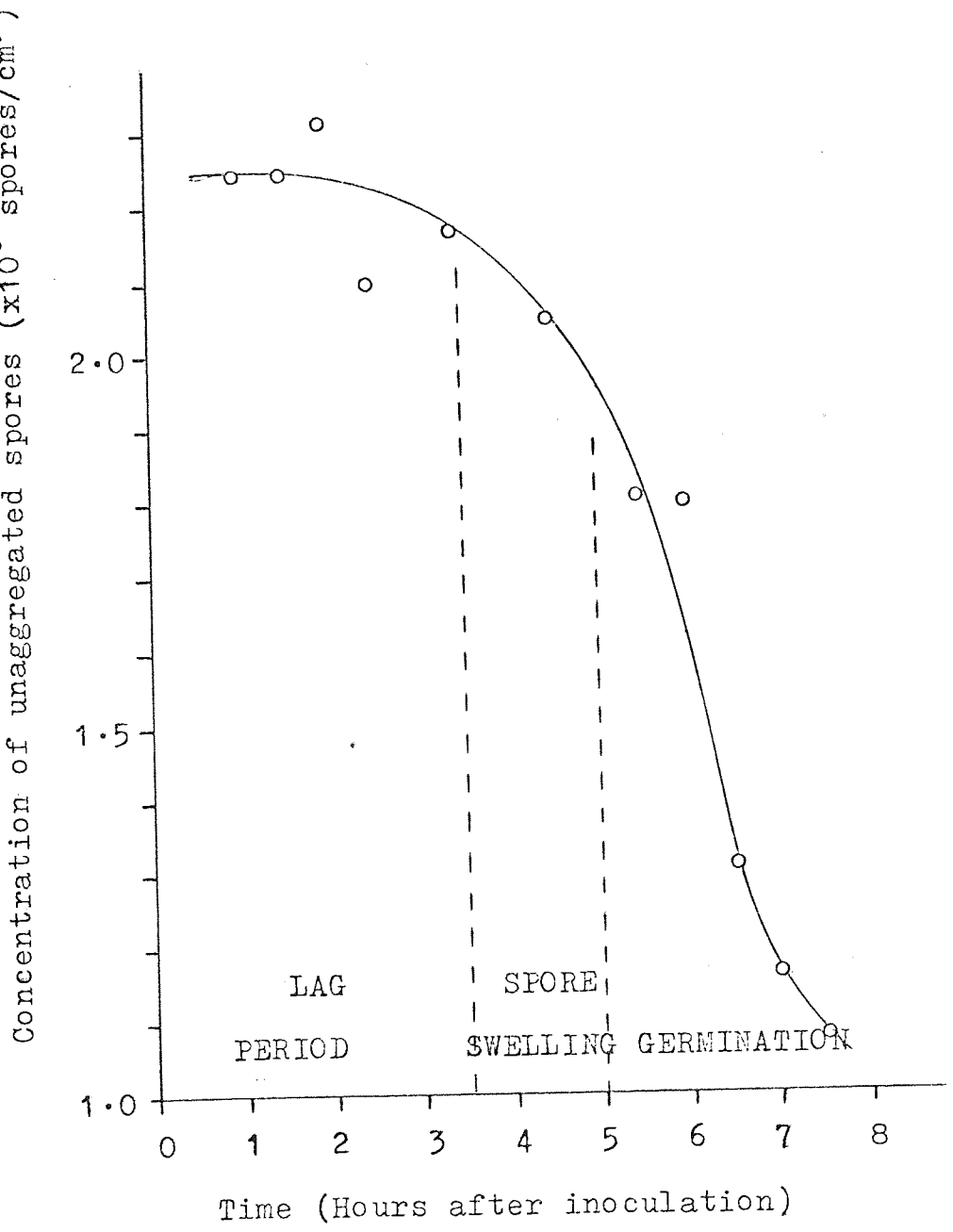
SPORE AGGREGATION - A.niger

(data from experiment I5)



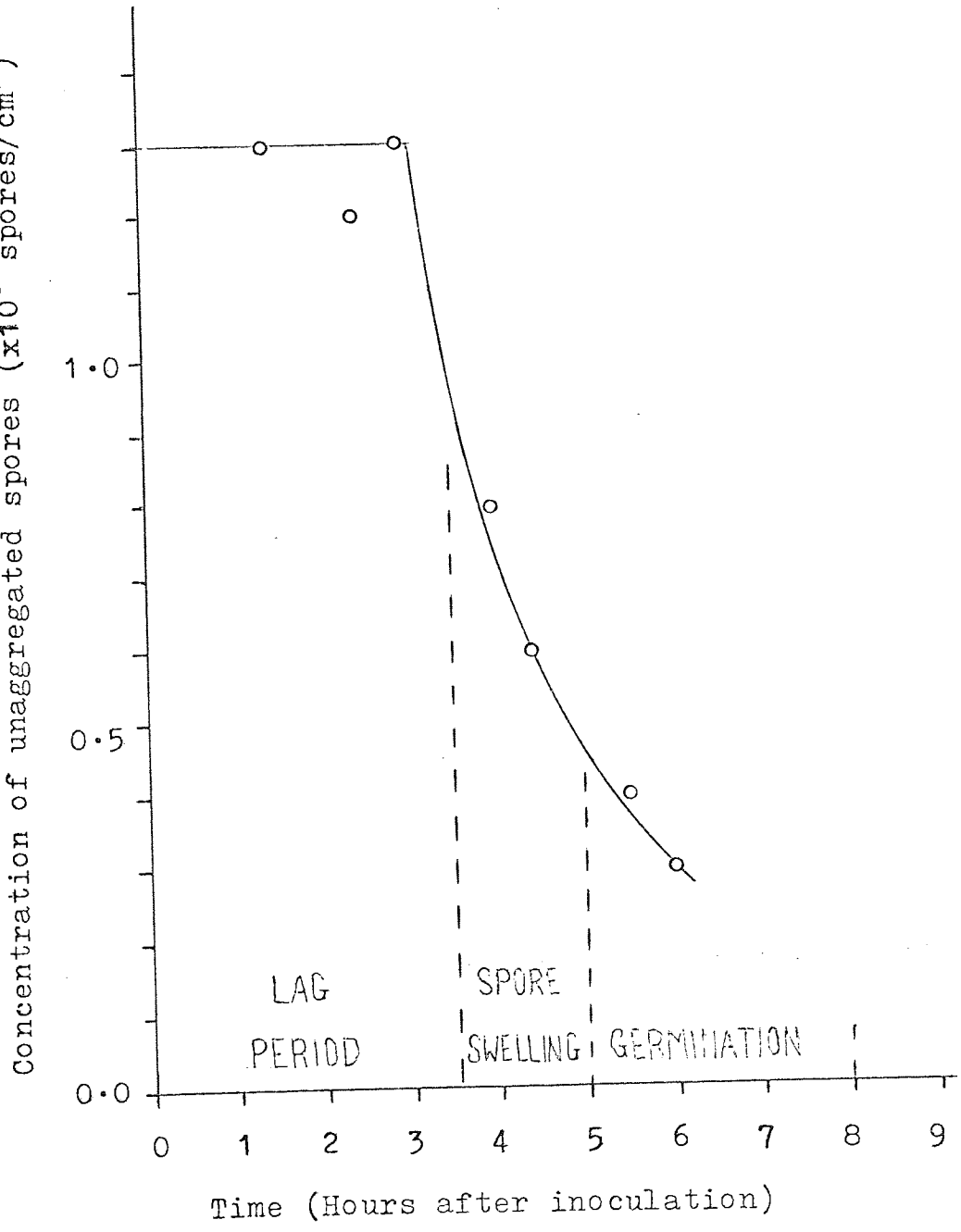
SPORE AGGREGATION - A.niger

(data from experiment I8)



SPORE AGGREGATION - A.niger

(data from experiment G2)



APPENDIX 7·3

AGGREGATE SIZE DISTRIBUTION

(Data from experiment I5, at 7·5 hours after inoculation)

