Hyphal Paramorphs of Ceratocystis adiposa. (Butl)

Thesis submitted for the degree of

Doctor of Philosophy

in the University of London

by

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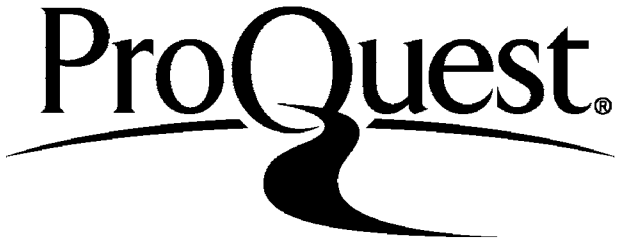
1980.



ABSTRACT.

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Elisabeth J. Skone.

The aim of this project was to study the biochemical and cell wall changes involved during the induction of paramorphs of the ascomycete fungus Ceratocystis adiposa by sorbose and bile salts and to find how early these effects could be detected during the formation of a mycelium from spores.

It was found that the reduction in radial growth rate (Kr) by sorbose was an osmotic effect. The rate of increase in dry weight with time was linear and not significantly reduced by the concentration of sorbose, glucose or polyethylene glycol which halved Kr ; 0.25M. Botrytis fabae was sensitive to much lower concentrations of sorbose, Kr being halved at 0.01M sorbose and 1.0M glucose. The bile salt, sodium deoxycholate, caused a specific 50% reduction in Kr at 0.25×10^{-4} M without reducing as growth rate In the paramorphs, Kr reduction was correlated with increased branch frequency.

Electron micrographs of the cell wall showed no differences between the normal and restricted forms : the cell wall had three layers . The structure of the hyphae and especially of the septum was similar to that found in the human pathogen Sporothrix schenckii which has been proposed as an imperfect stage of Ceratocystis (Nicot & Mariat,1973). Ungerminated and inhibited conidia showed the same cytoplasmic and cell wall structure reported by Hawes (1979). The cytoplasm contained many lipid droplets as energy reserves and these were not present in germinated conidia.

Chemical analysis of the cell walls revealed that when Kr was reduced to 50% by 0.25M sorbose and PEG* 200, the glucan content of the cell walls was decreased. Glucose increased the glucan level while Na deoxycholate caused only a slight decrease. The only sugars found on hydrolysis of the cell wall fractions were glucose, mannose and N-acetyl glucosamine. Melanin in the cell wall prevented extensive enzyme dissolution.

Only Na deoxycholate inhibited germination (50% at 7.5×10^{-4} M) although reversible inhibition occurred at concentrations of PEG* which prevented growth (1.0M). When Na deoxycholate was used in glucose solution, the conidia seemed to swell and show nuclear division earlier than those incubated in SSM.

*PEG = Polyethylene glycol SSM = Standard synthetic medium

ACKNOWLEDGMENTS.

My thanks are due to Professor K. Wilson and all the members of the Botany Department at Royal Holloway College who taught, encouraged and helped me during my seven years' of study.

I would like to thank especially my supervisor, Mr. P.A. Dixon, for his help in preparing this thesis and Dr. J.E. Kerr for her help as my tutor.

The photographs were taken and prepared by Mr. D. Ward and Miss L. Etherington. Miss C. Spurdon and Mrs. G. Anderson helped me with electron microscopy, Dr. J. Evans and Mrs. P. Andrews with using the Coulter Counter, Miss R. Mummery for liquid scintillation counting and Mrs. M. Frazer who made sure we kept the laboratories tidy.

Finally, I would like to thank Mrs. C.A. Sampson for typing the thesis.

This work was supported by studentships from the Science Research Council and Royal Holloway College.

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INTRODUCTION

Many fungi respond to changes in the nutritional condition of their environment and produce a paramorph. This usually takes the form of a reduction in extension rates of growth but an increase in branching. The mass may remain constant but the colony form is a small group of densely-packed hyphae.

This type of paramorph is formed in response to various sugar molecules, e.g., sorbose; to surfactant molecules, e.g., sodium deoxycholate; and to high osmotic conditions. The changes induced by sorbose have been shown to be due to changes in the enzyme level responsible for the initiation of branching.

The changes in enzyme level due to sodium deoxycholate and osmotic responses have not been investigated.

The ascomycete fungus used in this study, Ceratocystis adiposa, causes black rot of sugar cane. In culture on malt agar or a simple synthetic medium it is fast growing (Kr) and readily produces perithecia and two types of conidia.

Previous observations had shown that the growth rate could be reduced by sorbose and sodium deoxycholate, colony size was reduced, branching increased and this could be interpreted as a paramorphogenetic response.

In contrast to such fungi as Neurospora, high concentrations of sorbose were required suggesting that this organism did not respond in the same manner to low concentrations of sorbose but that paramorphogenesis was mainly osmotically controlled.

As paramorphs show abnormal morphology it is necessary to establish the normal ultra structure and chemistry of the ascomycete hypha before describing changes induced by paramorphogens.

The tips of septate hyphae contain dense cytoplasm and many cell organelles. Just behind the apex lies the Spitzenkörper (Girbardt 1957 and 1969) which is an aggregate of small vesicles and ribosomes. Apart from larger vesicles, some of which fuse with the plasma membrane, the apex is devoid of other organelles. Behind this region numerous mitochondria, nuclei, Golgi cisternae and rough endoplasmic reticulum can be seen. (Grove and Bracker 1970).

The Spitzenkorper is situated in the region of wall formation and McClure et al (1968) suggest that the vesicles migrate to the apex where they fuse with the plasma membrane liberating their contents to the cell wall. The contents of these vesicles vary, some being granular and others not. They are reported to be the only structure found in fungi and not in other cells, (Moore and McAlear, 1961) and termed lomasomes.

The occurance of lomasomes and endoplasmic reticulum cisternae near septa was reported by Brenner and Carroll (1968) who suggest that these structures are associated with cell wall synthesis.

Cell wall synthesis takes place mainly at the hyphal tip. This was shown using radioactive N-acetyl glucosamine. The label was incorporated into the hyphal tip and septa (Galun 1972), Gooday 1971). If the hyphae were subsequently treated with chitinase, most of the label was removed from the wall but not from the septa. Incorporation of label from uridine and leucine was along the length of the hypha.

Using Concanavalin A, Tkacz and Lampen (1972) established that new Mannan is inserted into the bud scar and also that parent

material is not used in making the bud cell wall. The distal tip of the bud was the major site of mannan and glucan insertion in Saccharomyces . However, Bartnicki -Garcia and Lippman (1969) reported that wall synthesis in the Y-form of dimorphic fungi took place all over the cell wall unlike the M - form where it took place only at the tip.

Under normal conditions of growth, the tip of Schizophyllum commune was filled with vesicles. If the mycelium was kept at 4°C for 24 hours which arrested growth the number of vesicles decreased remarkably. (Wang et al 1975). This phenomenon is said to support the hypothesis that vesicles are associated with hyphal extension.

According to Cook (1972) there are two kinds of wall growth:

a) Growth and extension involving the addition of wall material,

b) extension only which involves plasticizing of the wall.

In hyphae where (a) occurs, there are two sizes of vesicle but where (b) occurs only the smaller vesicles are present. This indicates that the smaller vesicles contain lytic enzymes and the larger ones synthetic enzymes or wall precursors.

The vesicles do not originate from the plasma membrane (Girbardt 1969), Grove and Bracker(1970) suggest that vesicles are produced by the endoplasmic membrane system including smooth surfaced cisternae in the sub-apical region and are incorporated into the expanding membrane at the cell surface.

Marchant et al (1967) postulate two vesicular systems to account for cell wall growth, one for primary wall synthesis and another for secondary wall synthesis. The vesicles for primary wall synthesis are produced by the endoplasmic reticulum and fuse with the plasmalemma at the hyphal apex. Multivesicular bodies form lomasomes and these are also produced by the endoplasmic reticulum and are suggested to be involved in chitin synthesis. Pythium lacks these multivesicular bodies and its cell wall is devoid of

chitin. However, this theory does not explain how wall precursors, lytic and synthetic enzymes are transported to the apex and how the precursors could be built into the wall apex. As will be discussed, there is evidence for secondary thickening of the cell wall but this involves the deposition of glucan (Trinci and Collinge 1975) rather than chitin which is present at the apex. (Gooday 1971 and Galun 1972).

Lytic enzymes like glucanase or chitinase are involved in splitting the polymers to allow the incorporation of new material into the cell wall. It was shown by Cortat et al (1972) that exoglucanase activity increased shortly before budding in yeast cells. The specific activity was twice as high in exponentially growing cells as in stationary phase cells and the enzyme was found inside and outside the cell. On cell fractionation, 85% of the protoplast enzyme was associated with vacuoles or sedimentable material. The enzyme associated with the cell wall was soluble and isolated cell walls were obtained free of enzyme. Mitchell and Sabar (1966) detected glucanase and protease in the cell walls of Pythium.

In exponentially growing yeast cells, three fractions have activity but in the stationary phase, only two fractions have activity. The heaviest fraction with glucanase activity was that containing the plasmalemma, the highest contained endoplasmic reticulum. The vesicle-containing fraction showed the highest specific activity of exoglucanase and also contained some endoglucanase and 50% of the cell mannan. Vesicles are never totally absent from a fungal cell as the septum requires the same components as does the apex for wall synthesis. The presence of glucanase activity with the plasmalemma fraction indicate that vesicles fuse with the membrane, liberating their lytic enzymes into the cell wall and external medium. Endoplasmic reticulum

shows some activity so this may be the origin of the vesicles.

Newly formed cell wall is highly susceptible to autolysis even when deposited close to old wall. The new wall of Aspergillus nidulans is thinner, takes up fluorescent dye and the tip is susceptible to osmotic change. The old wall is digested at a much slower rate (Polachek and Rosenberger 1975).

The incorporation of ^{14}C glucose into the cell wall was followed and most of the ^{14}C was retained. When ^{3}H ^{14}C was used, the ratio of H:C remained constant in the cell wall but decreased in the cytoplasm due to the higher metabolic loss of H. After a long time, autolysis breaks down some components of the cell wall of Aspergillus clavatus (Trinci and Righelato 1970).

The cell wall of Neurospora crassa has been investigated in detail. Mahadevan and Tatum (1967) removed the various fractions sequentially and found that each polymer on its own gives structural integrity to the wall. The glucan-peptide-galactosamine complex occurs on the outside of the wall and appears as thick fibrils (Mahadevan and Tatum 1965) accounting for 12 - 14 % of the cell wall. After this fraction of the wall had been removed, a layer of inner fibrils was exposed. Chitin fibrils appear to be protected by the glucan layer from enzyme and chemical attack.

The four layers of the cell walls are described by Mahadevan and Tatum (1965).

1) Peptide - polysaccharide complex (glucan-peptide-galactosamine)

11) Laminarin like β1,3 glucan (mannose, mannitol, glucosamine).

111) β 1,3 linked glucose polymer.

1V) Chitin.

Using enzyme dissolution combined with election microscopy, Hunsley and Burnett (1970) showed the structure of the cell wall of Neurospora to be five layered.

a) an outer layer of amorphous glucan 87 nm thick.

b) a glycoprotein reticulum 24 nm thick.

c) a protein layer 25 nm thick.

d) a protein layer 9 nm thick.

e) a chitin layer 8 nm thick.

The wall covering the hyphal apex is composed of protein, chitin and a little glucan. This forms an intact but not rigid structure. The increasing rigidity of the older wall is imparted by a reticulum and an outer glucan layer (Trinci and Collinge 1975).

The following statements can be made about the cell wall

1) Chitin fibrils, glucan and protein are formed at the apex.

2) They must be organized so that the sequence of polymers is glucan outside and chitin and protein inside.

3) Subapically the degree of aggregation of microfibrils must increase to account for the increased diameter of microfibrils in the mature wall. (Hunsley and Burnett 1968).

4) In the subapical region, new polymers must arise and be incorporated between the inner and outer region to form the glycoprotein reticulum.

Using longitudinal and serial transverse sections, Trinci and Collinge (1975) measured the wall thickness of Neurospora crassa and Geotrichum candidum. The wall thickness of N.crassa was a constant 50 nm for the apical 56 µm of a leading hypha. From 2 mm - 15 mm, the wall thickness increased to 270 nm and again remained constant. The extension zone measured as the length over which hyphal diameter increased was 28 µm. Geotrichum wall was 70 nm over the terminal 300 µm. It was shown that rigidification of the wall below the extension zone does not involve the deposition of significant quantities of wall material but probably the addition of small amounts of substances and the formation of cross-linkages. Vesicles appear to be fewer

compared to the tip so little softening of the wall takes place The inner layer of the mature wall has the same thickness as the primary wall.

Antisera were formed against three wall fractions of Neurospora crassa (Hunsley and Kay 1976). Using a fluorescent antibody technique the location of FRACTIONS 1, 111 and 1V on the cell surface were observed. Fig. 1.

1) Fluorescence at apex and subapex decreasing with distance from tip. Sometimes the apex did not fluoresce.

111) Faint fluorescence at tip increasing with distance.

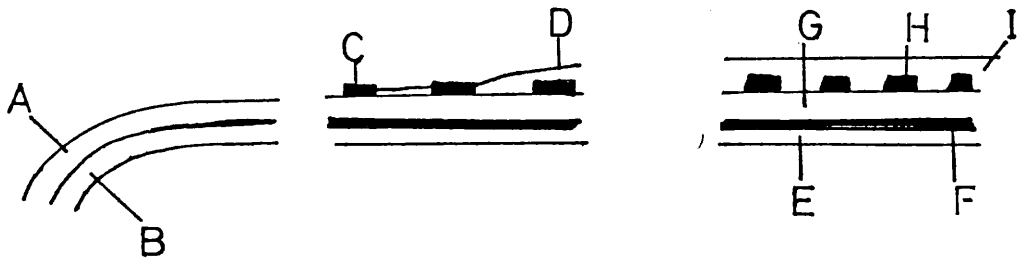
1V) Faint fluorescence except at hyphal fractures.

This would indicate that FRACTION 1V is on the inside of the cell wall and only exposed at breaks. FRACTION 1 covers the tip but is eventually covered by FRACTION 111.

Drastic chemical treatment revealed a microfibrillar layer at the tip while untreated hyphae showed an amorphous layer. Pronase also revealed microfibrils indicating that the overlying amorphous layer may be, or contain, protein. Increased binding of an uncharacterized antibody to the cell wall at the apical dome may indicate a high protein content and a similarity to the inner region of mature walls (Trinci and Collinge 1975)

FIGURE 1 (from Hunsley and Kay 1976).

STRUCTURE OF THE CELL WALL OF N. CRASSA.



Apex.

A. Amorphous layer which covers the apex. This gives a weak reaction to Antigen 1 and 111 or occasionally a strong reaction to Antigen 1.

B. Chitin microfibrils.

Subapex.

C. Reticulate structure of wall visible on surface. Strong reactor to Antigen 1.

D. Laminarin-like glucan covering reticulum. Moderate reaction to Antigen 111.

Mature. Strong reaction to Antigen 111 and weak reaction to Antigen 1.

E. Inner chitin protein region.

F. Discrete layer of protein.

G. Easily removable protein.

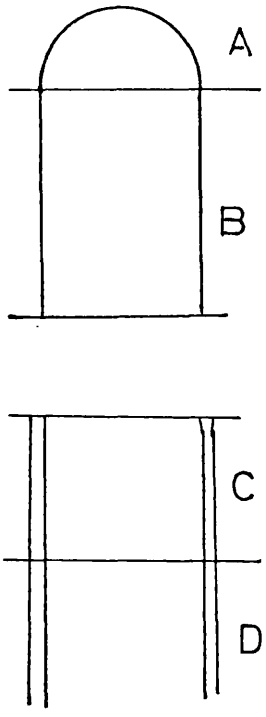
H. Reticulum embedded in outer glucan.

I. Outer amorphous layer of laminarin.

For this hypothesis of cell wall structure to work, new wall material is synthesized on top of the old wall away from the

cytoplasm. This confirms the model proposed by Trinci and Collinge (1975). See Figure 2 .

Figure 2 . Model proposed by Trinci and Collinge (1975) for regions of cell wall of Neurospora crassa.



A. Extension zone where primary wall contains microfibrils. The wall thickness is constant and extension is by fusion of vesicles with membrane.

B. Hyphal diameter is constant, wall inextensible and the same thickness as in zone A. Vesicles do not usually fuse with this area of cell membrane.

C. Wall increases in thickness to a mature wall by secondary wall formation.

D. Under carbon starvation, some wall polymers may be degraded for endogenous metabolism. (Zonneveld 1972).

Two sorts of fibril are reported to be present in Neurospora Mahadevan and Tatum 1965 and 1967) and in yeast cells of Paracoccidiodes (Carbonell et al 1970). Short thick fibrils on the outside can be digested in alkali indicating they are probably α, (1 → 3) glucan. Longer thin fibrils which are digested by chitinase are found on the inside of the wall.

The microfibrillar structure of Allomyces was investigated by Aronson and Preston (1960). The short chitin fibrils in the outer wall were arranged randomly. Beneath this layer fibrils were orientated longitudinally and the apex shows a preponderance of longitudinal fibrils.

The inner vegetative wall of Gilbertella persicaria sporangiospores is continuous with the germ tube wall. Fibrils 20 - 70 Å are found to be randomly orientated in a loose network within an amorphous matrix. This network is in three dimensions with fibrils extending from the inner to outer wall. The fibrils appear as light strands in an election dense matrix and resembles preparations of negatively stained polysaccharide. The dark matrix contains scattered pockets or granules of election transparent material. A possible interpretation may be that these are channels in the wall. (Bracker and Halderson 1971).

Yeast protoplasts in liquid culture produce nets of microfibrils 20 nm across which form flat straight bundles up to 500 nm wide and 4 μm long. The net disappeared in sodium hydroxide leaving microfibril clusters. They were found to be less than 15% chitin and a β-(1,3) - D - glucose polymer. In normal walls, microfibrils were 7.5 to 10 nm thick and the mesh was 20 - 60 nm wide forming the entire inner layer of glucan. (Kreger and Kopecka 1975).

Dimensions of microfibrillar elements in walls.