$(d_a')_i$ (μm)	$(d_a)_i$ (μm)	N_i	$N_i/\Sigma N_i$ (%)	$N_i \cdot (d_a)_i^2$	$N_i \cdot (d_a)_i^3$
29·8	–	(3)	0·4	–	–
40·8	–	(11)	1·8	–	–
51·8	–	(24)	5·0	–	–
62·8	–	(47)	11·1	–	–
74·0	–	(42)	16·6	–	–
85·0	–	(55)	23·8	–	–
96·0	–	(86)	35·0	–	–
107·0	–	(73)	44·5	–	–
118·2	2·2	77	54·6	$3·7 \times 10^2$	$8·2 \times 10^2$
129·2	13·2	78	64·8	$1·4 \times 10^4$	$1·8 \times 10^5$
140·2	24·2	80	75·2	$4·7 \times 10^4$	$1·1 \times 10^6$
151·2	35·2	52	82·0	$6·4 \times 10^4$	$2·3 \times 10^6$
162·2	46·2	45	87·9	$9·6 \times 10^4$	$4·4 \times 10^6$
173·2	57·2	29	91·6	$9·5 \times 10^4$	$5·4 \times 10^6$
184·4	68·4	18	94·0	$8·4 \times 10^4$	$5·8 \times 10^6$
195·4	79·4	20	96·6	$1·3 \times 10^5$	$10·0 \times 10^6$
206·4	90·4	6	97·4	$4·9 \times 10^4$	$4·4 \times 10^6$
217·4	101·4	7	98·3	$7·2 \times 10^4$	$7·3 \times 10^6$
228·4	112·4	2	98·6	$2·5 \times 10^4$	$2·8 \times 10^6$
239·6	123·6	3	99·0	$4·6 \times 10^4$	$5·7 \times 10^6$
250·6	134·6	1	99·1	$1·8 \times 10^4$	$2·4 \times 10^6$
261·6	145·6	3	99·5	$6·4 \times 10^4$	$9·2 \times 10^6$
272·6	156·6	1	99·6	$2·5 \times 10^4$	$3·8 \times 10^6$
283·6	167·6	1	99·7	$2·8 \times 10^4$	$4·7 \times 10^6$
294·8	178·8	0	99·7	$0·0 \times 10^4$	0·0
305·8	189·8	2	100	$7·2 \times 10^4$	$13·7 \times 10^6$
TOTAL		766		$9·3 \times 10^5$	$8·3 \times 10^7$

$(d_a')_i$ = aggregate diameter, $(d_a)_i = (d_a' - 116)_i$, N_i = number

APPENDIX 8.1

COMPUTER PROGRAM TO SIMULATE SPORE AGGREGATION

```
C   NOMENCLATURE:
C     IMAX    - MAXIMUM NUMBER OF SPORES PER AGGREGATE
C     X(I)    - NUMBER OF AGGREGATES CONTAINING I SPORES
C     COL(K)  - NUMBER OF SUCCESSFUL COLLISIONS OF K AGGREGATES
C               WITH J AGGREGATES
C     CONST   - EMPIRICAL CONSTANT IN LEVICH EQUATION
C     D(I)    - DIAMETER
C     DELTAT  - TIME INTERVAL
C     U       - PARTICLE VELOCITY TERM IN LEVICH EQUATION
C     VOID    - VOIDAGE
C
      DIMENSION D(1000),X(1000),COL(1000)
      IMAX=1000
      PI = 3.14159
      NEND=1
      TIME = 4.
      READ(1,100) D(1),VOID,X(1),DELTAT,U,CONST
C
C   THIS SECTION SIMULATES AGGREGATION ACCORDING TO THE
C   LEVICH MODEL
C
      D1CUB=D(1)**3/(1-VOID)
      DO2 I=1,IMAX
      D(I)=(I*D1CUB)**(1./3.)
    2 CONTINUE
      WRITE(2,200) TIME,X(1)
    1 TIME = TIME+DELTAT
      IF(TIME.GE.8.) GO TO 8
      MAX=NEND*2
      IF(MAX.GE.IMAX)MAX=IMAX
      DO 6 I=1,MAX
      K=I
      COL(K) = 0.
      IF(K.EQ.1)GO TO 4
      KUT = K-1
      DO 3 J=1,KUT
      COL(K)=COL(K)+X(J)*X(K-J)*PI/4.*(2*D(K-J)+D(J))**2*U*CON
     1ST*DELTAT/2.
    3 CONTINUE
    4 DO 5J=1,NEND
      COL(K)=COL(K)-X(K)*X(J)*PI/4.*(2*D(J)+D(K))**2*U*CONST*
     1DELTAT
    5 CONTINUE
    6 CONTINUE
      DO 7 I=1,MAX
      X(I)=X(I)+COL(I)
    7 CONTINUE
      WRITE(2,300)TIME,X(1)
      NEND=MAX
      GO TO 1
    8 CONTINUE
```

```
C
C   THIS SECTION DETERMINES A CUMULATIVE SIZE DISTRIBUTION
C   OF THE AGGREGATES
C
      DSET=10.
      I=0
      XTOT=0.
      WRITE(2,350)
   11 I=I+1
      IF(DSET.GT.D(IMAX)) GO TO 13
      IF(D(I).GE.DSET) GO TO 12
      XTOT=XTOT+X(I)
       GO TO 11
   12 WRITE(2,400) DSET,XTOT
      DSET=DSET+10.
      XTOT=X(I)
      GO TO 11
   13 CONTINUE
  100 FORMAT(F7.5,F5.2,F9.1,F5.2,F6.1,F9.6)
  200 FORMAT(/////' MODEL OF SPORE AGGREGATION'//6X,'TIME',10X
     1,'UNAGGREGATED SPORES'//F10.2,5X,F10.0)
  300 FORMAT(/F10.2,5X,F10.0)
  350 FORMAT(////' SIZE DISTRIBUTION - DIAMETERS'/)
  400 FORMAT(/F5.0,5X,F8.0)
      STOP
      END
```

APPENDIX 8.2

COMPUTER PROGRAM FOR STRUCTURAL SIMULATION MODEL

```
C  NOMENCLATURE:  (DATA ONLY)

C     DA,DB   - BOUNDS OF CUMULATIVE DISTRIBUTION
C               (BRANCH POSITION)
C     DINT    - INTERVAL WIDTH (BRANCH POSITION)
C     ND      - NUMBER OF INTERVALS (BRANCH POSITION)
C     GA,GB   - BOUNDS OF CUMULATIVE DISTRIBUTION
C               (GERMINATION TIME)
C     GINT    - INTERVAL WIDTH (GERMINATION TIME)
C     NG      - NUMBER OF INTERVALS (GERMINATION TIME)
C     DIST    - CUMULATIVE DISTRIBUTION(BRANCH POSITION)
C     GIST    - CUMULATIVE DISTRIBUTION(GERMINATION TIME)
C     TIMEO,TSET - STARTING AND FINISHING TIMES FOR
C               SIMULATION
C     HLBUD   - HYPOTHETICAL HYPHAL LENGTH AT GERMINATION
C     SGRATE  - SPECIFIC GROWTH RATE
C     TINT    - TIME INTERVAL
C     MAGG    - SWITCH : <1 FOR INDIVIDUAL GROWTH
C                        >1 FOR AGGREGATE GROWTH
C               (IF <1, NSS MUST BE A POSITIVE INTEGER >1)
C     NSS     - NUMBER OF SPORES TO BE SIMULATED
C     MGERM   - SWITCH : <1 FOR SINGLE GERMINATION TIME
C                        >1 FOR GERMINATION TME RANGE
C     RAGG    - AGGREGATE RADIUS
C     RSPORE  - SPORE RADIUS
C     VOID    - VOIDAGE WITHIN AGGREGATE

      DIMENSION FLEN(1000),SLEN(1000),XF(1000),YF(1000),XS(100
     10),YS(1000),NUMBRF(1000),NUMBRS(1000),NDIRF(1000),NDIRS(
     21000),BRLF(1000),BRLS(1000),DIST(20),GIST(20)
      COMMON DA,DB,DINT,DIST
      READ(1,150) DA,DB,DINT,ND
      READ(1,150) GA,GB,GINT,NG
      READ(1,250)(DIST(I),I=1,ND)
      READ(1,250)(GIST(I),I=1,NG)
      READ(1,350) TIMEO,HLBUD,SGRATE,TINT,TSET
      READ(1,450)MAGG,MGERM,NSS
      CALL OPENGINOGP

C  PREPARATION FOR THE DRAWING OF AGGREGATE STRUCTURES
      IF(MAGG.LT.1) GO TO 10
      READ(1,550)RAGG,RSPORE,VOID
      CALL SHIFT2(400.,370.)
      PI=3.14159
      SS=PI*(RAGG-RSPORE)*(1-VOID)/RSPORE
      NSS=IFIX(SS)
      THETA= 2*PI/FLOAT(NSS)
      A=(RAGG-RSPORE)*COS(THETA)
      B=(RAGG-RSPORE)*SIN(THETA)
      OVERY=A-(RAGG-RSPORE)
      OVERX=-B
      ALPHA=360.*THETA/(PI*2.)
```

```
   10 CONTINUE
      CALL SCALE(0.25)
      DO 17 ILOTS = 1,NSS
      IF(MGERM.GT.1) CALL DISTRIBUTE(TIMEO,GA,GB,GINT,GIST)
      WRITE (2,500)
      WRITE(2,2000)TIMEO

C  INITIALIZATION
      DO 11 II = 1,20
      FLEN(II)=0.
      SLEN(II)=0.
      XF(II)=0.
      YF(II)=0.
      XS(II)=0.
      YS(II)=0.
      NUMBRF(II)=0.
      NUMBRS(II)=0.
      BRLF(II)=0.
      BRLS(II)=0.
      NDIRF(II)=0.
      NDIRS(II)=0.
   11 CONTINUE
      HLSUMF=0.
      HLSUMS=0.
      NGT=1
      FLEN(1)=HLBUD
      AGE=0.
      SEGL=0.
      NTOTF=1

    1 AGE=AGE+TINT
      TIME = AGE + TIMEO

C  BRANCHING AND SECONDARY GERM-TUBE FORMATION
      CALL BRANCH(SGRATE,TINT,FLEN,NTOTF,SEGL,TIME,NUMBRF,YF,X
     1F,BRLF,NDIRF,HLSUMF,4)
      IF(MAGG.GT.1)GO TO 3
      IF(NGT.NE.1) GO TO 2
      CHANCE = GTPA  (AGE,TINT)
      RANDOM = G05AAF(X)
      IF(RANDOM.GT.CHANCE)GO TO 3
      NGT = 2
      WRITE(2,100) TIME
      NTOTS=1
      SEGL = FLEN(1)/2
    2 CALL BRANCH(SGRATE,TINT,SLEN,NTOTS, 0.-SEGL,TIME,NUMBRS,
     1YS,XS,BRLS,NDIRS,HLSUMS,2)
    3 IF(TIME.LT.TSET)GOTO 1


      WRITE(2,600)
      THL = HLSUMF + HLSUMS
      WRITE(2,400)TIME,THL,NTOTF,NTOTS
      NOUGH = NTOTF
      DO 4  K=1,NOUGH
    4 WRITE(2,500) K,FLEN(K),SLEN(K)
```

```
C   CALCULATION OF BRANCH COORDINATES
      DO 5  K=1,NOUGH
      N=2*(K-1)+1
      XTEMP=FLEN(N)
      CALL COORDFIX(N,XTEMP,NTOTF,4,NUMBRF,YF,XF,BRLF,NDIRF,FL
     1EN)
    5 CONTINUE
      IF(MAGG.GT.1)GO TO 9
      NOUGH = NTOTS
      DO 6 K=1,NOUGH
      N=2*(K-1)+1
      XTEMP=SLEN(N)
      CALL COORDFIX(N,XTEMP,NTOTS,2,NUMBRS,YS,XS,BRLS,NDIRS,SL
     1EN)
    6 CONTINUE

      WRITE (2,700)
      DO 16 K=1,NTOTF
   16 WRITE(2,800) K,YF(K),XF(K)
      WRITE (2,900)
      DO 7 K=1,NTOTS
    7 WRITE(2,800) K,YS(K),XS(K)
      WRITE(2,600)

C   DETERMINATION OF SPACE REQUIRED FOR DRAWING
      CALL SMALLEST(XF,1,NTOTF,0,MIN)
      XMIN = XF(MIN)
      CALL SMALLEST(XS,1,NTOTS,0,MIN)
      XMINS= XS(MIN)
      IF(XMINS.LT.XMIN) XMIN=XMINS
      CALL SMALLEST(YF,1,NTOTF,0,MIN)
      YMIN=YF(MIN)
      CALL SMALLEST(YS,1,NTOTS,0,MIN)
      YMINS=YS(MIN)
      IF(YMINS.LT.YMIN) YMIN=YMINS
      XMIN=ABS(XMIN) +10.
      YMIN=ABS(YMIN) +10.
      CALL BIGGEST(XF,1,NTOTF,0,MAX)
      XMAX=XF(MAX)
      CALL BIGGEST(XS,1,NTOTS,0,MAX)
      XMAXS=XS(MAX)
      IF(XMAXS.GT.XMAX) XMAX=XMAXS
      XMAX=XMAX+10.
    8 CONTINUE

C   PRODUCTION OF GRAPHICAL OUTPUT
      CALL SHIFT2(XMIN,YMIN)
      CALL MOVTO2(0.,0.)
      CALL SYMBOL(7)
      CALL SPOREPLOT(NTOTF,NUMBRF,XF,YF)
      CALL MOVTO2(0.,0.)
      CALL SPOREPLOT(NTOTS,NUMBRS,XS,YS)
      CALL SHIFT2(100.,-YMIN)
      GO TO 17
    9 CALL MOVTO2(0.,0.)
      CALL SYMBOL(7)
      CALL SPOREPLOT(NTOTF,NUMBRF,XF,YF)
      CALL SHIFT2(OVERX,OVERY)
      CALL ROTAT2(ALPHA)
```

```
 17 CONTINUE
    CALL DEVEND
100 FORMAT(//' TIME:',F6.2,' HOURS  -  SECOND GERM TUBE INIT',
   1IATED.')
150 FORMAT(3F10.0,I3)
250 FORMAT(10F0.0)
300 FORMAT('1'///' GENERATION OF SPURELING STRUCTURE'/)
350 FORMAT(5F0.0)
400 FORMAT(/' SUMMARY:    AT TIME: ',F5.2,' HOURS THE TOTAL H',
   1YPHAL LENGTHIS ',F10.2,' MICRONS'//20X,'MAIN GERM-TUBE',
   220X,'SECOND GERM-TUBE'/20X,'(MICRONS)',25X,'(MICRONS)'/2
   32X,I5,29X,I5)
450 FORMAT(3I2)
500 FORMAT(/' SEGMENT ',I4,8X,F10.4,24X,F10.4)
550 FORMAT(3F0.0)
600 FORMAT(//100('*')//)
700 FORMAT(//100('*')//' COORDINATES FOR SPURELING BRANCHES ',
   1 AND END POINTS ARE :'//23X,'Y',19X,'X'/)
800 FORMAT(I5,2F20.2)
900 FORMAT(' SECOND GERM TUBE :')
2000 FORMAT(//' TIME:',F6.2,'HOURS  -  SPORE GERMINATED.')
     STOP
     END
```

```