Phytophthora parasitica	114.75 - 128.08 Å	Hunsley and Burnett (1968).
Neurospora crassa	104.06 - 189.08 Å	
Schizophyllum commune	93.16 - 119.88 Å	
Gilbertella persicaria	20.70 Å	Bracker and Halderson(1971)
Yeast protoplast	200 Å	Kreger and Kopecka (1975).
normal cells	75 - 100 Å	

The wall of Neurospora crassa has chitin microfibrils embedded in a matrix of glucan and protein. Protein may constitute up to 14% of the wall (de Terra and Tatum 1963). Manocha and Colvin (1967) suggest that the protein may constitute a large part of the boundary of the pores which form a three dimensional network throughout the cell wall. Ferritin is incorporated rapidly into the cell and can be seen in the cell wall as lines of the same shape and size as the pores. As ferritin is a large molecule (25 - 30 Å) it may be assumed that it enters the lumen by pores and not diffusion through the matrix. (Manocha and Colvin 1967). Pores 40 - 80 Å have been shown in Pythium (Manocha and Colvin 1968).

Micropores 200 - 700 Å diameter were found in the microfibrillar framework of cross walls of Geotrichum candidum . (Hashimoto et al 1964).

The resistant sporangia of Allomyces have been shown to have pores in the wall 250 nm diameter. These can be seen when the amorphous material had been removed to expose the randomly arranged microfibrils which become concentric around the pore and form a thick rim on the inside of the wall. (Dodge and Lawes 1969)

Molecular sieving of invertase isozymes by the Neurospora crassa cell wall. (Trevithick and Metzenberg 1966). The increase in secretion and decrease in fractionation observed in mutants was explained by an increased porosity of the wall. The exclusion threshold of the wild type wall was found to be 4750 and of an osmotic mutant 18500. Trevithick et al (1966) proposed that the mutant had abnormally large pores.

These pores allow large molecules to enter or leave the cell e.g. in secretion of enzymes or uptake of vitamins.

Septa serve as an exoskeleton in that it gives support to cell wall and so maintains the form of the hypha. They protect the protoplasm from small mechanical injuries in that septal pores are plugged to isolate damaged cells. Turgor is maintained by septa opposing the outward pressure of the sap. (Buller 1933).

Ascomycete septa are simple and may taper toward a simple clear channel central pore (Moore and McAlear 1962). Reichle and Alexander (1965) suggest that the pore of Fusarium may be single or consist of a ring of pores which are plugged if adjacent cells are damaged.

The septa of Neurospora have a central pore 0.25 µm - 0.5 µm diameter (Trinci and Collinge 1973). The pores are plugged by spherical election densely staining bodies which do not have a membrane or well defined margin so are not Woronin bodies as described by Reichle and Alexander (1965) and Brenner and Carroll (1968). In fact, Woronin bodies were not observed in any of the strains of Neurospora examined.

Trinci and Collinge (1973) suggest that projections from the septum accumulate material which develops to form the plug. In open pores, lines of vesicles of the same size as those at the apex were observed and may carry wall precursors to the apex. If this is so, then the peripheral growth zone extends more than one cell back from the apex and the septal plug marks the extent of this. In compact colonies of mutants, the septa are plugged nearer the periphery than of wild type colonies of Neurospora.

The septa of Fusarium seem to be plugged with Woronin bodies (Reichle and Alexander 1965). There are four Woronin bodies associated with each septum.

The development of these Woronin bodies is described by Brenner and Carroll (1968) for Ascodesmis sphaerospora. Woronin bodies are formed from membrane bound sacs and have a lattice substructure and they are probably ergosterol. The membrane bound sacs originate from the endoplasmic reticulum involved in septum formation. (Thibaut 1969).

Peziza has several refractive granules each side of the septum, one central and the others peripheral. (Schrantz 1964). Ciliaria has only two bodies in the centre and evidence was found that they may pass through the pore. In Peziza the central pore may be sealed by the central body. These bodies are stained with haematoxyline, $KMnO_4$ indicating they are composed of protein and lipid.

Thibaut (1969) described the fine structure of the "pseudo synapsis" apparatus and the Woronin bodies associated with the septa of Sporothrix schenckii. The plasmalemma follows the septum and passes through the pore from one cell to another. The pore is covered by two convex discs and between them are "trabecules" which could be a bundle of microfibrils or concentric cylinders. (Bracker, 1967). No discs have been shown in transverse sections so the cylinder is an unlikely structure.

Connections between the cytoplasm of adjacent cells may be by plasmodesmata (Hawker et al 1966, Kirk and Sinclair 1966) in Rhizopus sexualis, Gilbertella persicaria, and Geotrichum candidum. They were seen as fine transcellular strands in newly formed septa which separate gametangia from their suspensors.

The plasmodesmata were continuous with the plasmalemma of each cell even in sections where the membrane had pulled away from the septum, e.g. in plasmolysis. Entire septa are restricted to Phycomycetes and Hemiascomycetes so plasmodesmata maintain contact between the two protoplasts. In Ascomycetes and Basidiomycetes contact is maintained throught the septal pore.

Early interest in the chemical composition of fungal cell walls was directed to discovering whether cellulose or chitin was the major component (Castle (1945), Blank (1954), Blank & Burke (1954)). Other analyses were done on pathogenic fungi (Blank & Burke (1954) & Noguchi et al (1975)). A review of sugars and amino acids found in fungal cell walls is given by Bartnicki-Garcia (1973 (b)), the most common sugars being :-

Glucose, Mannose, Galactose, Rhamnose,

N-acetyl glucosamine, Glucosamine and Galactosamine.

Mahadevan & Tatum (1965) described a sequential dissolution of Neurospora crassa cell walls by alkali and acid which separated the wall into 4 fractions.

Fraction 1. (11.8 - 16% of dried cell wall) dissolved in Na OH contained a peptide-polysaccharide complex which gave glucose and galactosamine on acid hydrolysis.

Fraction 11. (9.0 - 13.2% of dried cell Wall) was dissolved from the residue by H_2SO_4 and contained glucose, mannose, mannitol and glucosamine.

Fraction 111. (15.0 - 20.2% of dried cell wall) a second NaOH soluble fraction contained a glucose polymer.

Fraction 1V. (6.3 - 11.5% of dried cell wall) the final residue which, when digested with Helix pomatia juice released N-acetyl glucosamine from the chitin in this fraction.

Bull (1970 (b)) used the same technique on Aspergillus nidulans cell walls to obtain similar results in terms of the isolation of major components but with the addition of galactose in Fractions 1 and 11 and melanin associated with chitin in Fraction 1V.

de Vries & Wessels (1973) describe two glucans from the basidio-mycete Schizophyllum. The S glucan is alkali soluble and contains α - (1 - 3) linkages while the R glucan is insoluble and has β - linkages.

A more refined technique of sequential enzymic hydrolysis of Neurospora crassa cell wall was described by Hunsley & Burnett (1970) which led to a construction for the wall being deduced (Burnett 1976).

a) The outer layer 80-90 nm containing β - (1- 3), β - (1 - 6) and α - (1 - 3) linked glucans.

b) A coarse reticulum of glycoprotein embedded in an amorphous glucan matrix with protein increasing inwards 40 - 50 nm thick.

c) A layer of protein 8 - 10 nm thick.

d) Chitin microfibrils embedded in protein 20 nm thick.

Although there are four regions to the cell wall when determined chemically, only three layers are visible in walls treated by successive enzyme dissolutions and shadowing of ultra thin sections in the TEM.

It has proved possible to remove a number of components before the fungal wall loses its integrity (Manocha & Colvin (1967), Robertson(1968), Hunsley & Burnett (1970)).

The proportions of these components of the cell wall is important in determining the morphology of the fungus as alterations in the cell wall composition caused either by mutation or a paramorphogen lead to alterations in morphology, e.g., branch frequency (Mahadevan & Tatum (1965), de Terra & Tatum (1963)).

The colonial morphology exhibited by Neurospora mutants or paramorphs is due to an increase in branch frequency. de Terra & Tatum (1961) suggested that colonial growth is caused by a structural weakening of the cell wall as snail enzyme incorporated into the medium causes the same colonial morphology. The enzyme creates weak spots through which hyphal contents are forced by their internal pressure leading to an unusually large number of branches.

Colonial mutants were found to have less glucose and more glucosamine per unit weight of cell wall than the wild type. Sorbose induced paramorphs had 184% glucosamine and 68% of the glucose content of wild type cultures. (de Terra & Tatum 1963).

Brody & Tatum (1966) showed that the "col-2" mutant had an altered structure of G - 6 - P dehydrogenase which reduced its affinity for the substrate glucose - 6 - phosphate. The "ragged" mutants were deficient in phosphoglucomutase which caused an accumulation of glucose - 1 - phosphate to twelve times

the normal level and a β - glucan content of 50% of the wild type level (Mishra & Tatum 1970), (Brody & Tatum 1967).

Mutants "col-2," "balloon" and "frost" are caused by unlinked genes but all give distinct morphologies associated with abnormal G - 6 - P dehydrogenases. A correlation between the severity of enzyme defect and the degree of morphological abnormality was obtained in heterokaryons and double mutants. Therefore, the altered structures of enzyme molecules is responsible for the changes in the cell wall and consequently in morphology. (Scott & Tatum 1970).

Katz & Rosenberger (1970 & 1971) have described a mutant of Aspergillus nidulans which will grow at 41°C only if the medium is osmotically stabilized. IR spectrophotometry showed that the amount of chitin in the walls was only 7 - 15% of that normally found in wild type walls which is 20% (Katz & Rosenberger 1970(a). When mutant hyphae were transferred to conditions which allowed chitin synthesis, osmotically stable mycelium was formed at the tips but older regions became distorted and chitin was found to be incorporated into old walls. Normally chitin synthesis is restricted to the tip (Gooday 1971 & Galun 1972) as shown by the incorporation of labelled glucose and N-acetyl glucosamine or the deposition of other material during secondary thickening. (Trinci & Collinge 1975).

The synthesis of chitin by fungi has proved of interest as this is one feature by which the pathogenic fungal cell differs from the human host and therefore chitin synthesis may be a suitable target for anti fungal antibiotics. (Gooday 1977). In his review of the biosynthesis of fungal cell walls, Gooday (1977) deals with chitin synthesis in detail. The pathway of synthesis from glucose - 6 - phosphate to chitin is as follows (Edson 1972, McMurrough et al 1971 and Burnett 1968 for details of each step).

Fructose - 6 - P
↓
Glucosamine - 6 - P
↓
N-acetyl glucosamine - 6 - P
↓
N-acetyl glucosamine - 1 - P
↓
UDPN-acetyl glucosamine
↓
Chitin

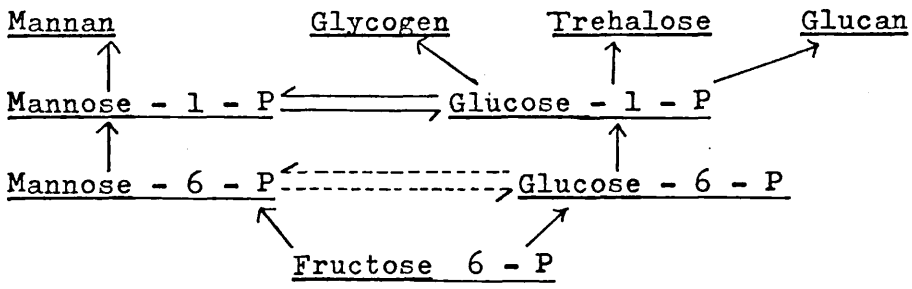
Chitin synthetase from Saccharomyces carlsbergensis requires Mg^{2+} , Mn^{2+} or Co^{2+} for enzyme activity for which Km = 0.6 - 0.9mM at pH 6.2 (Keller & Cabib 1971).
Km for Mucor rouxii chitin synthase is Km = 3.1 m Mol. l^{-1}). (McMurrough & Bartnick-Garcia 1971).
Aspergillus nidulans chitin synthase (EC 2.4.1.16) was obtained from a membrane preparation. Its optimum conditions were pH 7.5 and 30.5°C. Enzyme activity was greatly stimulated by Mg^{2+} and N-acetyl glucosamine while EDTA and Polyoxin D act as inhibitors, (Gooday et al 1976).
Ki for Polyoxin D is 3 μ mol l^{-1} for Coprinus cinereus enzyme.

When preparations of the A. nidulans enzyme were digested with trypsin for a short time, the activity was increased by 500% (Ryder & Peberdy 1977). Without the addition of N-acetyl glucosamine sigmoid reaction kinetics were obtained for Aspergillus and Coprinus (Gooday & Rousset-Hall 1975) indicating that chitin synthase may be an allosteric enzyme with more than one (possibly 4) binding site per molecule.

In yeasts, where chitin synthesis is restricted to the septum formed between mother and daughter cells, the enzyme in the cytoplasmic membrane is in a latent form. It is activated by a specific protease localized in cytoplasmic vesicles. An inhibitory protein binds to the activation protease when budding

is not taking place (Cabib & Keller(1971) & Farkas (1976)).

The other major component of fungal cell walls is glucan which may be present in different forms depending on the glycosidic linkages within the polymer. The major route of polysaccharide synthesis involves phosphorylation, Chung & Nickerson (1954) proposed the following scheme.



The glucan remaining in the wall of Aspergillus niger after a preliminary treatment with Na OH (Fraction 111) was analysed and found to be a linear glucan with 85 - 90% $1 \rightarrow 3$ linkages and 10 - 15% $1 \rightarrow 4$ linkages. The ratio of $\alpha : \beta$ was 4 = 1 (Stagg & Feather 1973). α - (1 - 3) glucan was also reported in Cryptococcus, Schizosaccharomyces versatilis Polyporus betulinus, P. sulphureus and Boletus edulis (Bacon et al 1968).

The glucan obtained from Saccharomyces cerevisiae was 85% insoluble and had β - (1 - 3) linkages while the soluble 15% had β - (1 - 6) linkages (Manners et al 1974), (Bacon & Farmer 1968). The alkali soluble fraction of the yeast cell wall which accounts for 20% of the wall has a molecular weight of 250000. It contains mainly glucose with traces of mannose. 80 - 85% was (1 - 3) - β - D linked, 8 - 12% (1 - 6) - β - D linked and 3 - 4% branched residues linked through C1, C3 and C6 (Fleet & Manners 1976).

When the yeast cell wall was digested with pronase, all the mannan and a third of the glucan were removed. The

residue was more susceptible to Na OH than before digestion indicating that the glucan may have been associated with protein which rendered it alkali insoluble (Hanly et al 1971).

Zonneveld (1972) followed the changes in the α (1 - 3) glucan fraction of Aspergillus nidulans during cleistothecium development and found it to be the main reserve polymer. When sufficient α glucan has been accumulated and the exogenous glucose supply depleted, then α - (1 - 3) glucanase activity increased and cleistothecium development started. If insufficient α -glucan had been accumulated, then no cleistothecia were formed.

The surface glucan (S glucan) of Schizophyllum commune has 1 - 3 linked α - D - glucosyl units and is not amorphous but has a microcrystalline rodlet structure like the surface of some conidia (Wessels et al, 1972; Ghiorse & Edwards, 1973).

Glucomannan complexes in association with protein have been reported in yeasts (Kessler & Nickerson 1959). In the glycosylation of mannoproteins in yeast, the initial sugar binding occurs while nascent polypeptide chains are still growing on ribosomes (Ruiz-Herrera & Sentandreu 1975).

The mannans of various species of Saccharomyces were acetolyzed and fragments consisted of α - (1 - 2) and α -(1 - 3) linked units. (Ballou et al 1974). Bretthauer and Chen Tsay (1974) found that in the yeast mannan protein complex, the mannan had an α -(1 - 6) backbone of mannose units with α -(1 - 2) and α - (1 - 3) side chains. The mannan was attached to the protein by N-acyl glycosylamine links between glucosamine and asparagine residues.

A specific stain for mannan in yeast cells was described by Tkacz et al 1971) and used to demonstrate sites of mannan insertion into the yeast wall. (Tkacz & Lampen 1972).

The distal tip of the growing bud was the major site of mannan insertion.

Nigeran, an alternating $\alpha(1-3)\ \alpha(1-4)$glucan has been found in several Aspergillus spp (Johnson 1965), (Bobbitt et al 1977). Carbohydrates composed of different sugar residues have been reported, e.g. galactosaminogalactan from A. niger (Bardalaye & Nordin 1976).

galactomannan from Trichosporon fermentans (Gorin & Spencer 1968, Gorin et al 1969).

peptido-rhamno mannan from Sporothrix schenckii (Lloyd & Bitoon 1971).

L-rhamno - D - mannan from Ceratocystis ulmi (Gorin & Spencer 1970.

D gluco - D - mannan from C. brunnea. (Gorin & Spencer 1970).

Cellulose has been found in phycomycete cell walls (Bartnicki-Garcia 1966) and in walls of some species of Ceratocystis (Smith et al 1967).

An investigation of cell wall chemistry within the genus Ceratocystis showed that the genus could be divided into two main groups; those species which contained cellulose and those which did not. (Rosinski & Campana 1964, Smith et al 1967, Jewell 1974). Rhamnose was also found in walls containing cellulose and the species containing these were found to be exoconidial in morphology. (Weijman & de Hoog 1975).

Ceratocystis adiposa cell walls contain glucose and mannose but not galactose. (Spencer & Gorin 1971). The structure of the D-gluco - D-mannan from Ceratocystis brunnea which gave the same type of spectrum as that from C. adiposa was further investigated. The ratio of glucose : mannose residues was 1 = 1.3, the free hexoses were in the D enantiomer form and

the specific rotation of the polysaccharide was + 93° indicating a preponderance of α - D linkages.

Carbohydrate is the major component of most walls and this is composed mainly of glucose with a little mannose galactose and amino sugar. Protein and lipid form most of the remainder with melanin when present.

The cell walls of Neurospora crassa were analyzed by Mahadevan & Tatum (1965), Edson (1972) and found to contain :-

C	44.1 %	C	39.34 %
H	6.41 %	H	6.66 %
N	2.06 %	N	1.88 %
P	0.31 %	P	0.92 %
		S	0.40 %

Bacteria also produce lytic enzymes, e.g. Bacillus cereus produces a chitinase and laminarinase. (Mitchell & Alexander 1963), consequently the hyphae of some fungi and other micro organisms act as carbon sources for these bacteria.

Hyphae of Aspergillus nidulans were found to be resistant to a mixture of glucanase and chitinase (Kuo & Alexander 1967) and Rhizoctonia solani and Cladosporium resistant to Streptomyces enzyme (Bloomfield & Alexander 1967). Conidia of Aspergillus phoenicus were resistant to Streptomyces enzyme until the melanin containing spicules were removed. The kinetics of melanin inhibition of glucanase, chitinase and cellulase have been described by Bull (1970 (b), (c)). Inhibition is of the non-competitive type, the melanin combining with groups other than the active centre of the enzyme molecule by electro static bonding. There is also evidence for an association of melanin with chitin and melanin bound chitin is extremely resistant to enzyme degradation.

The yeast Torulopsis aeris is resistant to enzymic lysis.

The outer coat of the cell wall contains a protein/polysaccharide complex which may contain xylan or a xylan/mannan complex to render it resistant (Ballesta et al 1969).