      SUBROUTINE DISTRIBUTE(R,RA,RB,GAP,CDIST)

C   THIS ROUTINE CHOOSES A PSEUDO RANDOM NUMBER TO A SPECIFIED
C   DISTRIBUTION
C   THE ROUTINE CALLS ON THE NAG LIBRARY
      DIMENSION CLASS(20),CDIST(20)
    1 A=G05AAF(X)
      NOUGH =(RB-RA)/GAP
      CLASS(1)=RA
      DO 2 J=1,NOUGH
      K=J
      CLASS(J+1)=CLASS(J)+GAP
      IF(A.GE.CDIST(J).AND.A.LT.CDIST(J+1))GO TO 3
    2 CONTINUE
      WRITE(2,100) A
    3 R= CLASS(K) + GAP * G05AAF(X)
  100 FORMAT('1'////' NUMBER GENERATED L',F5.3,'J IS OUTSIDE R',
     1ANGE OF DISTRIBUTION'/' COMPLETE CUMULATIVE DISTRIBUTION',
     2 IS REQUIRED.')
      RETURN
      END
```

```
      SUBROUTINE BRANCH(SGR,TI,XLEN,NUM,BIT,T,NUMBR,YA,XA,BRL,
     1NDIR,HLSUM,IDIR)

C   THIS ROUTINE DECIDES IF A BRANCH IS FORMED AND ALSO
C   CHOOSES WHERE THE BRANCH WILL GO
      DIMENSION XLEN(1000),NUMBR(1000),YA(1000),XA(1000),BRL(
     11000),CDIST(20),NDIR(1000)
      COMMON RA,RB,GAP,CDIST
      NEXTRA =0
      HLSUM=0.
      N=NUM

C   CALCULATION OF HYPHAL SEGMENT LENGTHS
      FIRSTL=XLEN(1)-BIT
      XOLD=XLEN(1)
      FIRSTL=FIRSTL *EXP(SGR*TI)
      XLEN(1)=FIRSTL + BIT
      DO 4 I=1,N
      XPOS=0.
      J=I+NEXTRA
      IF(I.EQ.1) GO TO 1
      XOLD= XLEN(J)
      XLEN(J)= XLEN(J) *EXP(SGR*TI)
    1 HLSUM=HLSUM+XLEN(J)

C   POSITIONING NEW BRANCH
      IF(BUD(XLEN(J),XOLD,SGR).LT.1.0) GO TO 4
    3 TEMPLE = XLEN(J)
      NEXTRA = NEXTRA+1
      CALL DISTRIBUTE(R,RA,RB,GAP,CDIST)
      XPOS   =XLEN(J)*R
      XLEN(J) = XLEN(J) - XPOS
      IF((I.EQ.1).AND.(BIT.GT.0.)) BIT =0.
      CALL COORDFIX(J,XPOS,NUM,IDIR,NUMBR,YA,XA,BRL,NDIR,XLEN)
      IF (IDIR.EQ.2) GO TO 10
      WRITE(2,300) T,XPOS,J,TEMPLE
      GO TO 4
   10 WRITE(2,200) T,XPOS,J,TEMPLE
    4 CONTINUE
  200 FORMAT(//' TIME:',F6.2,' HOURS  -  BRANCH FORMED ON SECO',
     1ND GERM-TUBE AT',F4.0,' MICRONS ALONG SEGMENT ',I3,' WHI',
     2CH WAS',F4.0,' MICRONS LONG.')
  300 FORMAT(//' TIME:',F6.2,' HOURS  -  BRANCH FORMED ON MAIN',
     U GERM-TUBE AT',F4.0,' MICRONS ALONG SEGMENT ',I3,' WHICH',
     2 WAS ',F4.0,' MICRONS LONG.')
      RETURN
      END
```

```
      SUBROUTINE COORDFIX(I,BPI,NBTOT,NDI,NB,Y,X,XLB,ND,HL)

C   THIS ROUTINE FIXES THE COURDINATES OF ALL BRANCHES AND
C   THE POINTS AT WHICH THEY JOIN THE MAIN HYPHA
      DIMENSION XLB(1000),HL(1000),ND(1000), NB(1000),Y(1000),
     1X(1000)
      M=1
      NBTOT=NBTOT+1
      NBA=NB(I)+1
      NDJ =NDI
      BPA=BPI
      J=2
      DO 1 K=1,I
    1 J=J+NB(K)
      SB=0.

C   DETERMINATION OF BRANCH DIRECTION
      IF(J.EQ.2) GO TO 6
      IF(NBA.EQ.1) GO TO 5
      NBEG=J-NBA+1
      NFIN=J-1
      DO 2 K=NBEG,NFIN
      M=K
      IF(BPA.LT.XLB(K)) GO TO 3
    2 BPA=BPA-XLB(K)
      NB(I)=0
      NDJ=ND(M)
    4 IF (G05AAF(X).LT.0.5)NDJ=NDJ+2
      GO TO 6
    3 NBA=NBA-(J-M)
      NB(I)= J-M
      J=M
      SB=XLB(M)
      XLB(M) = XLB(M)-BPA
      NDJ=ND(M)+3
      GO TO 6
    5 M=0
      NBSUM=0
      DO 21 K=1,I
      KZ=(I-K)
      NBSUM = NBSUM+NB(KZ)
      IF(NBSUM.GT.M) GO TO 22
   21 M=M+1
   22 M=J-1-M
      NDJ=ND(M) +3
    6 GO TO (11,12,13,14,11,12,13,14,11),NDJ
   11 STY=Y(M)
      STX=X(M)+(BPA-SB)
      NDJ=2
      GO TO15
   12 STY=Y(M)-(BPA-SB)
      STX=X(M)
      NDJ=3
      GO TO15
   13 STY=Y(M)
      STX= X(M)-(BPA-SB)
      NDJ=4
      GO TO15
   14 STY=Y(M)+(BPA-SB)
      STX=X(M)
      NDJ=1
```

```
C   FIXING COORDINATES
   15 IF(NBTOT.EQ.J) GO TO8
      DO 7 K=1,(NBTOT-J)
      LI=(NBTOT-K)+1
      Y(LI)=Y(LI-1)
      X(LI)=X(LI-1)
      XLB(LI)=XLB(LI-1)
    7 ND(LI)=ND(LI-1)
    8 Y(J)=STY
      X(J)=STX
      XLB(J)=BPA
      ND(J)=NDJ
      DO 9 K=1,(NBTOT-I)
      L=(NBTOT-K)+1
      HL(L)=HL(L-1)
    9 NB(L)=NB(L-1)
      NB(I)=NBA
      HL(I)=BPI
      RETURN
      END


      SUBROUTINE SPUREPLOT(NT,NB,X,Y)

C   THIS ROUTINE ORGANISES THE PLOTTING OF BRANCHES AND
C   HYPHAE
      DIMENSION NB(1000),X(1000),Y(1000)
      NTOT =1
      J=2
      DO 1 L=1,NT-1,2
      I=L
      IF(I.EQ.1) GO TO 23
      J=J+NB(I-2)
      M=0
      NBSUM=0
      DO 21 K=1,I
      KZ=(I-K)
      NBSUM = NBSUM+NB(KZ)
      IF(NBSUM.GT.M) GO TO 22
   21 M=M+1
   22 M=J-1-M
      XM=X(M)
      YM=Y(M)
      CALL MOVTO2(XM,YM)
   23 N=NTOT+1
      NEND=NTOT+NB(I)
   24 DO 25 JA = N,NEND
      XM=X(JA)
      YM=Y(JA)
      CALL LINTO2(XM,YM)
   25 NTOT = NTOT+1
    1 CONTINUE
      RETURN
      END
```

```
      SUBROUTINE SMALLEST (ARRAY,I1,I2,L,J)

C  THIS ROUTINE DETERMINES THE SMALLEST FROM A SET OF NUMBERS
      DIMENSION ARRAY(1000)
      SMALL= ARRAY(I1)
      DO 1 M= I1,I2
      IF(ARRAY(M).GT.SMALL) GO TO 1
      SMALL = ARRAY(M)
      J=M
    1 CONTINUE
      RETURN
      END


      SUBROUTINE BIGGEST(ARRAY,I1,I2,L,J)

C  THIS ROUTINE DETERMINES THE BIGGEST FROM A SET OF NUMBERS
      DIMENSION ARRAY(1000)
      BIG=0.
      DO 1 M=I1,I2
      IF(ABS(ARRAY(M  )).LT.BIG)GO TO 1
    2 BIG=ABS(ARRAY(M  ))
      J=M
    1  CONTINUE
      RETURN
      END


      FUNCTION BUD(XN,XU,SMU)

C  THIS FUNCTION REPRESENTS THE CHANCE OF BRANCH FORMATION
      BUD = 0.0
      BI = 0.09 * XU/(SMU**2) -5.64
      K= IFIX(BI) +1
      IF(BI.LT.0.)K=1000
      BI = 0.09 * XN/(SMU**2) -5.64
      L = IFIX(BI)
      IF(L.GT.100) GO TO 2
    5 IF(L.LT.K) GO TO 1
      CHANCE = 1/(100.-K)
      RANDOM = 605AAF(X)
      K=K+1
      IF(RANDOM.GT.CHANCE) GO TO 5
    2 BUD = 1.0
    1 CONTINUE
      RETURN
      END


      FUNCTION GTPA(T,TINT)

C  THIS FUNCTION REPRESENTS THE CHANCE OF SECONDARY GERM-TUBE
C  FORMATION
      BEGIN = 2.0
      XEND = 8.7
      RANGE=XEND-BEGIN
      STEPS = RANGE/TINT
      X= (T-BEGIN)/TINT
      GTPA = 1./(STEPS-X)
      IF(T.LT.BEGIN)GTPA = 0.
      RETURN
      END
```