The rate at which a fungus grows is limited by the rate of uptake of nutrients necessary for cell wall synthesis and metabolism. Therefore the kinetics of sugar transport and competitive inhibition of some sugars by others are important in determining normal and paramorph growth rates.

In a sorbose resistant mutant of N. crassa, there was no evidence found for altered sugar metabolism enzymes or the gain in ability to phosphorylate or utilize sorbose. (Klingmuller, 1967). An increase in the uptake of sorbose revealed that these mutants have a decreased active uptake system. Sorbose uptake showed saturation kinetics in both wild type and mutant strains. The values for Km were similar but Vmax was lower in some mutants. (Table 1). There was no evidence for a defective carbohydrate carrier or a specific sorbose carrier.

It was concluded that the mutants were permease mutants and it was found that a mutation of any one of 6 different chromosomal genes caused the formation of a sorbose resistant mutant. The mutation of any one of four of these genes resulted in a mutant with an altered sorbose permease system or one containing a suppressor which decreased the efficiency of the permease system. (Klingmuller,1967).

Scarborough (1970ab)) reported that 30 MG is not accumulated against a concentration gradient in glucose grown cells (Table 2) but agree that it cannot be phosphorylated by ATP dependent hexokinase. It appears that glucose uptake by system 1 involves a facilitated diffusion transport step followed by ATP phosphorylation and subsequent metabolism. This system

TABLE 1.

Kinetics of sugar transport.

Organism.	Sugar	Km	Vmax	Reference
Neurospora crassa	Sorbose	116 mM	15 nM ml min	Crocken & Tatum(1967)
Yeast	Sorbose	244 mM	900 "	
N. crassa	Glucose 1	25		Schneider &
	11	0.04		Wiley(1971)
"	Glucose 1	8		Scarborough
	11	0.01		(1970)
"	3.0 Methyl 1	10	12 nM	Neville et
	Glucose 11	0.07	4 nM	al(1971)
	Sorbose	3.5		
		10.0		Klingmuller
	3.0 Methyl			& Huh(1972)
	Glucose (30 MG)	0.03		
N. Crassa	Sorbose	3.6 - 7.1	0.051 - 0.157 mgm mgm^{-1} min^{-1}	Klingmuller (1967)
Yeast				
(resting)	Glucose	0.56	65 nM	van Uden
(chemostat)		0.72	mgm^{-1} min^{-1}	(1967)
Yeast	D-glucose	4.6	mgm/g/min 20 - 28	
	2-deoxyglucose	4.5	20 - 26	
	D-mannose	27	20 - 30	Kotyk(1967)
	D-galactose	21 - 46	10 - 13	
	L-glucose	10^2	1	
	L-rhamnose	10^3	3	
	D-xylose	95 - 170	22 - 32	
	D-arabinose	75 - 155	18 - 30	
	D-lyxose	80 - 110	15 - 22	
	D-ribose	300 - 800	3	
	L-arabinose	10^2	3	
	L-xylose	200 - 600	2.5	
	D-fructose	17	20 - 30	
	L-sorbose	21	7 - 16	

TABLE 2.

Characteristics of the two transport systems in Neurospora.
(Schneider & Wiley (1971(a)).

System 1.	System 11.
Synthesized constitutively.	repressed by growth in 0.1M glucose.
Km 25mM	Km 0.04 mM. Schneider & Wiley (1971).
8mM	0.01 mM. Scarborough (1970(a) & (b)).
Energy dependent.	energy input site is inside membrane preventing efflux.
*30 MG intracellular concentration. exceeds external at steady rate.	Efflux of α-methyl glucose is energy dependent. not inhibited by fructose, galactose, lactose.
	glucose is non-competitive inhibitor of fructose and galactose uptake.
* 3.0 methyl glucose	Properties similar to β-galactoside permease of E.coli.
	Activity increased by casein hydrolysate, tryptone and sugar deprivation. (Schneider & Wiley,1971(b)).

operates at high glucose concentrations. When cells are grown on fructose on low glucose media, a second system appears which catalyses the accumulation of unaltered 3O MG and L-sorbose against a concentration gradient. Repression of System 11 at high glucose concentrations conserves the energy used in uptake as facilitated diffusion requires less energy.

Both systems are temperature dependent, inhibited by Na N_3 and 2,4 dinitrophenol. There is no phosphorylation related to transport. Further evidence against this is given by Brown & Romano (1969) for Aspergillus nidulans. 2-deoxy-D-glucose, 6-deoxy-D-glucose and 1-deoxy-D-glucose are all transported into the cell but as they lack OH groups at C_1 and C_6, they cannot be phosphorylated at these positions. Radioactivity from labelled sugars appears first in the free sugar pool and then as sugar-phosphate. They suggest that intracellular kinases are responsible for the sugar-phosphate pool.

In ungerminated conidia (Neville et al,1971) of Neurospora crassa, a high affinity transport system which was relatively specific for D-glucose was found. The system also transports non-metabolised analogues, e.g., 2DG and 3OMG., the Km for 3OMG being 0.07 mM. The analogues are accumulated against a concentration gradient and uptake is inhibited by Na N_3. The results are consistent with the active transport mechanism for uptake. The system is repressed when conidia are germinated on glucose media but is restored when glucose is depleted or removed. Previously accumulated 3OMG stimulated subsequent influx and the stimulation was proportional to the internal substrate concentration. Efflux appeared to be a carrier-mediated process but was not inhibited by azide or sulfhydryl reagents.

A slime mutant of N. crassa which lacks a cell wall shows similar transport systems. (Schulte & Scarborough,1975). At 50mM, transport is glucose-facilitated diffusion while at very low concentrations, active transport occurs.

In Allomyces, 2 deoxy-D-glucose (2 DG) is not taken up until germlings are at the start of the rhizoid stage. Uptake increases and then remains constant. Glucose blocks the uptake of 2 DG. It appears that RNA synthesis during germination and protein synthesis are required for uptake of 2 DG. A protein requirement for lactose uptake in E. coli has also been observed. (Jones & Kennedy,1969) and lipid synthesis associated with the presence of membrane protein, (Fox,1969) which has 2 sugar binding sites. (Carter, Fox & Kennedy,1968).

When E. coli is exposed to azide, dinitrophenol, etc., the transport against a concentration gradient is abolished which may indicate a membrane carrier. The rapid equilibration of external and internal concentrations is inhibited by chemical analogues. This is consistent with the theory of a membrane

carrier involved in active transport by control cells and facilitated diffusion by poisoned cells. Winkler & Wilson, (1966) inferred from their data that energy coupling reduced the affinity of a carrier for its substrate on the inner surface of the plasma membrane.

Carter et al (1971) found a discontinuous relationship between glucose utilization rate and growth rate. This points to different methods of glucose metabolism. The reduced yield obtained at high growth rates may be the result of uncoupling catabolism and anabolism or a change to an energetically less efficient method of metabolism. Kr may therefore not be a particularly good method for estimating substrate affinity.

Certain Triton soluble substances (? carotenoids) are synthesized in the presence of sorbose and may have a role in sorbose stimulated sugar transport in Neurospora (Klingmuller,1971). They may be part of the transport system as they originate from cell membranes contained in washed sediment of disrupted cells. They are preferentially synthesized when transport is enhanced roughly in proportion to the increase in sorbose uptake. These findings are confirmed by others who report that lipids may be involved in active transport (Fox,1969 on E. coli).

Thornton et al (1976) found that 30 MG caused bursting of the hyphal tips of Dendryphiella salina at low concentrations but not at high concentrations. L-sorbose causes hyphal tip bursting in N. crassa. (Rizvi & Robertson,1965). At high concentrations, the rate of uptake is not sufficient to make a great difference in the water potential gradient between cell sap and medium. Metabolised sugars caused less bursting probably because they are converted into high molecular weight insoluble compounds which do not influence the osmotic pressure of cell sap. Bursting can be prevented by using 10^{-2} M buffer.

30 MG is used in transport studies to measure influx and efflux of non-metabolites. However, the tendency for tips to burst had not been reported. If this happens, then the amount of cytoplasm involved in uptake is reduced and so may account for some differences in rates of uptake in high and low sugar concentrations, i.e. System 1 and 11.

When fewer tips burst at higher concentrations, more labelled sugars can be absorbed for the same rate of uptake than when many tips burst at low concentrations of 30 MG. Competition by an unlabelled sugar would also reduce the rate of uptake of a labelled sugar. For the first 10 minutes of uptake, these two effects are probably indistinguishable but when hyphal tip bursting has ceased then the rate of uptake may be better assessed.

Thornton et al (1976) interpret the results of Neville et al (1971), Schneider & Wiley (1971) and others for two uptake systems as being the rates with intact and burst hyphal tips. A previous hypothesis (Scarborough 1970 (a), (b)) was that a rapid equilibrium was established by facilitated diffusion followed by removal of glucose repression of the active transport system. Thornton et al (1976) found that active transport occurred whether the mycelium was grown on (10^{-4}M) glucose or fructose and conclude that uptake cannot be explained by de-repression of the transport system. However, they state that after 150 minutes, the glucose was used up therefore the concentrations of glucose towards the end of the experiment would be very low and possibly de-repression did occur. They think that cyclic AMP may be involved which is possible as polyphosphates are involved in transport of glucose in yeast cells. (van Stevenick & Booij (1964)).

It may be concluded that there are two systems of sugar uptake in fungi, facilitated diffusion and active transport. Sorbose has been reported to inhibit both by competitive

inhibition. Phosphorylation may be involved in activating the carrier but does not bind to the sugar until it reaches the inside of the cell.

When sorbose combines with the carrier to prevent or decrease the rate of glucose uptake, glucose metabolism must be reduced. This would possibly result in slower growth rates and less efficient utilization of glucose especially if glucose transport is the rate limiting step of anaerobic fermentation in yeast. (van Uden). The effect of sorbose on glucose uptake may be one mechanism by which sorbose acts to produce the effects given in Table p. 60. (Trinci & Collinge,1973).

When sorbose is transported into the cell, it can inhibit enzymes like glycogen synthetase and glucan synthetase (Mishra & Tatum,1972) which are cell wall synthesizing enzymes. Cell wall growth has been shown to be a balance between lysis and synthesis of compounds like β - 1,3 glucan. If the synthesis of this is inhibited while lysis is unaffected, then the balance is upset and the growth rate decreased. Increased branching can partly be explained by increased wall softening caused by an excess of lytic enzyme in the mycelium. This would appear to be a second mechanism of sorbose paramorphogenetic activity.

"Thus growth and morphogenesis represent transport processes which, in common with the more popular membrane transport, must be described by vector quantities, having both magnitude and direction in space.

When we separate transport from morphogenesis, growth and movement in living organisms, the separation is not real but abstract." (Mitchell 1962).

Cirillo (1961) in the review of sugar transport in micro-organisms, names three types of transport and characterizes them as -

a) Simple diffusion. The rate of transport is a function of concentration difference across the membrane, molecular size and lipid solubility.

b) Facilitated diffusion characteristics.

1) Rate approaches saturation with increasing external concentration.

2) Rate is specific for stereo and optical isomers.

3) Structurally similar compounds act as competitive inhibitors.

4) Transport is specifically sensitive to certain inhibitors.

5) Rate is greater than that predicted from molecular size and lipid solubility.

6) Q_{10} is usually about 3.

7) Usually species or strain specific.

c) Active transport characteristics.

1) Requires metabolic energy.

2) Accumulation takes place against a concentration gradient.

3) Rate approaches saturation with increasing external concentration.

4) Rate is specific for isomers.

5) Competitive inhibition occurs between structurally similar compounds.

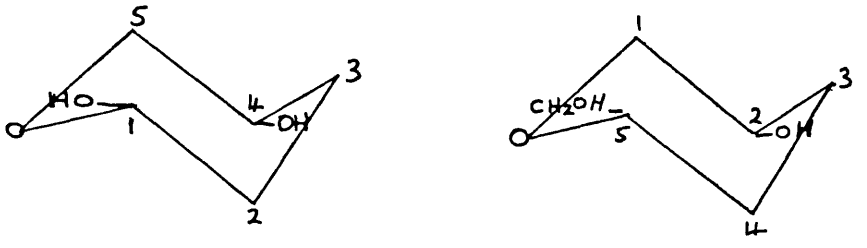
6) Sensitive to enzyme inhibitors.

Sugar transport into yeast cells is considered to be passive by Cirillo (1961) but to have a high Q_{10} . For most sugars, there appears to be an initial very rapid uptake followed by a slower rate. For sorbose, this slower rate was about 11 mgm/hr at 30° C, while at 4° C, no further uptake occurred.

The transport of hexoses into yeast cells was investigated

by Kotyk (1967). All monosaccharides tried were transported across the cell membrane but only those (Group 1) with equatorial OH groups at positions 1 and 4 of the C I chair conformation or with equatorial OH and CH_2OH at carbons 2 and 5 of the I C chair conformation reached an equilibrium in the entire volume of cell water. Group 2 sugars reached only 20 - 66% of this.

Group 1 sugars.



The sugars could be divided into 2 groups bases on their Km and V max values.

Group 1 Sugar.	Group 2 Sugar.
Km from 4 - 170 mM	Km 200 - 1000 mM
V max. 15 - 30 mgm/gm/min.	V max. 1 - 3 mgm/gm/min.

Sorbose and galactose are intermediate having V max 7 - 16 mgm/gm/min.

The two groups of sugars are considered to be transported by different carriers each showing counter transport and an activation energy of 6700 - 7800 cal/mol. See Table 3 .

The Group 2 carrier is probably shared by all sugars but because of different affinities, a competition can be observed in one direction but not the other.

TABLE 3 .

To compare the properties of sugar carriers (Kotyk 1967).

Feature investigated.	Carrier 1.	2.
Distribution of sugar in cell water.	100%	~ 50%
Effect of hypertonic medium on sugar distribution.	great	slight
Effect of uranyl ions on transport.	great	slight
Competition with glucose for uptake.	yes	no
Occurrence of counter transport.	yes	yes
Mobility ratio for loaded vs free carrier.	2 - 3	1
Apparent activation energy between 15 - 30°C.	6 - 8000 cal/mol.	6 - 8000 cal/mol.
Effect of 2,4 dinitro phenol.	no	yes

It would seem that Carrier 1 represents active transport and Carrier 2 facilitated diffusion.

Spoerl et al (1975) conclude that sorbose normally penetrates an intra-cellular compartment membrane by simple diffusion and that an increased rate of penetration brought about by metabolised sugars is the result of an increase in membrane permeability rather than a stimulation or initiation of a transport process. Sorbose uptake was increased 40 - 60% by glucose but uptake was not accounted for by growth or binding. Counterflow and efflux measurements indicate that access to an intra-cellular compartment (? vacuole) was restricted but efflux from the compartment was doubled by glucose addition. There was no evidence for facilitated transport in the compartment membrane as there is in the cell membrane. This may explain the differences in distribution of

sugar in cell water.

Pre-incubation of cells in D - galactose increased the uptake of L - xylose indicating that the carrier of Group 2 sugars can be induced or unmasked and the initial rate of uptake and space distribution are increased to the value of Group 1 sugars.

Cirillo (1968) investigated the role of sugars as inhibitors of L - sorbose and D - xylose transport by a constitutive monosaccharide transport system. D - glucose had the highest activity (Ki 5 mM) while others had Ki values of up to 2000 mM. Assuming that inhibition is due to the formation of a sugar-carrier complex, then the carrier has a broad specificety for pyranoses. Single changes at each carbon atom in the ring resulted in a decrease in activity.

There are two types of sugar transport reported in yeast (Cirillo(1961, 1962;) Van Steveninck & Rothstein (1965)). The first is facilitated diffusion which leads to an equilibrium of internal and external sugar concentrations. It is shown by starved cells with the uptake of sorbose or galactose and the uptake of glucose by poisoned cells therefore is independent of metabolic energy. Low concentrations of uranyl and nickelous ions do not effect transport while sorbose acts as a competitive inhibitor. Transport does not involve changes in the Ni and Co bound at the cell surface.

The second system of active transport occurs against a concentration gradient at the expense of metabolic energy. The uptake of glucose by unpoisoned cells and of galactose by induced cells are examples of this system. Transport is inhibited by low concentrations of UO_2^{++} and accompanied by a reduction in nickel and cobalt binding on the cell surface. This system has a low Km and high V max . Transport is mediated by an activated carrier formed from a non-active carrier by enzymic phosphorylation.

The non-activated glucose carrier accepts sorbose but the activated form will not, having a narrower range of substrate specificity. Sorbose is not transferred actively but in growing yeast, it is a competitive inhibitor of glucose transport.

Van Steveninck and Booij (1964) present data to suggest that one molecule of glucose is taken up with the disappearance of one polyphosphate monomer. Cations inhibit anaerobic fermentation of glucose by yeast. Hg^{++} penetrate into the cytoplasm while others are reversibly bound to a substance on the outside of the yeast; probably polyphosphate. Thorium ions are bound not only to polyphosphate but also to phosphatides in the cell membrane.

When glucose is transported into the cell, the amount of polyphosphate in the outer face of the membrane decreases. In yeast poisoned by monoiodoacetate, the number of glucose molecules that can still be taken up is equal to the original number of cation-binding sites.

The hypothesis is put forward that glucose uptake is associated with enzyme phosphorylation, possibly of the carrier with phosphate donated by polyphosphate. Glucose is not phosphorylated (see also Brown & Romano 1969) because glucose phosphates are not taken up by yeast cells. Glucose may be bound to the carrier by phosphate or the carrier may have to be enzymically phosphorylated prior to binding glucose. Uranyl ions bind to the outside of the cell and cause complete inhibition of glucose uptake indicating both bind to polyphosphates. During glucose uptake, there is a reduced cobalt and nickel binding capacity which supports the idea that inhibition is not by the formation of a glucose-metal complex. Cirillo (1961) reported that uranyl ions block sorbose influx, downhill efflux and counterflow. It immobilizes the sugar carrier system - perhaps combining with the carrier itself.

Wilkins & Cirillo (1965) put forward the concept that sugar

transport is a symmetrical carrier-facilitated diffusion in which net transport is the result of independent influx and efflux processes.

The rate for membrane transport (Vg) can be expresses as

$$Vg = Tg \left\{ \frac{G}{G + Kg} \right\}$$

Kg = Km for glucose transport.
Tg = Maximum transport velocity.
G = Concentration inside cell for efflux and vice versa.

The change in intra cellular concentration dGi/dt.

$$\frac{d\,Gi}{dt} = Tgo \left[\frac{Go}{Go + Kgo} \right] - Tgi \left[\frac{Gi}{Gi + Kgi} \right] - q.$$

q = rate of metabolism.
o = outside cell.
i = inside cell.

Because of cell metabolism which converts glucose into large molecular weight insoluble compounds like glucans, the internal concentration is always less than external concentration.

Anaerobic glucose uptake by resting cells of yeast followed unidirectional Michaelis-Menten kinetics and was competitively inhibited by L - sorbose; ($Km = 5.6 \times 10^{-4}$ M, $Ki = 1.8 \times 10^{-1}$ M, $V\,max = 6.5 \times 10^{-8}$ moles mg^{-1} min^{-1}.) Aerobic uptake was also inhibited. Glucose transport was concluded to be the rate limiting step of anaerobic fermentation of yeast and of growth of a respiration deficient mutant (van Uden, 1971).

Once nutrients have been absorbed by cells of the mycelium, they need to be transported to growing tips or fruiting bodies.

Buller (1933) considers protoplasmic streaming accounts for most of the transport within the mycelium. In Rhizopus nigricans, the simultaneous flow of protoplasm in opposite directions was clearly seen including simultaneous reversal of direction in one case. Streaming in Sordaria fimicola was so rapid that it dislodged vacuoles and carried them along. Granular cytoplasm appeared to pass through the septum without difficulty and vacuoles may pass through either whole or as smaller vacuoles. The general direction of flow is from old to young mycelium and the rate of movement proportional to the rate of growth.

Proposed causes of streaming are vacuolar pressure and/or an increase in the amount of protoplasm. Enlarging vacuoles drive protoplasm out of cells into ones which are growing rapidly and so making room for new protoplasm. Plugging of septal pores, however, restricts the movement forwards. As protoplasm is synthesized it increases in volume. Growth of the cell wall occurs at the tip and newly formed septa appear to bulge towards the tip.

The hypothesis of enlarging vacuoles requires that these be filled with fluid of high osmotic concentration to fill the old mycelium and Isaac (1964) found this not to be so. Isaac considered theories and calculated the pressure drop across a pore to be 0.5 atmos for a flow of $10\mu m sec^{-1}$ but was unable to demonstrate a pressure gradient in hyphae.

Rhizoctonia solani shows a drop in cytoplasm density from one side of a septum to the other, upstream the dry weight content of the cytoplasm was 24% and downstream only 8.5%. However, a greater proportion of the soluble material occurs downstream. (Isaac 1964)

Burnett (1968) sees good reason to suppose that some aspects of translocation could be accounted for by cytoplasmic streaming based on internal turgor pressure gradient but this mechanism is unlikely to be the only one.

It was shown that a 12 mm length of hypha would have to re-duplicate its cytoplasm to supply tips but in fact replacement of cytoplasm by vacuoles helps to establish rapid growth. (Zalokar 1959b). This confirms the results of Ryan et al (1943) that more than 10 mm was needed to provide cytoplasm for the growing zone. Shorter sections produced new cytoplasm at the same rate but could not produce enough to supply rapidly advancing tips and so slow down the growth rate.

Protein and RNA formation does not decrease away from the tip (Zalokar 1959b). Hyphal tips were richer in protein and sulphydryl groups. Glycogen was absent at the tip because it is hydrolysed into sugar and used for building the cell wall. Cytochrome oxidase and succinic dehydrogenase decrease at the tip while peroxidase increases at the tip and alkaline phosphatase behind the tip.

A strong reducing power was found in hyphal tips (Turian 1976) using reduction of Fe^{+++} to Fe^{++} and (Weiss and Turian 1966) using neutral red which was reduced to yellow at the tip. When differentiation into conidia was induced with phosphate buffer, the reaction faded. In conidia, succinic dehydrogenase had only one tenth the activity of young hyphae, and aldolase and β-galactosidase increased after germination. (Zalokar 1959a).

Turian (1976) pointed out that polarized growth was maintained only as long as proglycolytic conditions were maintained in the apex. Oxygen starvation and the inactivation of SH compounds

lead to the premature disappearance of the apical reducing zone which is then invaded by mitochondria. The apical dominance of the tip over the sub-apical oxidizing region maintains a redox gradient which could provide the electrochemical power postulated by Bartnicki - Garcia (1973) as a possible cause of polar migration of vesicles to the tip of actively growing hyphae.

There is no evidence that vesicles are guided by microtubules and they lack the means for self propulsion. Vesicles may be carried in the stream of cytoplasm from old parts to new parts of the hypha but the dense cytoplasm at the apex cannot carry them right up to the tip. It is postulated that a plausible mechanism could be electrophoresis.

The electropotential could be generated by reactions of the vesicle contents upon discharge. However, as during germination of a spore, wall synthesis occurs all over the surface, there must be a trigger which initiates polarized wall growth. This may be a possible role for the Spitzenkorper or Woronin body.

Growth kinetics reflect the rated of nutrient uptake, cell wall synthesis and the production of new mycelium. Changes in form without changes in mass imply an alteration in the basic growth kinetics of the system.

Originally, kinetic studies on micro organisms were restricted to the unicellular bacteria and yeasts. In these organisms, growth is autocatalytic, i.e. each cell produces more complete cells. Growth, measured as cell number or mass, can therefore be expressed exponentially once the lag phase of germination is completed and before an essential nutrient has been depleted. There is disagreement about whether filamentous fungi show autocatalytic growth; Righelato (1975) considers filamentous fungi to show autocatalytic growth while Mandels (1965) does not.

Filamentous fungi often have a slower growth rate than unicellular organisms. This may be because growth is by incorporation of new material into the cell wall. Gooday (1971) showed that this is mainly restricted to the tip of each hypha and does not occur along the length of each cell of a hypha except as secondary thickening in some fungi (Trinci & Collinge 1975). Therefore, the growth rate is limited by the number of binding sites into which new material can be incorporated at the tip. (Kubitscheck 1970).

When growth is exponential, the increase in cell mass or number is expressed by the following equation.

$$\frac{dN}{at} = \alpha N.$$

N = cell number or mass.
α = specific growth rate.
t = time.

This applies as long as there is no restriction on growth. However, this situation never lasts for long and lack of a suitable substrate, space, oxygen, etc., soon limit growth. Odum (1953) incorporated an expression into the equation to account for environmental resistance. The growth curve of a population is sigmoid.

(Rate of population increase) = (Maximum rate of increase) x (Degree of realization of maximum rate.)

OR.

(unrealized increase.)

The equation then becomes -

$$\frac{dN}{dt} = \alpha N \frac{(K - N)}{K} \quad (1) \quad \text{OR} \quad \frac{dN}{dt} = \alpha N - \frac{\alpha}{K} N^2 \quad (2).$$

K is the maximum carrying capacity of the environment, flask etc.

Integration of equation (1) gives -

$$N = No\ e^{\lambda t} \left(\frac{K}{K - N + Nae^{\lambda t}} \right) \qquad (3)$$

N_0 the number of organisms at t_0

The equations were derived to apply to individuals in a field situation but Koch (1975) applies them to cellular elements or the amount of protoplasm in a mycelium.

After a lag phase, during which the spore swells, a germ tube is produced and this may elongate at an exponential rate for a short while (Trinci 1971). The hypha then forms branches and the total increase in hyphal length is exponential. In Botrytis cinerea (Smith 1924), the exponential increase in total hyphal length continues for 10 hours and in Mucor hiemalis until 15 mm of hyphae have been produced (Trinci 1974).

When investigating apical branching in Geotrichum lactis, Trinci (1970) found that there was no reduction in growth rate prior to branch formation. In Aspergillus nidulans branching was preceded by an 18% reduction in growth rate followed by an increase until each branch approached the growth rate of the original parent hypha.

During this period of exponential growth, the mycelium is undifferentiated i.e., all the hyphae are equivalent and primary, secondary and tertiary hyphae cannot be distinguished. Trinci (1974) showed that the total hyphal length and the number of tips increase exponentially at the same specific growth rate. He points out that this is to be expected when increase in the length of an individual hypha is constant while the total mycelium increases exponentially (Zalokar 1959), (Trinci 1969).

Therefore, the total length of hypha divided by the number of tips should remain constant. This has been confirmed and the constant obtained referred to as the Hyphal Growth Unit (G). (Trinci 1973, Caldwell & Trinci 1973). The Hyphal Growth Unit can be considered as a physiological but not morphological entity. (Trinci 1974).

A temperature sensitive mutant of Neurospora crassa had a value of G of 193μm at 15°C and 38 μm at 30°C while the specific growth rate increased from 0.10 h^{-1} to 0.32 h^{-1}. At each temperature, the mean value of G. was about $4\frac{1}{2}$ times longer than the mean intercalary compartment. (Steele & Trinci 1977). Fiddy & Trinci (1976 a, b) have shown a relationship between septation and branch initiation in wild type Aspergillus nidulans and Geotrichum candidum and suggest that septa inhibit the transport of cytoplasmic vesicles containing lytic enzymes and wall precursors, which accumulate behind the septum and initiate a branch.

For an undifferentiated mycelium, the mean hyphal extension rate (E) is calculated from the following equation.

$$E = 2\,\frac{(Ht - Ho)}{Bo + Bt}\ (\mu m/tip/hr).$$

Ho = hyphal length at time 0.
Ht = hyphal length 1 hr later.
Bo + Bt = corresponding number of tips.

As a colony matures, differentiation into primary, secondary and tertiary hyphae takes place. The rates of elongation of these hyphae and their widths are not the same. (Table 4)

TABLE 4 .

Comparison of hyphal extension rates of primary, secondary and tertiary hyphae as % of primary hyphal extension rate.

	HYPHAE			
ORGANISM	1^{Y}.	2^{Y}	3^{Y}	Reference
Coprinus disseminatus	100	66	18	Butler (1961)
Neurospora crassa Spco 9	100	85	59	Steele & Trinci (1975)b
Neurospora crassa cot 3.	100	55	25	

The extension rate was found to be proportional to hyphal diameter and faster growing branches tended to originate from wider fast-growing hyphae. (Butler 1961).

The fast radial growth-rate of some fungal colonies is associated with the production of fast growing leading hyphae. However, the branches may have considerably slower growth rates. (Trinci 1974; Butler 1961: Steele & Trinci 1975(b)). Hyphae of undifferentiated Neurospora crassa mycelium have a lower Emax than the leading hyphae at the margin of differentiated colonies. (Steele & Trinci 1975b). In Geotrichum candidum, however, the Emax of undifferentiated and differentiated colonies is very similar. Steele & Trinci (1975(a)) showed that the different types of hyphae in N. crassa had extension zones of between 3.9 - 29.2 μm (see Table 5) but their extension zone expansion times were very similar.

$$\text{Extension zone expansion time} = \frac{\text{extension zone length } (\mu m)}{\text{rate hyphal extension } (\mu m/min)}.$$

The length of the extension zone and colony radial growth rate (Kr) are correlated in N. crassa and also for the species in Table 5 . (Steele & Trinci (1975 (a)).

r was calculated to be 0.9274, P. $<$ .01.

TABLE 5 .

Extension zone lengths and extension zone expansion times of leading hyphae at the margin of mature colonies at 25°C. (Steele & Trinci (1975 (a)).

SPECIES.	Extension zone length, μm	Kr, μm m. .	Extension zone expansion time, min.
Rhizopus stolonifera	28.5	24.0	1.2
Mucor hiemalis	10.2	8.3	1.2
Botrytis cinerea	14.2	7.8	1.8
Actinomucor repens	13.5	6.1	2.2
Fusarium moniliforme	4.5	3.3	1.4
Penicillium digitatum	3.9	0.8	4.8
Neurospora crassa spco 9.	18.6	16.5	1.1
Neurospora crassa cot 3.	15.4	14.8	1.0
Neurospora crassa SY7A.	29.2	38.7	0.8

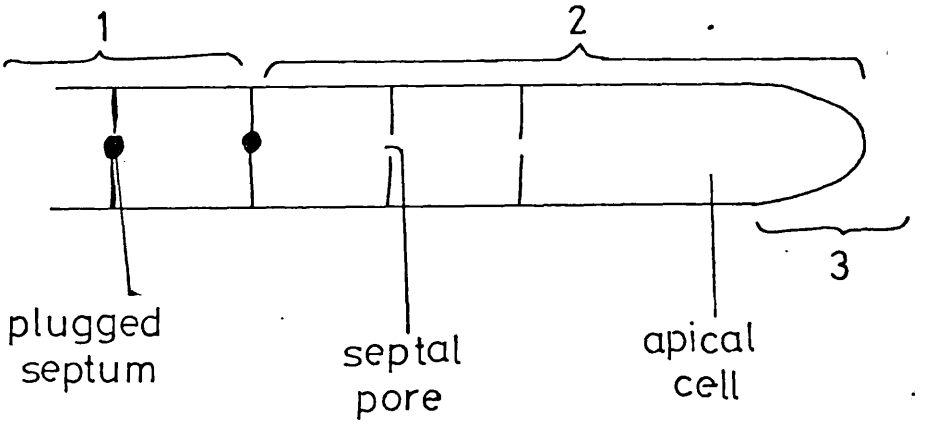
Because of the variation in the extension rates of individual hyphae, the measurement of individual extension rates is not a satisfactory method for obtaining Kr, the rate at which a colony advances across a substrate. If it is to be used, then many measurements should be taken and the relevant statistics calculated.

Experimentally determined values of Kr, α s, G, ω, extension zone and extension zone expansion times are given in Table 6 . .

FIGURE 3 .

Diagram to show the regions of a hypha.

(Trinci 1971).



1. Region not contributing to apical extension.
2. Peripheral growth zone.
3. Extension zone.

The extension rate of a hypha is influenced by many factors.

1. Hyphal diameter (Butler 1961).
2. Frequency of branching. This can be measured as the Hyphal Growth Unit, G. (Morrison & Righelato 1974; and Steele & Trinci 1975).
3. Peripheral Growth Zone (ω). (Trinci 1971). Fig 3 This is the region of the hypha which contributes protoplasm to the growing tip. It was found that $Kr \propto \omega$ (Trinci 1971, 1973, and Trinci & Collinge 1973).
4. Extension Zone. (Steele & Trinci 1975.) Fig 3 This is the tapered region at the hyphal apex. A direct relationship exists between the length of the extension zone and the rate of hyphal extension.

The method used to estimate ω. by cutting the colony margin

at an angle probably gives an over estimate of the true value. (Bainbridge 1976). It is concluded that the value for ω. lies halfway between the longest and shortest apical cells as about 72% of the longest cell contributes to growth and compartments behind short cells must contribute to growth.

TABLE 6.

Experimentally determined values of growth parameters of various fungi.

ORGANISM	Ref.	Kr μm/hr.	αs h-1	HGU μm	P.g.z (ω) μm	Extension zone μm	Extension Zone expansion time mins.
Absidia glauca (+)	6	379	.124		2487		
Actinomucor repens	6	486	.181		2479		
Aspergillus nidulans	7	146	.124				
	&8	127	.148				
A. nidulans BWB 224	10	277	.289				
		146 (25°C)	.148				
	14	297 (37°C)	.360				
BWB 480	10	163	.257				
A. niger.	6	133	.119		1137		
A. wentii	6	157	.147		1342		
Botrytis cinerea	1	468				14.2	1.8
Fusarium moniliforme	1	198				4.5	1.4
Geotrichum candidum	8	132	.173				
	&12		.385	102-112			
G. lactis.	6	200	.353		423		
Mucor hiemalis	8	377					
	1	498				10.2	1.2
	14	424	.099				

ORGANISM	Ref.	Kr μm/hr	α_s h	HGU μm	P.g.z (ω) μm	Extension zone μm	Extension Zone expansion time mins.
Mucor racemosus	6	433	.102		3406		
Penicillium chrysogenum	6	76	.164		496		
	14	53	.123				
P. chrysogenum							
(1)	3	38	.126	25	500		
(2)		96	.348	44	800		
(3)		110	.232	54	1200		
(4)		140	.116	101			
P. chrysogenum							
Q 176	9	172	.13	99			
BG_A		94	.21	53			
C 5		75	.20	51			
A		83	.30	44			
T 14		101	.24	43			
C 2		58	.17	38			
B		67	.28	29			
Penicillium digitatum	1	48				3.9	4.8
Rhizopus stolonifera	1	1440				28.5	1.2
Neurospora crassa	13		.217				
SY7A	1	350	.256	130		29.2	0.8
	5	2322	.274				
SYR-17-3A	11	2154				32.6	0.9
SPCO 1	8	343					
	4	350	.221	130			
	5	293	.256		871		
	5		.223				
	11	294				7.8	1.6
SPCO 3	11	216				5.8	1.6

ORGANISM	Ref.	Kr μm/hr	α_s h^{-1}	HGU μm	P.g.z (μ) μm	Extension zone μm	Extension Zone expansion time mins.
Neurospora crassa							
SPCO 9	1	990				18.6	1.1
	5	1001	.250		3930		
	5		.256				
	11	996				16	1.0
	2		.22	314		4.7	.28
	4		.303	325			
SPCO 12	11	72				2.2	1.9
Cot 3	1	888				15.4	1.0
	2		.15	201			
	5	1109	.132		5038	3.8	.20
	2		.156				
	11	1110				26.5	1.4

Key to references.

1. Steele & Trinci (1975)a
2. Steele & Trinci (1975)b
3. Righelato (1975).
4. Trinci & Collinge (1973).
5. Trinci (1973)b
6. Trinci (1971).
7. Trinci (1970).
8. Trinci (1974).
9. Morrison & Righelato (1974).
10. Bainbridge & Trinci (1969).
11. Collinge & Trinci (1974).
12. Caldwell & Trinci (1973).
13. Trinci (1973)a
14. Trinci (1969).

The linear rate of increase in the radius of a colony growing on a solid substrate is known as the Radial growth rate, Kr. (Trinci 1971).

It can be measured from the following :-

1) increase in the radius of a colony with time.

2) the rate of growth of a margin along a growth tube as described by Ryan et al (1943).

3) measuring the growth rate of individual hyphae microscopically. This last method is the least satisfactory because different hyphae of the same colony have different extension rates, so many measurements need to be done to obtain an accurate measure of Kr.

These measurements are easy to obtain, not destructive and therefore repeatable (Mandels 1965).

The increase in area and volume covered by a mycelium when growth is in one dimension, i.e., in a growth tube, is expressed in the following equations :-

$$\frac{dA}{dt} = Kr.\ b. \qquad \frac{dV}{dt} = h.b.\ Kr. \quad \text{(Koch 75) (1) (1a).}$$

A = area. b = breadth of colony.

V = volume. h = height.

t = time.

In two dimensions, i.e., a colony derived from a point inoculum the equation is as follows :-

$$\frac{dA}{dt} = \pi (Kr)^2 t. \qquad \frac{dV}{dt} = 2\pi\ h\ (Kr)^2\ t. \quad \text{(2) (2a).}$$

(Koch'75).