APPENDIX 8.3

COMPUTER PROGRAM FOR BIOMASS SIMULATION MODEL

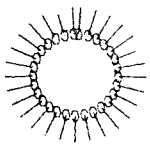
```
C  NOMENCLATURE
C
C     BIOMAS - DRY WEIGHT CONCENTRATION OF SOLIDS IN FERMENTER
C              [GRAMS/LITRE]
C
C     GCONC  - CONCENTRATION OF SPORES GERMINATING
C              [NUMBER/LITRE]
C
C     SGRATE - SPECIFIC GROWTH RATE [/HOUR]
C
C     SPCONC - SPORE CONCENTRATION IN TOWER [NUMBER/LITRE]
C
C     SPMASS - DRY WEIGHT OF A SPORE[GRAMS] (= 8.0E-11)
C
C     TGERM  - MEAN GERMINATION TIME [HOURS] (= 6.67)
C
C     TIME   - TIME FROM INOCULATION [HOURS]
C
C     TINT   - TIME INTERVAL [HOURS]
C
C     TSET   - TIME AT WHICH SIMULATION ENDS [HOURS]
C
C     VEGMAS - DRY WEIGHT OF HYPHAE [GRAMS]
C
C     VEGO   - THEORETICAL DRY WEIGHT OF HYPHAE PER SPORE AT
C              GERMINATION [GRAMS] (=0.614E-10, AT 25 MICRONS)
C
C*************************************************************
C
      READ(1,100)TSET,TINT,SPCONC,SGRATE
      WRITE(2,150)SGRATE,SPCONC
      SPMASS=8.0E-11
      VEGO=6.14E-11
      TGERM= 6.67
      IA=0
      IW=0
      TIME=0.
C
    1 TIME=TIME+TINT
      IF((TIME.GT.1.).AND.(IW.LT.1)) CALL WASHOUT(SPCONC,IW)
      IF((TIME.GE.4.).AND.(IA.LT.1))CALL AGGREGATE(IA,SPCONC,
     1GCONC)
      IF(TIME.GE.TGERM)CALL VEGROWTH(VEGMAS,VEGO,SGRATE,TIME,
     1TGERM,TINT,GCONC)
      BIOMAS=SPMASS*SPCONC+VEGMAS*GCONC
      WRITE(2,250)TIME,BIOMAS
      IF(TIME.LE.TSET)GO TO 1
C
  100 FORMAT(F4.1,F4.2,E8.2,F4.2)
  150 FORMAT('1'////' BIOMASS SIMULATION'//' SPECIFIC GROWTH'
     1,' RATE = ',F4.2/' INOCULUM CONCENTRATION = ',1PE8.2
     2////' TIME [HOURS]    DRY WEIGHT [GRAMS/LITRE]'/)
  250 FORMAT(/,5X,F4.1,17X,F5.2)
      STOP
      END
```

```
      SUBROUTINE WASHOUT(SPCONC,I)
C
C   THIS ROUTINE SIMULATES THE EFFECT OF WASHOUT ON THE NUMBER
C   OF SPORES IN THE FERMENTER
C
C     FRWASH - FRACTIONAL WASHOUT (= 0.5)
C
      I=1
      FRWASH = 0.5
      SPCONC=SPCONC*FRWASH
      RETURN
      END

      SUBROUTINE AGGREGATE(I,SPCONC,GCONC)
C
C   THIS ROUTINE SIMULATES THE EFFECT OF AGGREGATION ON THE
C   NUMBER OF SPORES GERMINATING
C
C     AGGNUM - MEAN NUMBER OF SPORES PER AGGREGATE (= 425)
C
C     AGGS   - NUMBER OF AGGREGATES FORMED
C
C     AGGTIM - TIME OVER WHICH AGGREGATION OCCURS (HOURS)
C              (= 4.0)
C
C     CFINAL - CONCENTRATION OF UNAGGREGATED SPORES AT END OF
C              AGGREGATION PERIOD (NUMBER/LITRE)
C
C     CONST  - SPECIFIC RATE CONSTANT FOR AGGREGATION EQUATION
C     CSURF  - CONCENTRATION OF SURFACE SPORES IN FERMENTER
C
C              (NUMBER/LITRE)
C
C     SMEANS - MEAN NUMBER OF SURFACE SPORES PER AGGREGATE
C              (= 110)
C
      AGGTIM =4.
      AGGNUM=425.
      SMEANS= 110.
      I=1
      CONST= 0.5E-03
      CFINAL = (1./(CONST*AGGTIM+(1./SPCONC**0.4)))**2.5
      AGGS=(SPCONC-CFINAL)/AGGNUM
      CSURF=AGGS*SMEANS
      GCONC=CSURF+CFINAL
      RETURN
      END

      SUBROUTINE VEGROWTH(VEGMAS,VEGO,SGRATE,TIME,TGERM,TINT,
     1GCONC)
C
C   THIS ROUTINE SIMULATES THE EFFECT OF GROWTH ON THE BIOMASS
C   IN THE FERMENTER
C
      IF(VEGMAS.GT.(1.12/GCONC))GO TO 1
      VEGMAS=VEGO*EXP(SGRATE*(TIME-TGERM))
      GO TO 2
    1 VEGMAS = VEGMAS + 0.504*TINT/GCONC
    2 RETURN
      END
```

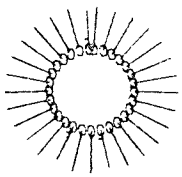
APPENDIX 8·4

COMPUTER SIMULATION OF COLONY GROWTH IN A.NIGER

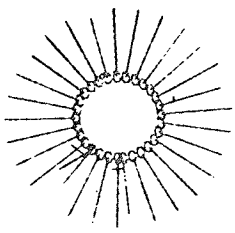
(Scale = 100 : 1)