When spores of Neurospora crassa were plated onto agar, the area covered by the mycelium plotted against time can be expressed by 3 linear segments containing 2 sudden changes in Kr during the first 24 hours of growth. (Rosen & Ryan 1960).

In three dimensions, the equation for rate of increase in volume -

$$\frac{dV}{dt} = 4\pi (Kr)^3 t^2 \qquad \text{(Koch 1975)} \qquad (2b)$$

The increase in mass with time can be expressed as

$$\frac{dM}{dt} = \alpha M. \qquad (3)$$

M = mass. t = time. α = specific growth rate.

Equation (3) integrates to give

$$\ln M - \ln Mo = \alpha t. \quad \text{or} \quad M = Mo\, e^{\alpha t}. \qquad (4) \quad (4a)$$

where Mo is the mass at t = 0.

From equation (4), Pirt (1967) obtained the following equations :-

a) For a colony considered to be a hemisphere -

$$\ln r = \frac{\alpha}{3} t + \ln ro. \qquad (5)$$

b) For a colony considered to be a disc -

$$\ln r = \frac{\alpha}{2} t + \ln ro. \qquad (6)$$

r = radius. ro = radius at time = 0.

From these equations (5) and (6), it would be expected that the radius of a colony should increase exponentially. However, it is usually reported to increase linearly and conforms to the following equations -

$$r = Kr.\, t + ro. \qquad (7)$$

$$r = ro + Kr\ (t - to). \qquad (8)$$

Exponential increase in colony size was found for Aspergillus nidulans colonies up to 1 mm radius by Trinci (1969). Zalokar (1959) reported exponential increase in submerged shake cultures of filamentous fungi.

The increase in radius of a pellet depends on the width of the peripheral growth zone as the inner core of the pellet does not contribute to growth. (Pirt,1966; Trinci, 1970).

$$\frac{dr}{dt} = w\alpha \qquad (9)$$

w = width of peripheral growth zone.

α = specific growth rate.

Integrating with respect to t gives -

$$r = w\alpha \cdot t + r_o \qquad (10)$$

r_o = radius at t_o.

The mass of a pellet may be expressed as -

$$M = \frac{4\pi r^3 \rho}{3} \qquad (11)$$

$$r = \frac{M^{\frac{1}{3}}}{\left(\frac{4}{3}\pi\rho\right)^{\frac{1}{3}}} \qquad (12)$$

ρ = assumed even density of pellet.

Substituting for r in equation

$$M^{\frac{1}{3}} = \left(\frac{4}{3}\pi\rho\right)^{\frac{1}{3}} \alpha\omega t + M_o^{\frac{1}{3}} \qquad (13)$$

This equation (13) indicates a theoretical cube root law for three-dimensional growth in pellet form. This has been confirmed experimentally by Emerson (1950), Marshall & Alexander (1960) and Machlis (1957). However, Zalokar (1959), Pirt & Callow (1960) and Borrow et al (1964) found certain fungi grew exponentially according to the equation -

$$M = M_o e^{\alpha t}$$

A better way of expressing growth than Kr is that of dry weight produced per unit of time. Any other parameter of growth should be related to this. (Mandels, 1965). The measurement of dry weight is usually obtained from liquid cultures incubated on an orbital shaker. As Mandels points out, this is not a "normal" environment but is employed to ensure a sufficient gaseous exchange and a uniform environment.

If solid media are employed, then problems will arise when it is desired to separate the mycelium from its substrate.

Other parameters which may be employed to measure growth are cell volume or cell number in unicellular organisms like yeasts but

these are not easily adapted to the study of filamentous fungi. Metabolic activity, e.g., respiration as measured in a Warburg apparatus, may also be used in certain circumstances if they are related to dry weight increase.

When growth is exponential, i.e., truly auto catalytic, the following phases may be easily recognised -

1. Lag phase - cell enlargement and increase in metabolic activity.
2. Acceleration phase. - increase in the rate of cell division to that of the exponential phase.
3. Exponential phase. - uniform rate of cell division.
4. Deceleration phase. - decrease in the rate of cell division.
5. Stationary phase. - cell division balanced by cell death, i.e., a constant number of living cells.
6. Decline. - a decrease in the number of living cells.

The growth curve for Geotrichum lactis (Trinci 1971) shows a close resemblance to the idealized growth curve. Under the conditions used, growth was filamentous rather than pelletted so there would be no region equivalent to the centre of a pellet which is starved of nutrients and oxygen. This filamentous type of growth shows a closer resemblance to the truly auto catalytic growth of unicellular organisms (Pirt 1967).

The type of growth obtained for a particular organism in shake culture depends partly on the size of the inoculum.

In Penicillium chrysogenum (Camici et al 1952) pellet formation occurred in cultures inoculated with fewer than $2^{1} - 3 \times 10^{5}$ conidia per ml of medium while filamentous growth occurred in cultures with a denser inoculum. If cultures are not shaken, then a mat of mycelium is produced on the

surface of the medium. The mat contains submerged hyphae which will become deprived of oxygen if they penetrate deeper than the level to which oxygen dissolves from the surface. Aerial hyphae often have a different morphology and anatomy from submerged hyphae.

When growth is pelleted, the resemblance to the idealized growth curve becomes less obvious. This is because a smaller proportion of the mycelium is contributing to growth. In truly auto catalytic growth, each cell replicates to form another cell. When growth is filamentous, it is restricted to each hyphal tip with the region immediately behind contributing cytoplasm. However, pellets do not continue to grow exponentially after they attain a certain diameter (Trinci 1970). Pirt (1967) suggested that a pellet consists of two zones; a peripheral growing zone and a non-growing, inner mass which lacks oxygen. The density of a pellet would have a considerable effect on growth kinetics. Pellets in which the hyphae are densely packed together would only be able to obtain nutrients at the centre of the pellet by diffusion from the external medium. The outer part of the pellet, where the rate of diffusion was greater than the rate of utilization of nutrients, would continue to grow. The central region, beyond the part where the rates of diffusion and utilization balanced, would be starved of nutrients and probably anaerobic. In less densely packed pellets, the liquid medium would be able to circulate within the pellet and so the zones previously described would be less well defined and the central zone would be comparatively smaller.

Cube root growth is associated with pellet morphology and exponential growth with filamentous morphology. This is only a generalization and intermediate stages between the two conditions probably occur.

Emerson (1950) has shown that a cube root plot gave a

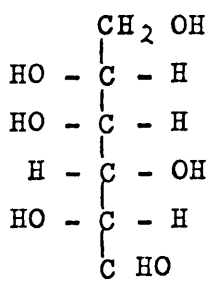
straight line relationship between dry weight and time. This indicates that the diameter of a pellet increases at a constant rate and the density of the hyphae must be almost uniform. Trinci (1970) reported that pellets of Aspergillus nidulans and Penicillium chrysogenum increased in radius at a constant rate which was, however, much slower than the Kr on solid media.

Emerson (1950) investigated the $3\sqrt{\ }$, logarithmic and linear relationship of dry weight and time for Neurospora crassa. He concludes that for N. crassa, a $\sqrt[3]{\ }$ plot is better than a log plot because a straight line relationship continues for longer than with the log plot. Marshall and Alexander (1960) report a cube root relationship between oxygen consumption and time for a number of fungi and actinomycetes. The use of oxygen consumption has the advantage over dryweight in that repeated observations can be made on the same culture. With none of the organisms they used, did logarithmic, square root or arithmetic graphs of data appear linear during active growth.

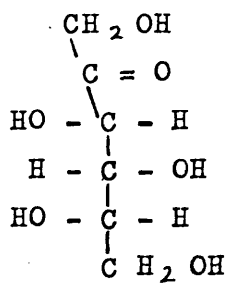
Growth kinetics may be altered by various chemicals and external factors eg sorbose, sodium deoxycholate and water potential. The most frequently investigated paramorphogen is sorbose and has already been shown to be a competitive inhibitor to glucose uptake.

L-sorbose is a monosaccharide with the same empirical formula $C_6 H_{12} O_6$ as glucose but is structurally different.

D-glucose



L-sorbose



L-sorbose is synthesized from D-glucose during the early stages of ascorbic acid production. (Geissman 1968).

D-glucose $\xrightarrow[\text{catalyst}]{H_2}$ D-sorbitol $\xrightarrow[\underline{\text{Suboxydans}}]{\underline{\text{Acetobacter}}}$ L-sorbose.

In a large survey of "the utilization of sugars by fungi", Lilly & Barnett (1956) reported that "quantitative data on the utilization of L-sorbose by fungi is less abundant than for other hexoses." They found that "many fungi either do not use sorbose or do so slowly".

The following fungi use sorbose as a carbon source and produce $>$ 45% of the dry weight of mycelium in glucose when grown in sorbose medium. The basal medium used contained 2g per l asparagine which provides enough carbon for most fungi to show some growth. Table 7

TABLE 7.

Ability of certain fungi to use sorbose.

Expressed as % yield in glucose.

Fungus.	% yield in glucose.
Alternaria solani	52
Aspergillus clavatus	82
Aspergillus elegans	45
Aspergillus niger	72
Aspergillus rugulosus	79
Botrytis cinerea	47
Collybia velutipes	53
Endoconidiophora adiposa	148
Fusarium conglutinans	111
Fusarium culmorum	114
Fusarium lycopersici	100
Fusarium medicaginis	108
Fusarium niveum	119
Fusarium oxysporum	59 +
Fusarium tracheiphilum	120
Gliomastix convoluta	91
Glomerella cingulata	68
Lenzites saepiaria	103
Melanconium fuligineum	82
Penicillium expansum	87
Penicillium spiculisporum	79
Phoma beta	128
Polyporus versicolor	74
Sphaeropsis malorum	78

+ Cochrane (1958) otherwise from Lilly & Barnett (1956).

In the experiments to determine the effect of sorbose in conjunction with other sugars, the concentrations used were 20g/l of each sugar. The values for Endoconidiophora adiposa are as follows. (Lilly & Barnett 1956).

Sugars	glucose	glucose sorbose	maltose	maltose sorbose	sorbose	double glucose
Yield mgm per 25 ml culture	183	268	158	247	240	169

On these results, E. adiposa is placed with the group of fungi which utilize sorbose well and are not inhibited by sorbose in mixtures with maltose or glucose.

Their other experiments confirm the results of Tatum et al (1949) that Neurospora sitophila grown on sorbose or glucose produced almost equal masses of mycelium although colony size was restricted by sorbose.

Endoconidiophora fimbriata was found to be one of the most sensitive fungi tested by Lilly & Barnett (1956). When examined microscopically, a severe toxic effect was observed. Hyphae were thicker and excessively branched as opposed to the slender, sparsely branching hyphae of normal growth on maltose. After 6 days, 77% of the hyphal tips were dead in sorbose medium compared to 15% in maltose medium. Less sensitive fungi showed excessive branching but few dead hyphal tips. The yield of mycelium of E. fimbriata was reduced to 10% of the yield of 2% maltose medium by adding 2% sorbose.

Lilly & Barnett reported that autoclaving did not effect the inhibitory effect of sorbose and so conclude that the inhibition is not caused by a toxic product formed during autoclaving. Sorbose media turn brown if autoclaved especially if mixed with the other components. It is less sensitive to change if autoclaved on its own and then mixed with other components when cool.

Viability of conidia (de Serres et al,1962) of N. crassa depended on time of autoclaving and composition of medium. In Westergaard's medium with 0.1% sucrose, germination was reduced to 20% by 1% sorbose and to 5% by 2% sorbose.

The inhibition of radial growth rate caused by sorbose may be modified by the other components of the medium, e.g. carbon source, nitrogen source etc. Care must also be taken when interpreting results from media where sucrose has been used as autoclaving will hydrolyse sucrose to glucose and fructose.

Brockman and de Serres (1963) found great variation in colony diameter of Neurospora not only with the concentration of sorbose but also with the medium into which sorbose was incorporated. By increasing the concentration of sorbose from 1% (0.055M) to 5% (0.278M) the colony diameter was reduced by $\frac{2}{3}$ on the three types of media. However, at each concentration of sorbose, the colony diameters were in the ratio of 1.5 = 3.0 = 5.0 on Westergaard : Fries : Vogel containing 0.05% each of glucose and fructose.

To obtain distinct colonial morphology, the ratio of sugar : sorbose was found to be 1 : 1 for fructose but 100 : 1 for glucose. Rizvi and Robertson (1965) also found glucose $>$ sucrose $>$ fructose in overcoming sorbose inhibition of an albino form of Neurospora crassa.

When N. sitophila was grown on sorbose, colony size was restricted but the dry weight produced in liquid media did not diminish compared to glucose. (Tatum et al 1949). A similar effect was observed when maltose was used. When Chaetomium globosum was grown on malt extract medium (Lilly & Barnett 1956), it was found that the malt extract overcame the effect of sorbose. A similar but less marked effect was shown by Sordaria fimicola and Endoconidiophora fimbriata. Yeast extract

however, failed to reverse sorbose inhibition. Lilly & Barnett suggest that the effect of malt was not wholly due to its sugar content but the nitrogen content or vitamin content. When vitamins were removed from malt extract, the fungi grew better on the untreated malt but the activity of the treated malt against sorbose was not completely destroyed. The addition of inositol (also Tatum et al 1949) pantothenic acid, nicotinic acid, pyridoxine and coprogen did not reduce sorbose inhibition but the addition of peptone caused a slight decrease in inhibition.

Nitrogen sources tested for their effect on sorbose inhibition were KNO_3 , Asparagine, glutamic acid, arginine, urea and casamino acids.

With maltose as the carbon source, casamino acids and glutamic acid were generally most effective in reducing sorbose inhibition although with Chaetomium globosum and Endoconidiophora fimbriata asparagine was most effective.

Acidity generally had little effect on the action of sorbose in maltose media.

Temperatures at or above the optimum for growth tend to increase the inhibitory effect of sorbose while at lower temperatures, less inhibition was observed.

Matsushima & Klug (1958) found that the growth of 3 strains of Ustilago maydis on glucose and asparagine medium was 70 - 75 mgm/10 ml medium while on sorbose/asparagine medium, three strains gave 0, 40 and 11 mgm/100 ml. When glutamic acid was used with sorbose, one strain gave 78 mgm/10 ml while another strain showed no growth at all.

The growth of melon-1 mutant of N. crassa is stimulated by sorbose and this is accompanied by a decrease in the frequency of branching (Murray & Stb 1960).

Trinci and Collinge (1973)a investigated in detail the effect of sorbose on the growth of Neurospora crassa spco 1 and spco 9, using Vogel minimal medium with sorbose and without.

Morphology in batch cultures.	spco 9 was filamentous in both. spco 1 sorbose increased pellet concentration. no difference in hyphal diameter, fine structure or length of growth unit.
On Solid Media.	increased branch initiation. decreased hyphal growth unit. cell length reduced. diameters of leading hyphae reduced. intra-hyphal hyphae more frequent. hyphal tips contained fewer vesicles. diameter of vesicles smaller. plugged septal pores occurred nearer margin. peripheral growth zone narrower.
Specific growth rate.	lag phase increased. 2% sorbose decreased α_s by 18% in spco 1. spco 9 not affected.
Yield.	spco 9 yield slightly decreased. spco 1 not affected by 1% and increased by 2%.
Germination. α_g.	lag phase increased. reduced by 13-14% by 2% sorbose.
Kr Radial growth rate.	reduced to 10-13% by 2% sorbose.

Peripheral growth zone. reduced to 12.5%.

Crocken & Tatum (1968) found that sorbose caused less efficient utilization of glucose in Neurospora in that less glucose was incorporated into the cell wall but there was an increase in carbon dioxide production. In the cell wall, the β 1, 3-glucan level was decreased. Low trehalose levels were related to the inability of the colony to conidiate. (Hanks & Sussman 1969). Crocken & Tatum (1967) reported that sorbose did not enter the cell against a concentration gradient. Sorbose equilibrated with the Neurospora in 9 hours at 30°C but in yeast after only 1 hour. Michaelis Menten kinetics of uptake were found to be for Neurospora Km = 116mM V max 0.015 μM/ml/min.

Yeast. = 244mM V max 9.0 μ M/ml/min.

When glucose was added to sorbose equilibrated cells, it caused an efflux of sorbose against a concentration gradient (counter) flow for which they postulate a carrier mechanism - probably facilitated diffusion.

Mishra & Tatum (1972) showed that the enzymes glycogen synthetase (EC 2.4.1.11) and glucan synthetase of Neurospora crassa wild type are inhibited by sorbose in vivo and in vitro. Sorbose grown cultures showed a marked decrease in the specific activity of both enzymes compared to sucrose grown cultures. Enzymes from the sorbose resistant mutant patch were not inhibited by sorbose. They conclude that the paramorphogenic action of sorbose seems to result from its inhibition of cell-wall synthesising enzymes.

Sorbose is not metabolised by yeast but Neurospora metabolises it to glucose via sorbitol.

The enzyme α -glucosidase from yeast synthesized the

following disaccharides by transglucosylation : 1 -α - , 3 -α - and 4 -α - D - glucosyl - L - sorbose. These disaccharides were able to accept glucose to produce trisaccharides. Two trisaccharides synthesized under the co-existence of L-sorbose and Maltose were 1, 4-di-α-D-glucosyl-L-Sorbose and 1-α- isomaltosyl - L - Sorbose (Matsusaka, Chiba & Shimomura (1975).

This may provide an alternative mechanism by which sorbose is utilized by fungi to that of metabolism to glucose.

The inhibitory action of sorbose is greater in the presence of sucrose and maltose than in glucose. Lilly & Barnett (1953) suggest that the size or complexity of the sugar molecule may be a factor in inhibition. As inhibition is greatest at or above the optimum temperature for growth, they suggest that enzymatic activity may be a key to understanding sorbose inhibition as this would involve size and complexity of the sugar molecule and also temperature -

Mutants which are relatively resistant to sorbose may have changed synthetase enzymes or altered methods of sorbose uptake (Klingmuller 1967).

The mutant "patch" of Neurospora crassa has enzymes which are not affected by sorbose and it is not deficient in the uptake of sorbose (Mishra & Tatum 1972). It is not known whether the mutation causes a primary defect in enzyme structure.

Glycogen synthetase (α - 1, 4 glucan synthetase) and β 1, 3 glucan synthetase from wild type Neurospora are inhibited by sorbose while those "patch" enzymes are not affected by sorbose. Glycogen synthetase occurs in two forms, glucose - 6 - phosphate (G - 6 - P) dependent and G - 6 - P independent. These two forms differ in their affinity for the substrate, the independent form having a greater affinity for UDPG that the

G - 6 - P dependent form. It was concluded that G - 6 - P has a specific binding site on the enzyme and when bound reshapes the molecule resulting in a greater substrate affinity. This is supported by the facts that sorbose specifically inhibits the dependent enzyme activity in vitro and the effect of sorbose is reduced by high concentrations of G - 6 - P. The dependent enzyme of patch may be conformationally different and so not affected. (Traut & Lippman 1963).