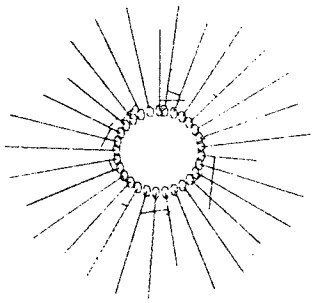
1 hour after germination



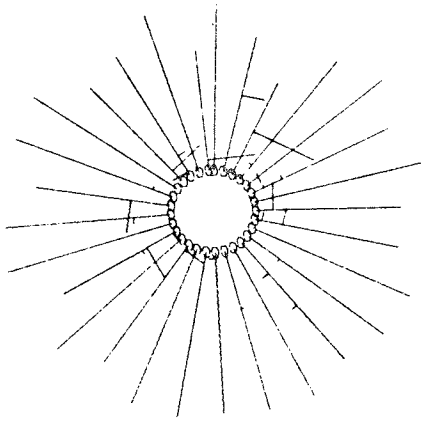
2 hours after germination



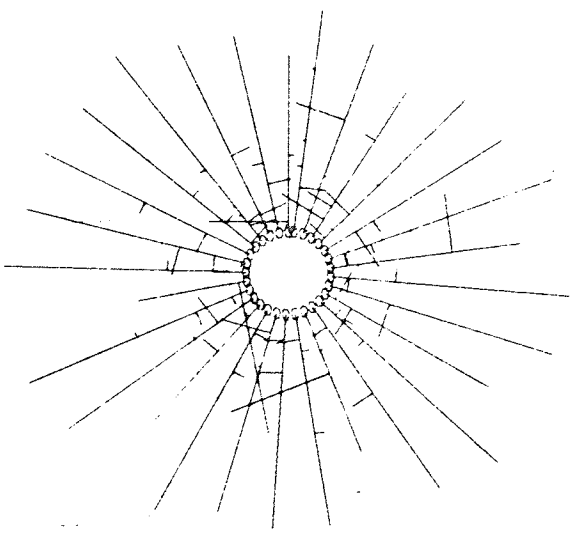
3 hours after germination



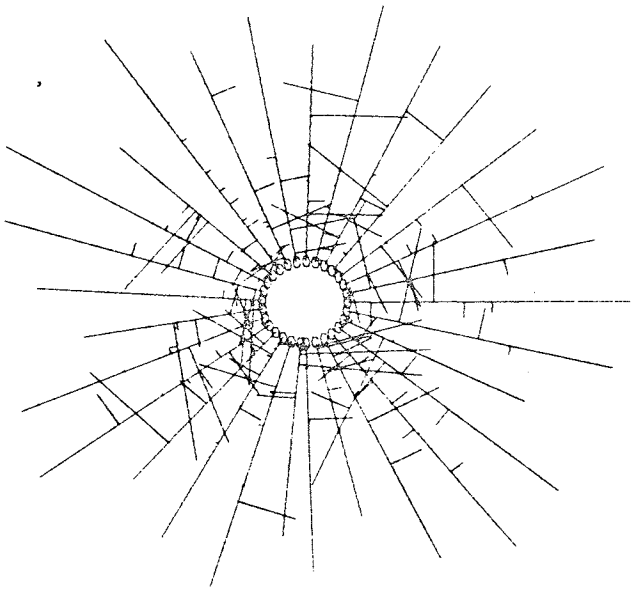
4 hours after germination



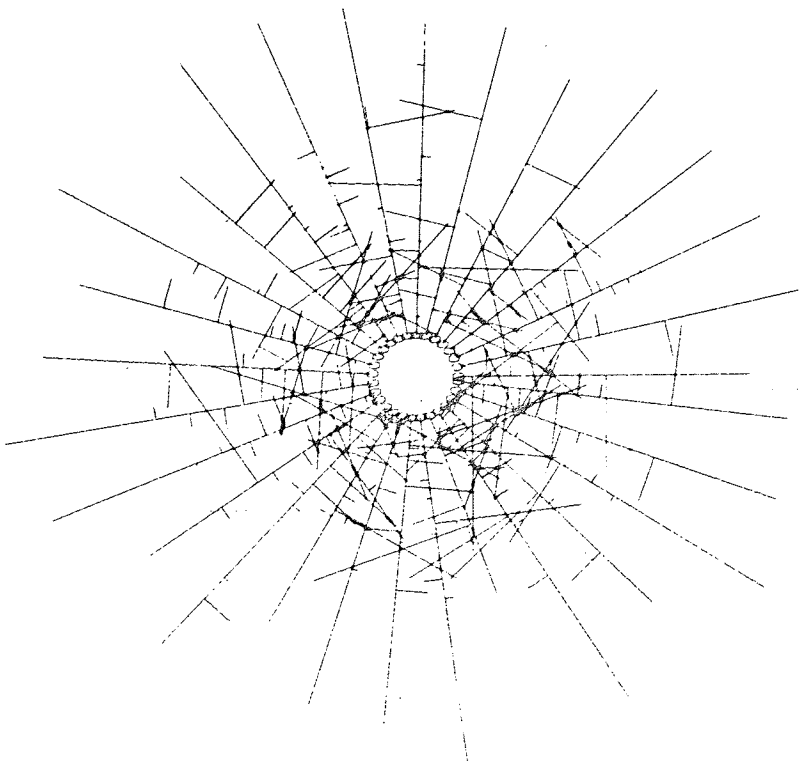
5 hours after germination



6 hours after germination

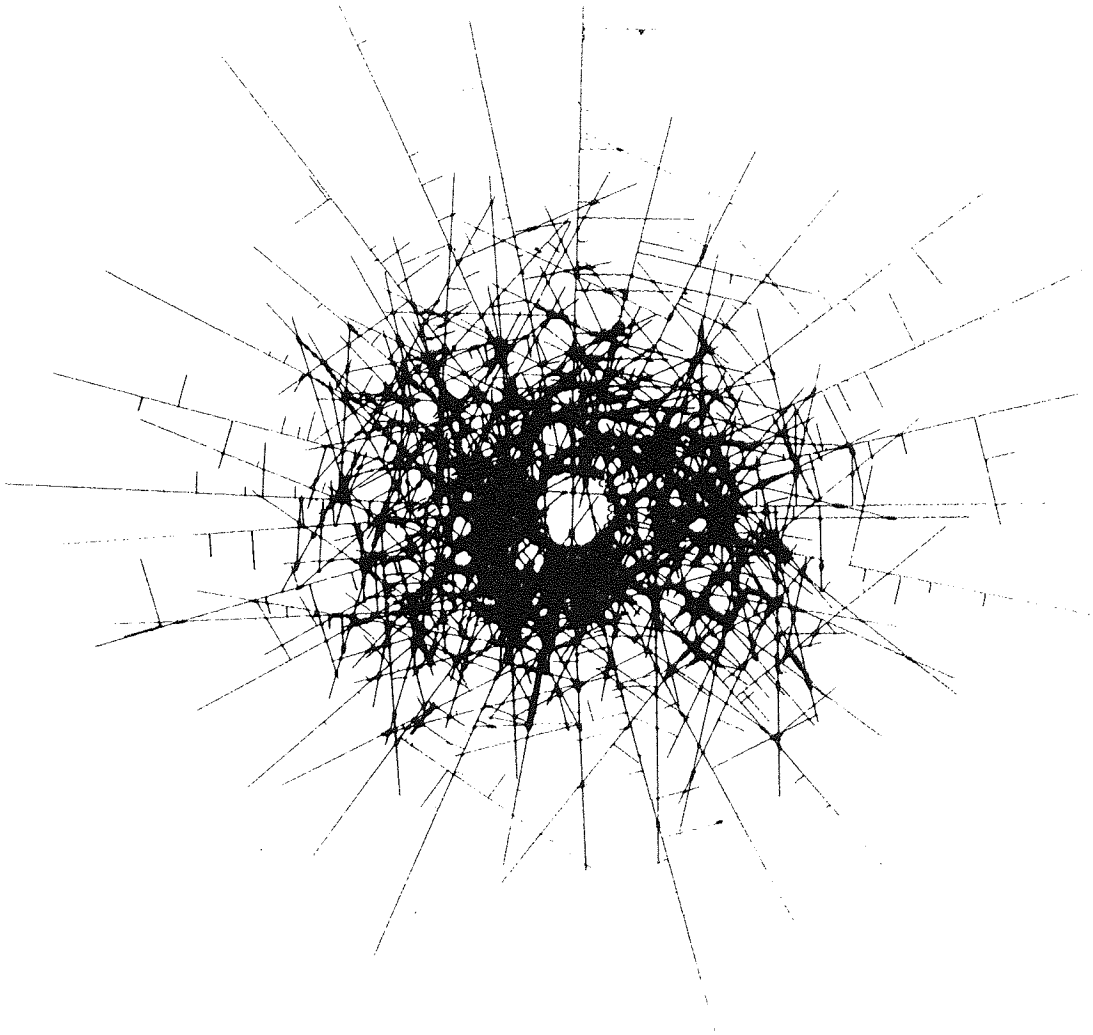


7 hours after germination

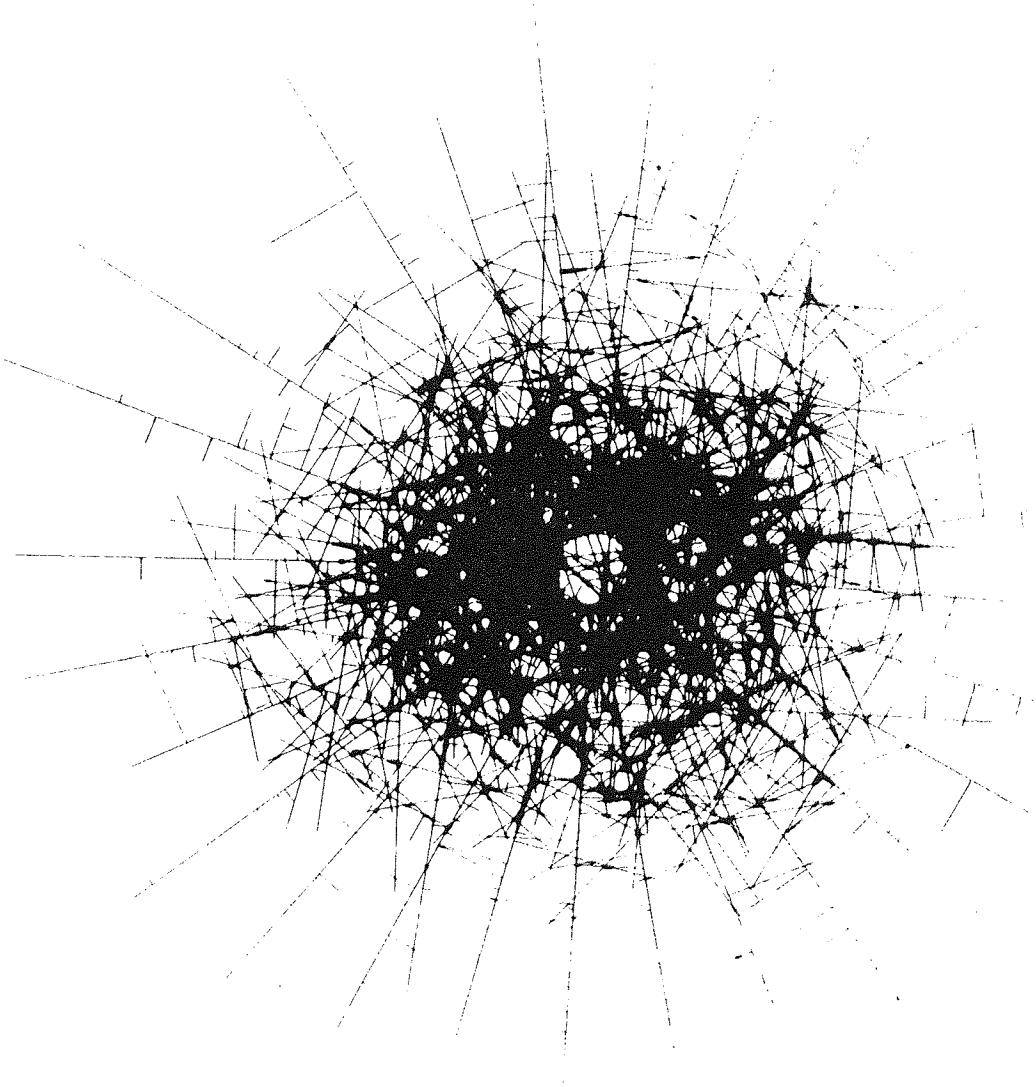


8 hours after germination

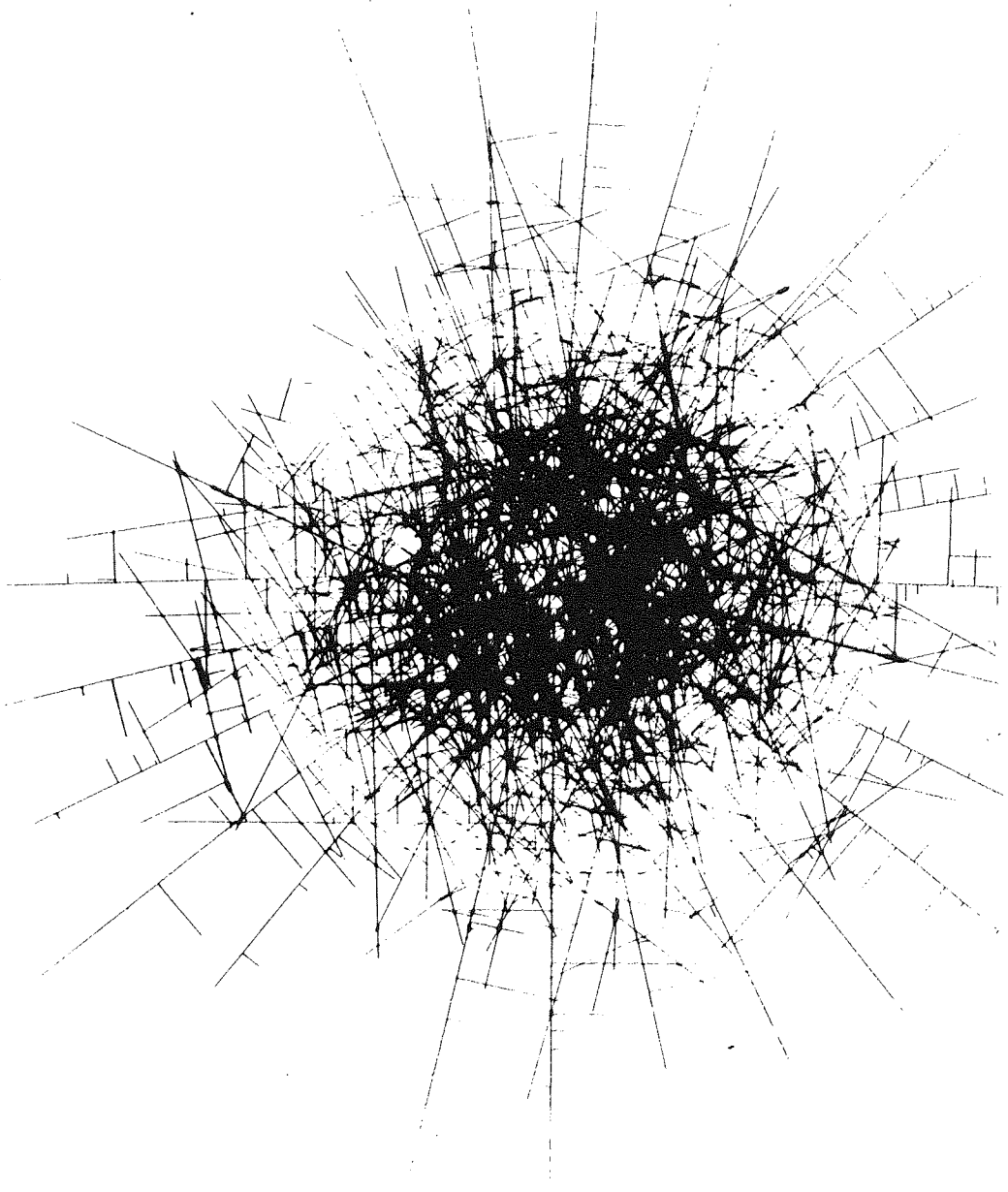
11 hours after germination



12 hours after germination



13 hours after germination



REFERENCES

ALLEN, P.J. (1965). Metabolic Aspects of Spore Germination in Fungi. Annual Review of Phytopathology 3, 313-342.

ATKINSON, B. & DAOUD, I.S. (1976). Microbial Flocs and Flocculation in Fermentation Process Engineering. Advances in Biochemical Engineering 4, 42-121.

BARTNICKI-GARCIA, S. (1968). Cell-wall Chemistry, Morphogenesis and Taxonomy of Fungi. Annual Review of Microbiology 22, 87-108.

BARTNICKI-GARCIA, S. & LIPPMAN, E. (1969). Fungal Morphogenesis - Cell-wall Construction in Mucor rouxii. Science 165, 302-304.

BLAKEBROUGH, N. (1967). Biochemical and Biological Engineering Science - Vol. 1. Academic Press. (London).

BORROW, A., BROWN, S., JEFFREYS, E.G., KESSELL, R.H.J., LLOYD, E.C., LLOYD, P.B., ROTHWELL, A., ROTHWELL, B., & SWAIT, J.C. (1964). The Kinetics of Metabolism of Gibberella fujikuroi in Stirred Culture. Canadian Journal of Microbiology 10, 407-443.

BOYLE, W.C., CRABTREE, K., IACCARINO, E.P., LIGHTFOOT, E.N. & ROHLICH, G.A. (1968). Flocculation Phenomena in Biological Systems. in Advances in Water Quality Improvement. pp.287-313. Eds. E.F. GLOYNA & W.W. ECKENFELDER. University of Texas Press.

BURKHOLDER, P.R. & SINNOTT E.W. (1945). Morphogenesis of Fungus Colonies in Submerged Shaken Cultures. American Journal of Botany 32, 424-431.

BURNETT, J.H. (1976). Fundamentals of Mycology - 2nd Ed. Edward Arnold (London).

BUTLER, G.M. (1966). Vegetative Structures. In The Fungi Vol. 2 pp.83-112. Eds. G.C. AINSWORTH & A.S. SUSSMAN. Academic Press (London).

CALAM, C.T. (1969). The Evaluation of Mycelial Growth. In Methods in Microbiology - vol. 1 pp.567-593. Eds. J.R. NORRIS & D.W. RIBBONS. Academic Press (London).

CALAM, C.T. (1976). Starting Investigational and Production Cultures. Process Biochemistry 11, (3), 7-12.

CALDWELL, I.Y. & TRINCI, A.P.J. (1973). The Growth Unit of the Mould Geotrichum candidum. Archives of Microbiol. 88, 1-10.