Mishra (1977) has reviewed the changes in the cell wall of morphological mutants of Neurospora and the altered morphology was found to be related to an increase in fraction 1 or a decrease in fraction 111 or both. On biochemical screening, many of the morphological mutants have been found to be defective for enzymes of carbohydrate metabolism. The enzymes 6 - phosphogluconate dehydrogenase and glucose - 6 - phosphate dehydrogenase show increased values of Km in mutants while phosphoglucomutase shows a decreased specific activity in mutants.

Lardy et al (1950) found hexoKinase activity inhibited by sorbose and suggested that inhibition was due to the formation of L - Sorbose - 1 - phosphate.

Decreases in the amounts of specific glucan polymers in the cell walls of fungi grown in sorbose medium could be explained by the inhibition of glucan synthetases (Crocken & Tatum 1968, Mahadevan & Tatum 1965).

When Neurospora crassa was grown in sorbose medium the proportions of glucosamine and glucose released by acid hydrolysis of the cell wall were 184% and 68% respectively compared with a control. Four colonial mutants also were reported to have less glucose and more glucosamine than did the wild type. The mutant melon - 1 had 15% less glucose and 24% less glucosamine per unit weight when grown on sorbose than without sorbose,

therefore the total polysaccharide content of the cell wall was reduced.

It was suggested that colonial growth is caused by a structural weakening of the cell wall (de Terra & Tatum 1961). Snail digestive juice causes colonial growth at low concentrations and forms protoplasts at high concentrations. The enzyme creates weak spots in the cell wall through which the hyphal contents are forced by turgor pressure thus leading to increased branching (Wilson 1970).

The phenomenon of hyphal tip disintegration when colonies are flooded with KCl, sucrose and other solutions is described by Robertson and Rizvi (1968).

When colonies of Neurospora crassa are flooded with 0.5M sucrose, the cells plasmolyse and cease growth. This is followed by resumption of growth at the apex (Rizvi & Robertson 1965). Their interpretation is that sucrose molecules pass into the cell and an osmotic equilibrium is established. When colonies are flooded with sorbose, the tips, after recovery from plasmolysis, grow normally or as dichotomous branches for some minutes and then burst. If hyphae are continuously bathed in sorbose, a repeated sequence of branching and disintegration is found at the colony margin and growth is retarded.

High concentrations of snail enzyme cause the formation of dense colonies with lots of aerial hyphae and the hyphal tips do not burst even when flooded with water. The wall of apical cells grown on 2% - 5% enzyme medium appeared thin but otherwise normal. In older hyphae, the wall and a large part of the septum appeared to be eroded. However, the hyphae retain their original shape which indicates that a rigid component of the cell wall remains.

Sorbose-restricted colonies have thin hyphae and thicker

walls. When enzyme was added to sorbose medium, there was an increase in colony diameter and a decrease in the number of disintegrated tips. The snail enzyme splits the sucrose into glucose and fructose so the glucose counters the sorbose effect. However, this does not explain why the apex is protected by the enzyme from osmotic shock.

When an auxotrophic mutant of Saccharomyces cerevisiae is starved of inositol, spheroplasts in an isotonic medium begin rapid lysis at 2½ hours. (Atkinson et al 1976). Increasing the osmolarity of the medium delays lysis and prevention can be achieved by raising the osmolarity just before the spheroplasts are due to lyse. Osmotic rescue suggests that lysis is due to an increase in cytoplasmic osmotic pressure and not to inhibition of metabolism. These results may help to explain the bursting of hyphal tips when the cell wall is weakened at the tip by sorbose.

The growth of vegetative and aerial hyphae of Neurospora crassa were investigated by McGuire & Siegel (1975). Vegetative hyphae showed 90% viability in minimal medium, 79% on solid medium and 85% on solid sorbose medium. Aerial hyphae showed 11%, 27% and 11% viability respectively. Viable aerial hyphae seemed to be resistant to sorbose during the 6 hours after isolation but resistance is lost by the time the colony is 24 hours old. McGuire & Siegel (1975) suggest that aerial hyphae contain modified cell wall enzymes. The wall has two layers, the outer one not covering the apex. They propose that the inner layer is rigid and the outer layer mediates cellular-environmental interactions. Aerial hyphae differ from vegetative hyphae in their amino acid content of the outer layer and the susceptibility of the inner chitin layer to hydrolysis.

The reduction of radial growth rate of Coprinus lagopus by 2 - deoxy - D - glucose, D - glucosamine and L - Sorbose is reported by Moore & Stewart (1972).

Paramorphogen	Concentration which gave 50% reduction (mM). 2-deoxy-D-glucose	D-glucosamine	L-Sorbose.
control	.04	.30	0.80
5mM acetate	.05	.20	1.50
fructose	.08	.20	1.00
mannose	.40	.70	9.00
glucose	4.00	9.00	60.00

At low concentrations, the effects were additive but competition and interaction occur at higher concentrations.

Normally, circular colonies became lobed with an irregular outline and hyphal compartments became wider and shorter. D-glucosamine caused the apical compartment to balloon, regrowth was by a single branch to the side of the tip. 2-deoxy-D-glucose caused the apical cell to vacuolate and burst. Sorbose caused swelling but the apex did not burst and growth was resumed by the formation of multiple branches at the tip.

It is unlikely that sugar transport is affected as the greater inhibition occurred on media without a sugar carbon source. Therefore, inhibition must occur after they have entered the hyphal cell. The effects are not osmotic but related to molecular structure.

Moore & Stewart (1971) reported that 2-deoxy-D-glucose caused outgrowths of Coprinus lagopus dikaryotic hyphae destined to become clamps to continue to grow away from the parent hyphae as monokaryotic branches.

It was observed by Megnet (1965) that only growing cells were killed by 2-deoxyglucose and fragments of lysed cells

appeared in the culture of Schizosaccharomyces pombe. A resistant mutant was found which was unable to use glucose as a carbon source and it was also deficient in hexokinase. It is concluded that in this organism, 2-deoxyglucose inhibits some reactions in the synthesis of cell wall polysaccharides. Johnson (1968) found that sites of 2-deoxyglucose induced lysis coincided with regions of growth of the glucan layer of the wall of yeast cells. Working with Saccharomyces cerevisiae, Farkaš et al (1969) narrowed the site of inhibition down to the fibrillar glucan component. 2-deoxyglucose is not incorporated into the cell walls and cells lyse at sites normally of extensive wall synthesis.

In 1947, Littman described a medium for isolating fungi from speciments on which the spread of fast growing fungi was restricted making isolation for pure culture easy. The medium contained 1% dextrose, 1% peptone, 1.5% dehydrated oxgall, 2% agar with crystal violet and streptomycin to inhibit bacterial growth. The 34 tested fungi grew as non-spreading, well separated colonies.

Tatum et al (1949) found that 9×10^{-4} M sodium deoxycholate produced discrete colonies of Neurospora crassa and Syncephalastrum racemosum. The effect was not influenced by inositol and there was no difference in the inositol content of the fungus. Colonies tended to form aerial mycelia earlier than on sorbose media.

Various detergents were tried and the paramorphogenic activity varied.

Anionic agents which showed paramorphogenetic activity were sodium lauryl sulphate, dioctyl sodium sulphosuccinate, sodium 3, 9 diethyl tridecanol-6-sulphate (Tergitol 7), sodium deoxycholate while sodium oleate was inactive. Cationic and Non-ionic agents were inactive and cationic agents were also toxic.

Colony diameter of Aspergillus nidulans was inversely proportional to concentration of sodium deoxycholate (Mackintosh and Pritchard 1963) and the number of colonies per plate. The concentration used in media for replica plating was 0.08% (2×10^{-5} M) as germination was not affected and marginal hyphae appeared normal. Sodium lauryl sulphate was found to behave like sodium deoxycholate.

Marked restriction of colonies of Chaetomium aureum was achieved with 0.025% - 0 - 1% (6×10^{-4} - 2×10^{-3} M). At 2×10^{-3} M restriction was greater at pH 6.5 than at pH 7.0 and the fungus showed abnormal growth at this concentration. Ghora(1973)

Threlfall (1972) investigated the effect of sodium deoxycholate on wild type and pentachloronitro benzene (PCNB) resistant Aspergillus nidulans. 2×10^{-3} M reduces the colony diameter of wild type to 60% of control and of PCNB resistant strain to 53%. Conidiation remains normal and there is no effect on either hexosamine or hexose content of the cell wall.

Sodium deoxycholate was used to dissolve membrances of animal microsomes to obtain ribosomal preparations. (Klein at al 1967). It was also used in experiments with m RNA of yeast to release 80S monomers and polysomes from membrances. It was found that sodium laurylsulphate binds to protein. The binding is a function of the polypeptide moiety of protein and is not influenced by large amounts of carbohydrate. It depends on the ability to unfold the protein molecule and is restricted by S - S groups, influenced by salt concentration and the charge on the protein molecule. (Pitt-Rivers & Impiombato 1968).

As sodium deoxycholate exhibits detergent properties, it is not surprising that lipids are affected. The level of lipase activity in Candida lipolytica was increased with an increase in the phosphorus content and the introduction of sodium deoxycholate,

cholate and acetate into the medium. The action of sodium deoxycholate may be to decrease the surface tension at the boundary of the lipid and aqueous phase and promote an improvement in the enzyme contact with substrate. (Zvyagintseva 1972).

Ksandopulo (1974) reported that sodium deoxycholate stimulated lipolytic activity of Geotrichum as a result of an increase in absorption of lipase on the oil water interface.

Sodium deoxycholate caused an increase in toxin yield from Vibrio cholerae due to the release of cell bound toxin.
Fernandes + Smith (1977)

Hyphal paramorphs are initiated by the effect of the paramorphogen on the germ tube. Spore germination describes the events which occur when a dormant spore initiates normal metabolic activity to form a mycelium. Reviews on spore germination have been written by Gottlieb, 1950; Allen, 1965; Sussman & Halverson,1966 and Schmit & Brody, 1976.

Sorbose does not appear to inhibit germination but reports on sodium deoxycholate vary. Kaplan & Walls (1971) found a reduction in the viability of Coprinus lagopus when grown on sodium deoxycholate medium but Mackintosh & Pritchard (1963) reported that sodium deoxycholate was not mutagenic and did not affect the viability of Aspergillus nidulans conidia.

Neurospora crassa conidia released a ninhydrin positive factor which was found to be essential for germination when incubated in buffer at low water potential. The damage to the spores is not lethal as in most cases conidia recover when transferred to nutrient medium. Charlang & Horowitz (1971 & 1974).

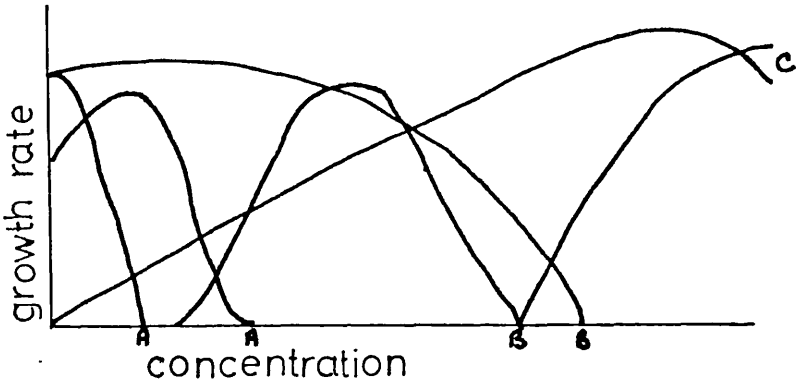
Water is necessary for spore germination to leach out inhibitors from the cells (Gottlieb 1973) and to rehydrate the spores (Yarwood 1950). The limits of water activity at which spores will germinate varies with the fungus concerned.

Stachybotrys atra requires aw > .95 while Aspergillus chevalieri will germinate after a long lag phase at aw 0.7 (Ayerst 1969). Conidia of Erysiphe spp. will germinate at relative humidities approaching zero and not in free water, the optimum being 100% RH (Yarwood 1950). These spores have a high moisture content 52 - 75% compared to other species whose spores contain between 6 and 25% water. Yarwood suggests that spores need to rehydrate to the levels of Erysiphe conidia before they will germinate.

The first phase of germination is shown by the spores swelling and this has two stages. The first is physical and dead spores show swelling to some extent (McRobbie et al 1972) while the second stage is metabolic and requires glucose in Rhizopus (Ekundayo 1966). Although spores of Rhizopus fail to swell in water, ^3H_2O and H_2O are freely exchangeable showing that the spore wall is permeable to water although glucose is needed before swelling and accumulation of water can take place.

Studies of the growth of fungi at low water potentials have been stimulated by the use of controlled environmental conditions in the preservation of foods with high concentrations of sugar or salt. (Ayerst 1968). Other areas where water relations have been of interest are those of soil ecology and host-parasite relationships of fungi. Fungi which grow at low water potentials are usually osmotolerant but a few may be true osmophiles. The relationship between the three groups of fungi, intolerant (A), facultative or tolerant (B) and obligate (C) (Ingram 1957) is shown below.

The relationship between concentration and growth rate for(A) intolerant, (B)facultative and (C) obligate osmophilic fungi.



The water requirements of organisms are influenced by other environmental factors (Scott 1957).

a) Nutritional value or toxicity of the solutes used to lower a_w.

b) Temperature.

c) Oxygen is less soluble as the a_w of a solution decreases.

d) pH.

e) Presence of inhibitors, e.g. CO_2.

f) Ability to adapt to a low water potential environment.

The osmotic pressure or osmotic potential of a solution, at a given temperature is defined as that pressure which must be exerted on the solution to prevent any net movement of solvent between the solution and its pure solvent, when these are separated by a perfect semi-permeable membrane. (Morris 1968).

$$\overline{\Pi} \propto \frac{n_2}{V} \quad T$$

$\overline{\Pi}$ = osmotic pressure in atmospheres.

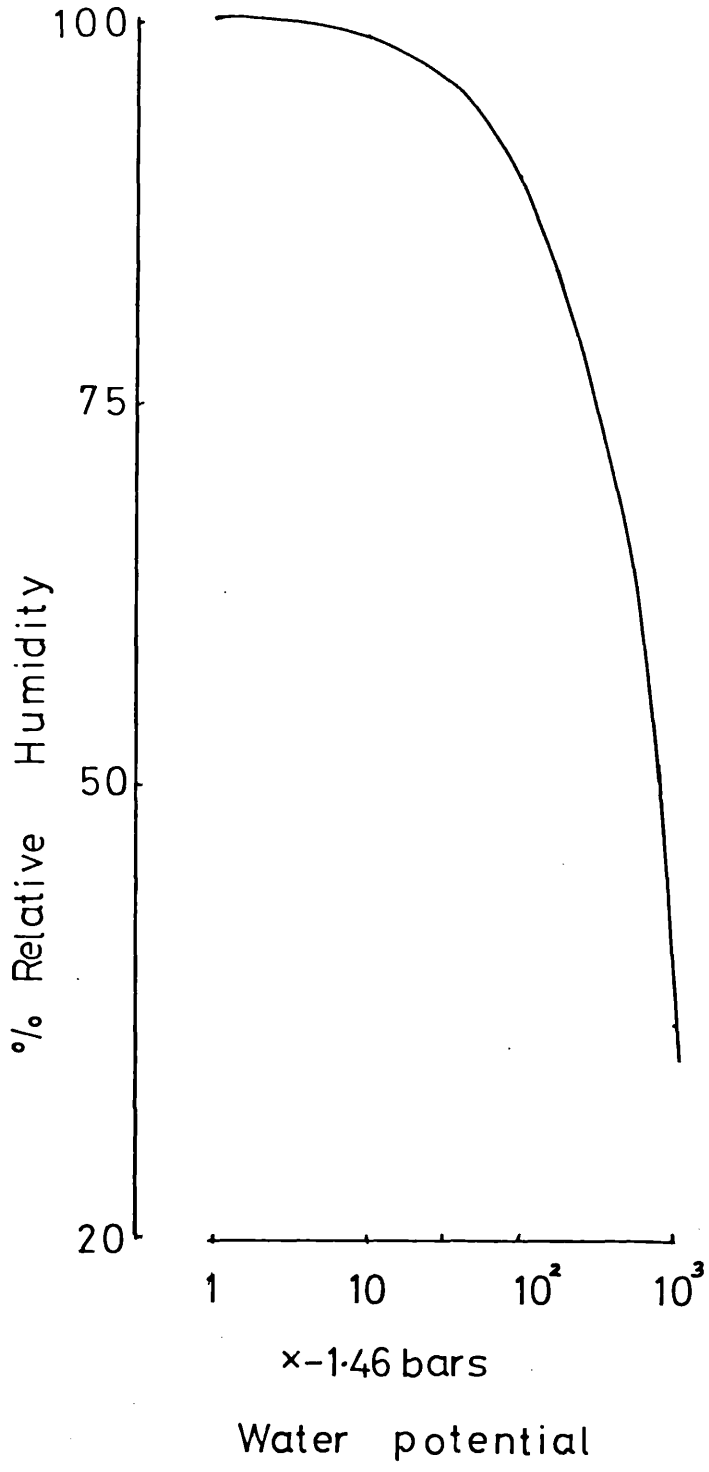
n_2 = number of moles of solute in V litres of solution.

T = temperature. ^{o}K.

Vapour pressure of a solution or solvent is the partial pressure of its vapour when at equilibrium with the liquid at a

Fig 4

The relationship between Relative Humidity and Water Potential (Leyton, 1975)



given temperature and pressure.

Water activity $a_\omega = {}^{P}/_{Po}$. P = vapour pressure of solution.

Po = vapour pressure of solvent.

Relative humidity (%) = ${}^{P}/_{Po}$.

$\pi = -4.555 . T . \log_e a_\omega$. (Curran 1971).

The diffusion of water molecules into a cell causes the volume of the vacuole to swell and press the cytoplasm against the cell wall. This pressure is Turgor pressure (Galston 1964).

Much of the work cited in biological literature gives pressure measurements in bars (or atmospheres) -

1 bar = 10^6 dyne cm^{-2} = 10^5 Pascals.

1 atmos. = 1.01325×10^6 dyne cm^{-2} or 760 mm Hg.

1 Pascal = 1 M^{-1} Kg S^{-2} = 10 dyne cm^{-2} .

101325 Pa. = 1 atmos.

while the current S I unit is the Pascal. (Leyton 1975). Fig 4

Osmotic pressure can be measured by methods using the depression of the freezing point of the solvent by the dissolved solute. This method is not satisfactory for concentrated solutions or solutes of high molecular weight as the solutions do not freeze rapidly. Various methods of determining water activity by a dew point technique have been reported. (Anagnostopoulos 1973, Ayerst 1965, a & b). The water potential measurements of various concentrations of polyethylene glycol 4000 were compared by Mexal & Reid (1973) and they found considerable variation between different methods and authors. The range of water potential (- bars) for a 16% solution was 5 - 11 bar and for a 24% solution 12 - 26 bar.

The ability of micro organisms to grow at low water potentials enables them to colonise substrates which are stored dry to preserve them, e.g. grain or food with a high sugar content. Reviews on.

the water relations of food spoilage micro organisms have been made by Christensen, 1957; Ayerst, 1968; 1965a; Panasenko,1967, Scott, 1957 & Ingram, 1957).

Soil micro organisms have to contend with both matric and osmotic water potential. (Griffin, 1963). The dominant fungi in the soil are species of Penicillium and Aspergillus at 75% - 90% R H. (Chen & Griffin,1966).

A table of a_w at which the growth of pathogenic fungi is optimal, reduced to 50% and prevented by osmotic matric potentials and relative humidity in air, is given by Cook and Papendick (1972).

Temperature/water potential interactions are discussed by Cook and Christen (1976). Maximum growth rates were obtained at the following water potentials of the medium when adjusted, using salts.

	20°C to 30°C.	35°C.
	(- bars)	
Fusarium roseum Graminearum.	10 - 28	28 - 55
" " culmorum	8 - 14	28
Gaeumannomyces graminis tritici.	8 - 12	no growth.

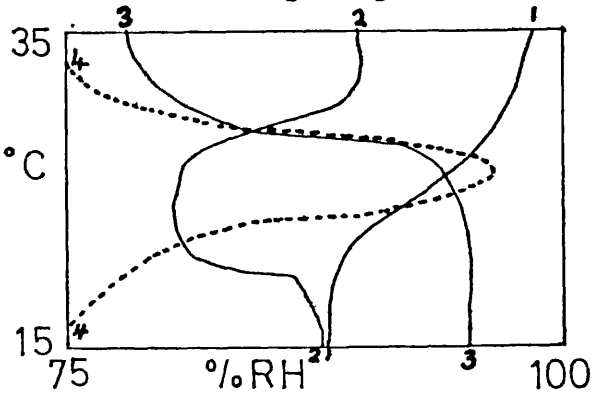
For these fungi, drier conditions were required for maximal growth as the temperature was increased from 10° - 35°C.

Thakur (1973) investigated the effect of osmotic-concentration and temperature on species of Aspergillus found in alkaline or fertile soils. 0.3M - 0.5M NaCl or cane sugar stimulated pigment production while 1.8M - 2.4M was toxic and inhibited pigmentation. Species from alkaline soils were more tolerant of higher temperatures (35°C), pH (8 - 9) and osmotic concentrations 1.8 - 2.0M compared to fertile soil species.

Growth of Fusarium roseum f. sp. cerealis "Culmorum" on media of different osmotic and matric potentials is reported by Cook et al (1972). Although stimulation was observed on media

from 0 to - 8 bars, there was no such effect on the fungus grown on straw in the same range of matric potentials.

There are four main groups of fungi based on their reaction to relative humidity and temperature (Chen & Griffin 1966) which is shown in the following diagram -



Group 1. - survive lower RH values at lower temperatures.

Group 2. - survive lower RH at optimum temperatures.

Group 3. - survive lower RH at higher temperatures.

Group 4. - Aspergillus glaucus group - survive high RH values at optimum temperatures.

The radial growth rate responses of Phytophthora cinnamoni and Alternaria tenuis to osmotic and matric water potential were compared. Osmotic potential was controlled with KCl or sucrose and matric potential by using three texturally different soils. Optimum growth in agar and soil was obtained at between -5 to -15 bars. With decreasing water potential, both fungi were less tolerant to matric than to osmotic stress. The matric potential at which growth ceased was $\frac{1}{2}$ to $\frac{1}{3}$ of the corresponding osmotic potential.

The osmotic pressures of pathogenic fungi have been reported as being much greater than the osmotic pressure of their hosts' cells (Ronsdorf (1934) in Thatcher (1939)).

Uredinio spores of Puccinia triticina have an osmotic pressure of 49.5 atmos, the germ tubes of P. simplex an osmotic pressure of 30.4 atmos, while the cells of barley are only

9.5 atmos. Using plasmolytic methods with $CaCl_2$, Thatcher (1939) found that the osmotic pressure of each parasite was greater than its host. Rust infection is reported to increase the permeability of the plasma membrane of the host cell (Thatcher 1935 in 1939). As the osmotic pressure of the fungus is higher than the neighbouring host plant cells, water can be obtained from them even when they are not completely turgid. In non-turgid cells with a more permeable membrane, solutes diffuse out into the free water in the cell wall so the fungus can obtain nutrients before haustoria etc., have been formed.

An account of the effect of osmotic pressure on mycorrhizal fungi is discussed by Mexal & Reid (1973). There is a time lag associated with initiation of growth at low a_w. Thelephora has a 6 week lag and Cenococcum 3 - 4 week lag at -10 bars.

Salts and sugars have been used to adjust osmotic potentials of media but as these are metabolised, their osmotic effects cannot be distinguished from toxic or increased substrate effects.

Gee et al (1965) reported that using Na Cl in extracting chloroplasts caused a loss of the limiting membrane, ground substance and inter grana lamellae. In 1.0 M Na Cl, a Staphylococcus aureus mutant autolysed and released osmotically sensitive protoplasts. This was due to the activation of N - acyl - muramyl - L - alanine amidase which is bound to the cell wall (Gilpin at al 1972).

High sugar concentrations maintain a mycelium in a fermentative condition. The more sugar provided, the more ethanol is produced and conidiation prevented. This catabolite repression is manifasted in both Krebs and glyoxalate cycles. The metabolite flow is directed through glycolysis and the energy spilled into fermentation in Neurospora vegetative hyphal tips and yeast vegetative buds (Turian 1973). Yeast grown on glucose from

1 - 50g / litre (up to 03M) shows aerobic fermentation while on galactose up to 5g / litre respiration is aerobic but at higher concentrations, metabolism becomes increasingly fermentative. (Brown & Johnson 1970). They suggest that high sugar concentrations may be associated with repression of mitochondrial structures. The fatty acid content of yeast grown at low concentrations is 8% of the dry weight but at high sugar concentrations, it is 5%.

When the osmotic pressure of media was adjusted with glucose, glycerine or both, the growth response at the same osmotic pressure was different on different media. On glycerine, the growth rate was the same at all osmotic pressures (up to 29.3 atm) and slower than on media containing glucose. The effect of osmotic pressure in reducing radial growth rate was more marked on glucose media.

The strong inhibitory effects of very low concentrations of Carbowaxes on the rate of elongation of root hairs suggest that it does not only reduce the osmotic pressure of the medium. The effect of Poly-ethylene-glycol on indole acetic acid-induced elongation of oat coleoptiles was similar to that obtained by equivalent concentrations of mannitol. The loss of anthocyanin from beet tissue was not increased by Poly-ethylene glycol and this suggests that Poly-ethylene-glycol may be suitable for multicellular systems such as coleoptiles but not suitable as osmotic agents in studies using single cells, e.g., root hairs. (Jackson 1962).

In higher plants, the effect of Poly-ethylene glycol 200 and Mannitol was different from that of Poly-ethylene glycol of molecular weights of 1000 or more. All molecular weights affected respiration to the same extent and it was not the detergent properties of Poly-ethylene glycol which caused toxity.

High molecular weight Poly-ethylene glycol causes blocking of the pathways of water movement, a reduction in water absorption and so causes desiccation of the plant. Poly-ethylene glycol 4000 does not enter roots unless they are damaged. (Lawlor 1970).

Poly-ethylene glycol is an unsuitable carbon source for mycorrhizal fungi (Mexal & Reid 1973). When Cenococcum was grown on agar containing ^{14}C Poly-ethylene glycol, labelled Poly-ethylene-glycol was found in the hyphae but only at 5% of the original concentration and it was found to be inert in the fungal cell. At -20 bars, Cenococcum grows on the sides of flasks before growth is seen in the liquid medium, possibly because oxygen is the limiting factor as only 25% oxygen is dissolved when distilled water is present in Poly-ethylene-glycol solution.

Kidd et al (1977) describe a method of growing Ceratocystis montia on liquid medium which minimizes the problems usually associated with reduced oxygen tension of Poly-ethylene glycol media and avoids using agar to solidify medium which alters the matric and osmotic properties. Glass beads were placed in a petri dish and nylon netting laid on top to support the mycelium. The netting was soaked in liquid medium which was added to the petri dish so that it covered the beads but did not submerge the netting.

The lags observed are not due to Poly-ethylene glycol absorption as it would only take a few days to induce the necessary permease system.

Anand & Brown (1968) report that increased size of Poly-ethylene glycol causes a decrease in growth rate and suggest that this may be caused by other physico-chemical properties of the molecular size rather than osmotic pressure.

The specific growth rate of yeast was measured at different

water potentials (a_w) using Poly-ethylene glycol 200 to adjust the medium to the required a_w (Anand & Brown 1968). The yeasts were less tolerant of low a_w in the presence of Poly-ethylene glycol than sugar. The minimum a_w required for growth with Poly-ethylene glycol was the same for osmo-tolerant and intolerant strains but not the same when sugar was used. The tolerant strains had a broad a_w optimum and intolerant strains a sharp optimum. Neither group had a general requirement of $a_w < 0.997$. In this case, osmo-tolerant is better termed sugar-tolerant.

Using Salts to adjust osmotic pressure, 50% reduction in growth rate occurs at -20 to -30 bars in Endothia (Hunter et al 1975) and at -49 bars in Verticillium (Congley & Hall 1976). Growth of Verticillium is completely inhibited at -119 bars. Optimum growth of Aspergillus niger occurs at 40°C and 93% relative humidity. At 100% humidity, the optimum temperature is 30°C. The range over which growth will take place is 76 - 100% RH and 15°- 47°C but at the extreme conditions, a lag of 100 hours occurs (Bonner 1948).

When Fomes fomentarius was grown at high osmotic pressures (-21.1 to-29.3 bars, Vincent 1969) growth was retarded and the hyphae meandering rather than straight. As the osmotic pressure was increased the hyphae were shorter, cells bulged and the cell wall was thicker. The cells produced vacuoles earlier and in greater numbers, resembling old forms of the mycelium. This was confirmed by the accumulation of large quantities of glycogen. These changes in morphology are characteristic of known aging processes and can also be brought about by extremes of pH.

Yeast colonies on solid media tend to change from smooth to rough colony form or in liquid media, from round to long cells

with increasing osmotic pressures. These changes are associated with staling. (Ingram 1950).

In Achlya bisexualis (Darnaud 1972) an increase in osmotic pressure of the medium from 3.2 to 7 atmospheres reduces the growth rate and causes changes in morphology which point to an increase in the osmotic pressure of the cell. The hyphae become irregular in outline, thicker, more branches and the tips dichotomise or form lateral branches.

Ceratocystis montia grown at -20 bar showed only 2 mm of growth after 12 days and even at -2bars, the colony diameter was reduced to 60 mm from 70 mm after 12 days. (Kidd et al 1977).

In studies on the water relations of fungi, care must be taken to ensure that the substances used to alter the water potential of the medium are not toxic and do not alter the metabolism of the organism. If this is found to happen, then reduction in growth rate and other changes cannot be solely attributed to the osmotic effect of the medium.

Brown & Johnson (1970) reported that the fatty acid content of yeast varied with the type of metabolism. By increasing the concentration of glucose from 2 to 10 g/litre, the total fatty acid composition of Saccharomyces cerevisiae was reduced from 8% to 5% of the dry weight with a disproportionate decrease in the unsaturated component. This was accompanied by a decrease in oxidative degradation of glucose and a possible decrease in mitochondrial lipid (Brown & Johnson 1971). In Candida utilis there was accumulation of deposited fat because oxidative metabolism of glucose is not repressed. The increased amounts of fatty acid correlate well with an increase in triglycerides.

In 3.22M ethylene-glycol or glycerol media, Neurospora crassa conidia grow as single cells which are disrupted by an abrupt decrease in osmotic pressure of the medium. Ethylene-

glycol inhibits germ tube emergence by either metabolic inhibition of germ tube formation (but not of single cell growth) or by insufficient internal pressure of the germ tube. Mitochondrial fractions of osmotically disrupted cells have a low cytochrome content. (Bates & Wilson 1974).

Penicillium baarnense produces abnormal fruit bodies on media where the osmotic pressure has been increased by diethylene glycol (Bouvier 1973). Sexual reproduction is also affected by a mutation governing the transfer of homo cysteine to methionine. Restoration of normal fruiting is obtained when the osmotic pressure is lowered by diluting the medium. Bouvier (1973) suggests that this is likely to remove some permeability barrier in the intra-cellular medium.

The growth of four soil fungi Geastrum sp, Fusarium moniliforme, Penicillium canescens and Phytophrhora cinnamomi was studied by Wilson & Griffin (1975). The osmotic potential of the medium was increased with KCl. At high osmotic potentials (up to -201 bars) radial growth rate and respiration were decreased, the lag increased (24 hours at -81.2 bar) and more oxygen was required to produce unit growth. Growth was retarded by the inability to maintain sufficient turgor pressure in the cells. More energy was required either for osmo regulation or because respiration became partially uncoupled from energy production. Osmo-regulation may be by a water pump mechanism, accumulation of solutes or the increase in concentration of small molecules, e.g., amino acids by altered metabolism. Thatcher (1939) also suggests that these osmo-regulatory processes may be present in cells which rapidly recover from plasmolysis. Recovery is not necessarily due to the uptake of the substances used to lower water activity, as Carbowax 4000 gave similar results to mannitol and glycerine, but to absorbing solutes of the growth medium or to

internal mobilization of osmotically active substances.

The osmotic equivalent of growing tips is greater than the growth medium resulting in the presence of a hydrostatic pressure differential between the interior of the hyphal tip and the environment. Normal extension growth is dependent on this differential (Park & Robinson 1966). Hyphal tips are able to equilibrate to applied hypertonic or hypotonic solutions so as to re-establish a pressure differential consistent with the rate of wall synthesis at the apex. Apices that are caused to cease extension growth by applied hypertonic solutions occlude the apex by continued formation of wall substances in the absence of a sufficiently high pressure differential to push growth forward.

Links et al (1957) describes the "Bulging factor" of an antibiotic-like streptothricin which causes hyphal tips to swell. As water diffuses into young growing hyphae, that in excess for growth is removed by an energy requiring mechanism. The "Bulging factor" may interfere with this or act on the cell wall material or its biosynthesis apparently changing the directed plasticity of the growing apex.

The turgor pressure of cells of Nitella (Green 1956) in dilute soil extract was 0.28M. When the turgor pressure was reduced to 0.14M by Carbowax 750, the growth rate was reduced and cellulose synthesis was shown to be inhibited by the initial reduction in turgor.

The morphology of Aspergillus wentii showed little change until the media osmotic pressure was -61.2 (Adebayo et al 1971). However, in Mucor hiemalis the apparent lack of involvement of turgor pressure in growth retardation and the relationship between substrate water potential and Kr : and internal osmotic water potential and Kr indicate that solute induced growth retardation involves an inhibitory influence of low internal

osmotic water potential on growth.

In substrates of matric controlled water potential there is a lack of solutes for uptake to attain the low internal water potential. This may be the reason why growth is inhibited at matric potentials of $\frac{1}{2}$ to $\frac{2}{3}$ that osmotic potential (Adebayo & Harris 1971).

When investigating the adaption of sugar-tolerant yeasts to high sugar concentrations (Brown & Simpson 1972) it was found that the permeability of the cell to sucrose was different for tolerant and intolerant yeasts. Intracellular accumulation of polyols, hexitol, arabitol and glycerol was found in all tolerant yeasts investigated but not in the intolerant strains. The polyols were present when the yeasts were grown on synthetic medium and were not washed out of the cell so are probably bound.

Although yeasts synthesize the polyols, they probably function like K+ does in halophilic bacteria to increase the osmotic pressure of the cell. The kinetics of inhibition for sucrose are similar to those for Na+. (Davis et al 1973).

Brown (1974) proposes two explanations of sugar tolerance. There may be different proteins present in the sugar tolerant yeasts as there are in halophilic bacteria (Brown 1964). Intracellular composition may be different so the effect of the environment is lessened, e.g., NADP - specific isocitrate dehydrogenase of Saccharomyces rouxii is indistinguishable from S. cerevisiae in kinetics, water relations, and electrophoretic properties. The primary determinant of the water requirements of these enzymes is not a_w but the type and concentration of solutes.

Differences in metabolism of S. rouxii (tolerant) and S. cerevisiae (intolerant) were further investigated (Brown 1974). S. cerevisiae produced invertase and ^{14}C sucrose was recovered only as breakdown products. In S. rouxii, however, ^{14}C Sucrose

was recovered and no invertase produced. The equilibrium concentration of sucrose in S. rouxii is about 40% of the external concentration. Lactose is not metabolized and is accumulated to 70% of the external concentration in S. cerevisiae and only 10% in S. rouxii but this may be only in the cell wall and not in the protoplasm. Isolated cell walls accumulated 16% in both strains.

S. rouxii accumulated arabitol, glycerol, hexitol and mannitol. Arabitol accounted for 38% of the dry weight of cells grown in basal medium and 45% of those grown in synthetic honey broth. Although less polyol was produced in basal medium, more was retained on washing than from those grown on supplemented medium. Yeast grown in Poly-ethylene-glycol lost a lot of polyol on washing.

Experiments with amphoteracin indicate that membrane integrity is a major requirement for retaining the polyols.

The differentiation of vegetative mycelium to form fruit bodies is also subject to water relations. In the Aspergillus glaucus group, cleistothecium production is inhibited by osmotic pressures greater than-450 bar. Conidial production is enhanced up to-150 bar and then decreases (Curran 1971).

No perithecia are formed by Neurospora tetrasperma at 37°C on synthetic medium but if sucrose is replaced by xylitol or ribitol then perithecial production is stimulated up to 0.5M. Poly-ethylene-glycol, Na Cl or increased sucrose concentrations did not induce perithecia at 37°C so this is probably a metabolic effect rather than an osmotic effect. (Viswanath - Reddy & Turian 1975).