CAMICI, L., SERMONTI, G. & CHAIN, E.B. (1952). Observations on Penicillium chrysogenum in Submerged Culture. World Health Organisation 6, 265-275.

CHOUDHARY, A.Q. & PIRT, S.J. (1965). Metal Complexing Agents as Metal Buffers in Media for the Growth of Aspergillus niger. Journal of General Microbiology 41, 99-107.

COCKER, R. (1975). The Morphological Development of Aspergillus niger in Tower Fermenter Culture. Ph.D. Thesis, University of Aston in Birmingham.

COCKER, R. & GREENSHIELDS, R.N. (1977). Fermenter Cultivation of Aspergillus. In Genetics and Physiology of Aspergillus pp.361-390. Eds. J.E. SMITH & J.A. PATEMAN.

DOWEN A.W. (1979). Oxygen Mass Transfer in Tower Fermenters. Ph.D. Thesis, University of Aston in Birmingham.

DUCKWORTH, R.B. & HARRIS, G.C.M. (1949). The Morphology of Penicillium chrysogenum in Submerged Culture Fermentations. Transactions of the British Mycological Society 32, 224-235.

EKUNDAYO, J.A. & CARLILE, M.J. (1964). The Germination of Sporangiospores of Rhizopus arrhizus : Spore Swelling and Germ-tube Emergence. Journal of General Microbiology 35, 261-269.

EMERSON, S. (1950). The Growth Phase in Neurospora Corresponding to the Log Phase in Unicellular Organisms. Journal of Bacteriology 60, 221-223.

FIDDY, C. & TRINCI, A.P.J. (1976a). Mitosis, Septation, Branching and the Duplication Cycle in Aspergillus nidulans. Journal of General Microbiology 97, 169-184.

FIDDY, C. & TRINCI, A.P.J. (1976b). Nuclei, Septation, Branching and Growth of Geotrichum candidum. Journal of General Microbiology 97, 185-192.

FLOWERS, T.H. & WILLIAMS, S.T. (1977). Measurement of Growth Rates of Streptomycetes : Comparison of Turbidimetric and Gravimetric Techniques. Journal of General Microbiology 98, 285-289.

FOSTER, W.J. (1949). Chemical Activities of Fungi. Academic Press (London).

GABINSKAYA, K.N. (1976). Influence of the Composition of the Nutrient Medium on the Nature of the Growth of Tieghemella hyalospora. Microbiology 45, 281-283.

GALBRAITH, J.C. & SMITH J.E. (1969). Filamentous Growth of Aspergillus niger in Submerged Shake Culture. Transactions of the British Mycological Society 52, 237-246.

GEMMELL, R.S. (1975). Modelling and Simulation of the Aggregation of Suspensions. In Mathematical Modelling for Water Pollution Control Processes. pp.67-85. Eds. KEINATH & M.P. WANIELISTA. Ann Arbor Science Publishers.

GOODAY, G.W. (1971). An Autoradiographic Study of Hyphal Growth of Some Fungi. Journal of General Microbiology 67, 125-133.

GRAY, W.A. (1968). The Packing of Solid Particles. Chapman & Hall Ltd. (London).

GREENSHIELDS, R.N. & SMITH, E.L. (1971). Tower Fermentation Systems and Their Applications. The Chemical Engineer 249, 182-190.

GULL, K. (1975). Mycelial Branching Patterns of Thamnidium elegans. Trans. British Mycological Society 64, 321-363.

HAWKER, L.E. (1966). Germination : Morphological and Anatomical Changes. In The Fungus Spore. Ed. M.F. MADELIN.

HONDA, H. (1971). Description of the Form of Trees by the Parameters of the Tree-like Body. Journal of Theoretical Biology 31, 331-338.

JAMES, A. (1973). The Properties of Microbial Aggregates and their Behaviour in Tower Fermenters. Ph.D. Thesis, University of Aston in Birmingham.

KATZ, D., GOLDSTEIN, D. & ROSENBERGER, R.F. (1972). Model for Branch Initiation in Aspergillus nidulans Based on Measurements of Growth. Journal of Bacteriology 109, 1097-1100.

KIER, I., ALLERMANN, K., FLOTO, F., OLSEN, J. & SORTKJAER, O. (1976). Changes of Exponential Growth Rates in Relation to Differentiation of Geotrichum candidum in Submerged Culture. Physiologica Plantarum 38, 6-12.

KUBITSCHEK, H.E. (1970). Evidence for the Generality of Linear Cell Growth. Journal of Theoretical Biology 28, 15-29.

LEOPOLD, L.B. (1971). Trees and Streams - The Efficiency of Branching Patterns. Journal of Theoretical Biology 31, 339-354.

LEVICH, V.G. (1962). Physico-chemical Hydrodynamics. Edgewood Cliffs, Prentice-Hall (London).

MACHEK, F. & FENCL, Z. (1973). Differentiation as a Basis for the Understanding of Product Formation. Biotechnology and Bioengineering Symposium Series 4, 129-142.

MACHLIS, L. (1957). Factors Affecting the Lag Phase of Growth of the Filamentous Fungus Allomyces macrogynus. American Journal of Botany 44, 113-119.

MANDELS, G.R. (1965). Kinetics of Fungal Growth. In The Fungi - vol. 1. Eds. G.C. AINSWORTH & A.S.SUSSMAN. Academic Press (London). pp. 599-612.

MANNERS, J.G. (1966). Assessment of Germination. In The Fungus Spore, pp165-174. Ed. M.F. MADELIN.

MARSHALL, K.C. & ALEXANDER, M. (1960). Growth Characteristics of Fungi and Actinomycetes. Journal of Bacteriology 80, 412-416.

MARTIN, J.F., URUBURU, F. & VILLANUEVA, J.R. (1973). Ultrastructural Changes in the Conidia of Penicillium notatum during Germination. Canadian Journal of Microbiology 19, 797-801.

METZ, B. (1976). From Pulp to Pellets. Ph.D. Thesis, Delft, Holland.

METZ, B. & KOSSEN, N.W.F. The Growth of Molds in the Form of Pellets. Biotechnology and Bioengineering 19, 781-799.

MEYRATH, J. & SUCHANEK, G. (1972). Inoculation Techniques - Effects Due to Quality and Quantity of Inoculum. In Methods in Microbiology, vol. 7b pp.159-209. Eds. J.R. NORRIS & D.W. RIBBONS. Academic Press (London).

MITHCHISON, J.M. (1963). The Cell Cycle of a Fission Yeast. Symposia for the International Society for Cell Biology 2, 151-167.

MOORE-LANDECKER (1972). Fundamentals of the Fungi. Prentice-Hall (Hemel Hempstead).

MORRIS, G.G. (1972). The Kinetics of Mould Growth in Aerobic Tower Fermenters. Ph.D. Thesis, University of Aston in Birmingham.

PIRT, S.J. (1966). A Theory of the Mode of Growth of Fungi in the Form of Pellets in Submerged Culture. Proceedings of the Royal Society - B 166, 369-373.

PIRT, S.J. & CALLOW, D.S. (1960). Studies of the Growth of Penicillium chrysogenum in Continuous Flow Culture With Reference to Penicillin Production. Journal of Applied Bacteriology 23, 87-98.

PLOMLEY, N.J.B. (1959). Formation of the Colony in the Fungus Chaetomium. Australian Journal of Biological Science 12, 53-64.

PROSSER, J.I. & TRINCI, A.P.J. (1979). A Model for Hyphal Growth and Branching. Journal of General Microbiology 111, 153-164.

ROBERTSON, N.F. (1965). The Mechanism of Cellular Extension and Branching. In The Fungi, vol. 1, pp. 613-623. Eds. G.C. AINSWORTH & A.S.SUSSMAN. Academic Press (London).

ROBERTSON, N.F. (1968). The Growth Process in Fungi. Annual Review of Phytopathology 6, 115-136.

SMITH, E.L. & GREENSHIELDS, R.N. (1974). Tower Fermentation Systems and Their Application to Aerobic Processes. The Chemical Engineer 281, 28-34.

SMITH, J.E. (1975). The Structure and Development of Filamentous Fungi. In, The Filamentous Fungi vol. 1, pp.1-15. Eds. J.E. SMITH & D.R. BERRY. Edward Arnold.

SMITH, J.E. & GALBRAITH, J.C. (1971). Biochemical and Physiological Aspects of Differentiation in the Fungi. Advances in Microbial Physiology 5, 45-134.

SMITH, J.H. (1923). On the Apical Growth of Fungal Hyphae. Annals of Botany 37, 341-343.

SMITH, J.H. (1924). On the Early Growth Rate of the Individual Fungus Hyphae. New Phytologist 23, 65-79.

SMOLUCHOWSKI M. (1916). Drei Vortrage Uber Diffusion, Brownsche Molekulerbewegung und Koagulatim von Kolloid-teilchen. Physik Z. 17, 557.

SMOLUCHOWSKI, M. (1917). Versuch einer Mathematiscen Theorie der Koagulationskinetic Losungun. Z. Physik Chem. 92, 129.

STEEL, R. MARTIN, S.M. & LENTZ, C.P. (1954). A Standard Inoculum fo Citric Acid Production in Submerged Culture. Canadian Journal of Microbiology 1, 150-157.

STEEL, R., MARTIN, S.M. & LENTZ, C.P. (1955). Submerged Citric Acid Fermentation of Sugar Beet Molases : Increase in Scale. Canadian Journal of Microbiology 1, 299-311.

TRINCI, A.P.J. (1969). A Kinetic Study of the Growth of Aspergillus nidulans and Other Fungi. Journal of General Microbiology 57, 11-24.

TRINCI, A.P.J. (1970a). Kinetics of Apical and Lateral Branching in Aspergillus nidulans and Geotrichum lactis. Transactions of the British Mycological Society 55, 17-28.

TRINCI, A.P.J. (1970b). Kinetics of the Growth of Mycelial Pellets of Aspergillus nidulans. Archiv fur Mikrobiologie 73, 353-367.

TRINCI, A.P.J. (1971a). Influence of the Width of the Peripheral Growth Zone on the Growth Rate of Fungal Colonies on Solid Media. Journal of General Microbiolgy 67, 325-344.

TRINCI, A.P.J. (1971b). Exponential Growth of the Germ-tubes of Fungal Spores. Journal of General Microbiology 67, 345-348.

TRINCI, A.P.J. (1972). Culture Turbidity as a Measure of Mould Growth. Transactions of the British Mycological Society 58, 467-473.

TRINCI, A.P.J. (1973). The Hyphal Growth Unit of Wild Type and Spreading Colonial Mutants of Neurospora crassa. Archives of Microbiology 91, 127-136.

TRINCI, A.P.J. (1974). A Study of the Kinetics of Hyphal Extension and Branch Initiation of Fungal Mycelia. Journal of General Microbiology 81, 225-236.

TRINCI, A.P.J. & BANBURY, G.H. (1967). A Study of the Growth of the Tall Conidiophores of Aspergillus giganteus. Trans. of the Brit. Mycol. Soc. 50, 525-538.

TRINCI, A.P.J. & COLLINGE, A.J. (1974). Occlusion of the Septal Pores of Damaged Hyphae of Neurospora crassa. Protoplasma 80, 57-67.

VAN SUIJDAM, H. (1979). Private Communication.

VOLD, M.J. (1963). Computer Simulation of Floc Formation in a Colloid Suspension. Journal of Colloid Science 18, 684-695.

WHITAKER, A. & LONG, P.A. (1973). Fungal Pelleting. Process Biochemistry 8, (nov), 27-31.

YANAGITA, T. (1957). Biochemical Aspects on the Germination of Conidiospores of Aspergillus niger. Archiv fur Mikrobiologie 26, 329-344.

ZALOKAR, M. (1959). Enzyme Activity and Cell Differentiation in Neurospora. American Journal of Botany 46, 555-559.