There was no differentiation into primary spores by Aphanomyces euteiches at less than -5 bar. Spores were formed at -4 bars but were not active until -2 bars with best production at

-0.5 bars. As the water activity of the solutions decreased, the osmotic pressure of the mycelium increased to -14 bars at which concentration the turgor pressure was nought. (Hoch & Mitchell 1973).

The effect of humidity and moisture on spore germination is reported by Snow (1949) and Ayerst (1969). Schein (1964) points out that at RH $>$ 90% condensation occurs readily on the walls of the chambers used in germination experiments because a drop of 1°C will cool the atmosphere to below its dewpoint. Most incubators are only able to be controlled to $\pm$ 1°C. If condensation takes place, the germination probably occurs in free water. (100% RH). This effect is less of a problem $<$ 90% RH.

When NaCl or KCl was used to reduce a_ω of media, germination of Verticillium dahliae was reduced by 50% at -11 to -14 bar and completely inhibited at -98 bar. If sucrose was used to adjust a_ω the values were -40 bars and - 114 bars respectively. (Congley & Hall)(1976). Timofeeva (1971) reports that microsclerotia of V. dahliae germinate on substrates at 14.07 - 44.2 atmos. The lag is increased to 7 days on substrates of 58.9 atmos. and a torulose growth of the germ tube is observed.

The most rapid germination of V. albo-atrum occurred at 25°C in basal medium a_ω .9964 and was inhibited at 15°C and 30°C. If a_ω was reduced to .9778 then germination was greater at 30°C than at 25°C. (Mozumder et al 1971).

Four species of the Aspergillus glaucus group of fungi were tested for their ability to germinate at low humidities. (Snow 1949). The critical humidity for A. chevalieri was 73%. Germ tubes produced at these very low humidities were often badly misshapen, swollen and had thicker tips.

Alternaria spores germinate best in 0.3M sucrose. The half times in 1.0M sucrose are 4 hours and in 2.0M sucrose 16 hours.

(Waggoner & Parlange 1976). Changes in the concentration of the medium during germination retard germination in proportion to the number of changes. The spores require at least 87% relative humidity (-190 bars) to germinate.

In 3.22M ethylene glycol or glycerol Neurospora crassa conidia grow as single cells and do not form germ tubes. (Bates & Wilson 1974). At lower concentrations 0.82 - 2.7M conidia germinate to form colonies with diameters inversely proportional to concentration. When placed in 3.22M glycol, conidia plasmolyse rapidly and recover within 5 minutes suggesting rapid entry of glycol into the cell. The walls of conidia cultures in glycol have a refractive index of 1.41 which is the same as for hyphal tips. Untreated conidia and old walls have a refractive index of 1.36. Concentrations of ethylene glycol and propylene glycol above 3.22M caused extensive cell death but glycerol at 6.4M did not.

When a_w of a medium is lowered with Na Cl or non-electrolyes, the main effects on micro organisms are :-(Charlang & Horowitz 1971)

a) prolongation of lag phase.

b) reduction of growth rate. (Wilson & Griffin 1975).

c) reduction in total amount of growth.

Solutes show different toxicities to Neurospora crassa. At a_w.98 or less, glycerol $<$ NaCl $<$ glucose $<$ sucrose but at higher a_w metabolism of the solute interferes with the water activity effect, e.g., at $a_w > .985$ glucose accelerates growth.

The addition of Ca^{2+} or Mg^{2+} to NaCl medium increases the growth rate and yield but does not shorten the lag phase. The solubility of oxygen decreases with increased solute concentration so oxygen starvation may be partly the reason for low

effects.

A heavy inoculum was found to reduce the lag phase (Charlang & Horowitz 1971) and they proposed that a substance necessary for germination was lost at low a_w. This was confirmed by inoculating media from which germinated conidia had been removed and obtaining a higher yield. This germination factor was released at a_w.936 and can be extracted from young mycelium. Chloroform and antibiotics known to increase cell membrane permeability release the germination factor which suggests that low a_w increases the permeability of the membrane. However, most conidia recover from a_w damage when transferred to nutrient medium.

Penicillium chrysogenum and Aspergillus nidulans which are more xerotolerant than Neurospora crassa retain this germination factor and germinate at $a_w < .9$ although it is released by chloroform. It is suggested that the ability to germinate at low a_w is related to resistance to permeability changes.

A spore enzyme from Bacillus cerues causes non-viable spores previously treated with urea and thioglycollic acid to germinate. The enzyme was released from spore debris by an increased pH or raising the solute concentration. Chemical release of the enzyme was by polyamine spermine which has a strong affinity for electronegative structures, e.g. cell membranes and probably releases the enzyme by displacing it. (Gould et al 1966).

The most extreme manifestation of paramorphogenesis is dimorphism where the change from a mycelial phase to a yeast phase of growth may be induced by environmental conditions.

Dimorphism is defined by Romano in his review (1966) as "an environmentally controlled reversible interconversion of yeast (Y) and mycelial (M) forms of a fungus and is denoted as $Y \rightleftharpoons M$."

The transformation from Y to M, or M to Y, may be induced by various external conditions - see Table 8 including the composition of the substrate on which the fungi are growing. Therefore, dimorphism may be considered as a paramorphic response to a changed environment.

Much work on the phenomenon of dimorphism has been done using pathogenic fungi, e.g., Sporothrix schenckii, Paracoccidioides brasiliensis, Blastomyces dermatitidis, and Histoplasma capsulatum or Mucor rouxii as the Y phase is the usual form in which the fungus enters and spreads through the host.

Table 8

Factors which influence the growth form of some fungi.

Factor	Organism	Reference
Temperature	Histoplasma capsulatum	Salvin 1949a
	Blastomyces dermatitidis	" "
	"	Levine and Ordal 1946.
Carbon dioxide	Trichophyton mentagrophytes	Chin and Knight 1957.
	T. rubrum	" " "
	Mucor rouxii	Bartnicki - Garcia and Nickerson 1962a.
Temperature and CO_2	Sporothrix schenckii	Drouhet and Mariat 1952.
Carbon source	Trigonopsis variabilis	Matthewson and Barnett 1974.
	Candida albicans	Nickerson and Mankowski 1953.
Hexoses	Mucor rouxii	Bartnicki Garcia 1968.

Factor	Organism	Reference
Amino acids	Sporothrix schenckii	Drouhet and Mariat 1952.
	Trigonopsis variabilis	Senthe Shanmugathan and Nickerson 1962a.
	" "	Sasek and Becker 1969.
	Candida albicans	Nickerson 1950.
- SH group.	Histoplasma capsulatum	Salvin 1949a.
Biotin	" "	" "
Manganese	Chaetomium globusum	Barnett and Lilly 1966.
	Aspergillus parasiticus	Detroy and Ciegler 1971.
	Penicillium claviforme	Tinnell et al 1977.
	P. clavigerum	"
Osmotic pressure	Saccharomyces rouxii	Koh 1975.
Crowding	Glomerella cingulata	Lingappa and Lingappa 1969.
	Septoria spp	Stevens and Hall 1909.
	Fusarium	
	Colletotrichum	
	Gloeosporium	
KCN	Mucor rouxii	Friedenthal et al 1974.
Age	Trichophyton gypseum	Wilhelm 1947.
	Epidermophyton floccosum	"
Antibiotics	Paecilomyces viridis	Barathova et al 1972.

a) Temperature.

Temperature is the most important factor in determining the growth form of Blastomyces dermatitidis (Salvin 1949b). At 25°C, growth is mycelial while at 37°C it is yeast-like and this is independent of the composition of the medium.

Sporothrix is effect by temperature and Co_2 level (Drouhet and Mariat 1952) and Histoplasma by temperature and the presence of an -SH group (Salvin 1949a).

b) Nutrition.

Both forms of Candida are obtainable at 25°C depending on the composition of the medium (Nickerson and Mankowski 1953). The yeast form grows well on glucose, ammonium sulphate, inorganic salt medium. On glucose, glycine, yeast extract agar filaments are formed at the edges of colonies. When glucose is replaced by soluble starch, glycogen or dextrin growth is reduced but of a filamentous form. Phosphate and cysteine prevent filaments forming.

High levels of hexoses stimulate Mucor to exhibit yeast-like growth, glucose being most effective followed by fructose, manrose, galactose in decreasing order of efficiency. However, 10% glucose did not prevent the elongation of preformed hyphae but inhibited hyphal development from germinating spores. (Bartnicki-Garcia 1968). Friedenthal et al (1974) report that on 2% glucose growth is yeast-like while at 0.1% glucose, growth is mycelial. The Y form appears to require high levels of readily metabolised carbon source, e.g. glucose. Low levels of glucose or a less readily metabolised carbon source, e.g. starch, favours the M form.

In Trigonopsis, the proportion of three-cornered cells in a culture may be varied by the carbon source. (Matthewson and Barnett 1974) or the amino acid used as the nitrogen source (Senthe Shanmugathan and Nickerson 1962), (Sasek and Becker 1969). Hydroxyproline, alanine, proline and methionine caused more than 80% of the cells to be triangular.

Manganese deficiency may be expressed by a change of form in some fungi, e.g. Chaetomium globosum forms short swollen globose or irregular-shaped cells (Barnett and Lilly 1966). The absence

of Manganese prevents coremia formation in two species of Penicillium (Tinnell et al 1977). Yeast-like cells of Aspergillus parasiticus form at 7.3×10^{-4} M Manganese while hyphal cells grow at 7.3×10^{-3} M Manganese (Detroy and Ciegler 1971). However, the yeast-like cells of A. parasiticus do not closely resemble those of true dimorphic fungi in ultrastructure (Garrison and Boyd 1971).

Cysteine, glutathione and thioglycollate prevent filamentous growth in Candida (Nickerson and Mankowski 1953) and in Histoplasma (Salvin 1949a). It was reported that no single amino acid but a sulphide or sulphydryl group in a small organic molecule e.g. amino acid, was necessary for yeast growth. Biotin was necessary for the development of the yeast phase of Histoplasma. (Salvin 1949a).

In Sporothrix, the yeast phase is formed when casein hydrolysate or amino acids are included in the medium. (Drouhet and Mariat 1952). This has the effect of increasing the CO_2 tension in the medium and it is this which causes the transformation of M to Y.

c) Carbon dioxide.

In Sporothrix, 5% CO_2 was sufficient to maintain the yeast phase. (Drouhet and Mariat 1952).

Anaerobiosis does not prevent the elongation of preformed hyphae in Mucor but hyphal to yeast transformation is favoured by anaerobic fermentation conditions (Bartnicki - Garcia and Nickerson 1962a).

The formation of macrospores in Trichophyton was induced by growing the fungus in air x 12 - 24% ($^{v}/_{v}$) CO_2 (Chin and Knight 1957). Some enzymes concerned with glucose metabolism were found to be stimulated by CO_2 (Chin and Knight 1963b) which is not surprising considering that high glucose levels also encourage yeast formation; possibly because a higher rate of metabolism gives off

more Co_2 than the slower utilization of starch.

d) Inhibitors.

Potassium cyanide was found to influence the form of Mucor (Friedenthal 1974) and the antibiotics Cyanein and Azalomycin F induce the Y form of Paecilomyces viridis (Barathova et al 1972).

Cyanide induces anaerobiosis in cells (Burnett 1968) and as CO_2 and anaerobiosis maintain and induce Y in Sporothrix (Drouhet and Mariat 1952) and Mucor (Bartnicki-Garcia and Nickerson 1962a) this may be the reason for the cyanide effect. The effect of antibiotics is probably very complex.

e) The dual phenomenon described by Wilhelm (1947) is attributed to prolonged cultivation which induces an abrupt change in the growth form. Pleomorphic overgrowth by the M form is a response to physiological aging by the conidial form. In Trichophyton gypseum and Epidermophyton floccosum both forms are present and it was suggested that the mycelial form arises as a mutation in old conidial cultures.

f) Crowding.

When Glomerella cingulata was streaked onto potato - sucrose agar growth was of the Y form with streaks of fluffy mycelium at the edge. The Y form was obtained at densities of 3000 conidia per mm^2 or 2×10^6 per ml when secondary conidia were formed to give the Y phase. Unlike Mucor, aeration favoured conidial development on appropriate media and CO_2 did not induce Y phase. Diffusible substances associated with the conidia are considered responsible for inhibition of mycelium and preferential development of Y phase. (Lingappa and Lingappa,1969).

These changes from Y $\rightleftharpoons$ M are accompanied by changes in the cell wall and also in the proportions of RNA, DNA and protein. (Table 9). The changes are not specific, e.g., mannose is increased in the M form of Histoplasma and Paecilomyces but

decreased in Mucor rouxii and Saccharomyces rouxii.

Chitin or amino sugar occurs at a higher level in the Y form than the M form of dimorphic fungi. (See Table 9) except in Candida and Mucor rouxii. The M form of Candida contains three times as much chitin in the alkali insoluble fraction of the cell wall as does the Y form. This may be due to the removal of differential amounts of glucan by the alkali as there appears to be twice as much glucan in the Y form.

The higher level of chitin found in Y form is brought about by a longer lived chitin synthetase due to a modification to the enzyme molecule or more favourable conditions within the cell. (Ruiz Herrera and Bartnicki-Garcia 1975). To maintain the spherical shape of the yeast cell, synthesis of wall material takes place over all the cell surface while in the M form, synthesis is restricted to the tip. (See p 3 of ultrastructure) (Bartnicki - Garcia and Lippman 1969).

The composition of the cell wall of the Y and M forms has been shown to differ. The proportions of chitin and FRACTIONS 1, 11, 111, and 1V vary. In the Y form, FRACTION 1 and glucan are higher and chitin is lower than in the M form. The glucan of Y form shows more α - linkages than the M form of Blastomyces (Kanetsuna and Carbonell 1971) and Paracoccidioides. (Kanetsuna et al 1972).

The protein and amino acid content tends to be higher in the M form accompanied by differences in the amino acid composition.

The ratio RNA / DNA reported for Paecilomyces and Sporotrichum contradict each other although it would be expected that a higher value for RNA would be associated with a higher protein content as in Sporotrichum.

The importance of cysteine and especially SH groups in maintaining Y form is confirmed by a 12 times greater content of

disulphide in the M form of Paracoccidioides and a lower protein sulphide reductase level.

These changes in the cell wall are reflections of altered enzyme activities.

TABLE 9.

DIFFERENCES IN COMPOSITION.

Organism and Component	Yeast	Mycelium	Reference
Blastomyces dermatitidis			
TCA precipitated nitrogen	15-30%	5%	Taylor (1961)
RNA	peak at log phase	constant low level	"
Fraction 1 of cell wall	34.5%	27%	Kanetsuna and Carbonell (1971).
Hexose (% of total cell wall)	36.2%	51%	"
Amino sugar	37.0%	22.8%	"
Amino acid	7.8%	10.9%	"
Glucan	95%	60%	"
Paracoccidioides brasiliensis			
Glucan	Mainly α	Mainly β	Kanetsuna et al (1972)
Disulphide (proportions)	1x	12x	"
Protein disulphide reductase	5x	1x	"
Chitin	37-48%	7-18%	Kanetsuna et al (1969)
Protein	7-14%	24-41%	
Histoplasma capsulatum			Domer et al (1967)
Chitin	25%	4%	
wall thickness	.05-.08	.01-.02	
Mannose	1.2%	5.1%	
Glucose	21.1%	5.5%	
Amino acids	5%	10%	
Candida albicans cell wall.	μ mole / 1000 amino acid	μ mole	
Hydroxyproline	-	31.2	Chattaway et al (1968).
Glutamic acid	65.5	90.7	
Proline	63.1	36.9	
Methionine	79.3	21.6	
Histidine	112.2	-	
Arginine	-	53.4	
Glucosamine	1.8 - 4.3%	6.7 - 10.8%	
glucanase released glucose	39.2%	18.8%	
Cell wall Fraction 11, 111 and 1V.			
Chitin	1x	3x	
Protein	3x	1x	
Sporotrichum schenckii			
RNA/DNA	.7-1.4	9.3	Mariat (1959) (1960)
NA/Protein	.38-.54	.50-.98	
RNA/Nitrogen	.16-.32	.41-.93	
RNA/Nitrogen	.18-.22	.05-.09	
Respiration	lower	higher	Mariat (1968)

TABLE 9

Organism and Component	Yeast	Mycelium	Reference
Paecilomyces viridis			
Melanin	4 %	13.5 %	Barath and Betina(1972).
Glucose	11.2	14.0	
Galactose	10.0	5.0	
Mannose	2.6	4.8	
Glucosamine	11.1	7.5	
Galactosamine	9.7	1.5	
RNA/DNA	13.8 -) 14.7)	3.9 -) 8.6)	Barathova et al 1972.
DNA	0.6 x	1x	
RNA	2.2 x	1x	
Protein	0.7 x	1x	
Mucor rouxii.			
Cell wall synthesis	over all surface	restricted to tip.	Bartnicki-Garcia & Lippman(1969)
Chitin synthetase	longer lived	shorter lived	Ruiz Herrera & Bartnicki-Garcia(1975).
Cell wall. Chitin	8.4 %	9.4 %	Bartnicki-Garcia & Nickerson(1962c).
Chitosan	27.9	32.7	
Mannose	8.9	1.6	
Protein	10.3	6.3	
Lipid	0.8	2.0	
M.racemosus.			
Mannan contains % polypeptide	25%	15%	Borgia and Sypherd(1975).
Hydrolysis gives ratio of mono: di: tri: oligo saccharide.	3:3:3:2	6:2:2:1	
Saccharomyces rouxii.			
Cell wall hexosamine	3.8%	0.8%	Koh 1975
Glucose :mannose ratio	1.2: 1.0	1.9: 1.0	
Cordyceps militaris.			
Cell wall glucose	60%	46%	Marks et al (1971).
hexosamine	6.8%	11.0%	
Protein	9.7	6.0	
Readily extractable lipid	6.6	10.7	

Genetic analysis of Candida albicans showed that the breakdown in an intracellular - SH maintenance reaction caused filamentous growth of a mutant which normally shows yeast morphology under the same conditions. The addition of cysteine partly restores division in the mutant and an enzyme which diffuses from the parental strain releases a -SH substances into the medium. The block effects cell division and not cell growth as the growth rate is only slightly less than that of the parental strain. (Nickerson & Chung 1954).

The control over chitin and mannan synthesis which is important in determining morphology (Chattaway et al 1968) may be partly provided through control of the activity of phosphofructo - kinase by adenosine phosphates.

Some of the enzymes which occur early in the pathway of glucose metabolism are stimulated by CO_2 (Chin & Knight 1963b) e.g. G - 6 - P dehydrogenase and glucose phosphate isomerase. Hexokinase, phosphoglucomutase and 6 - P gluconate dehydrogenase are only weakly, if at all, stimulated so dimorphism seems to depend on levels of activity of G - 6 - P dehydrogenase and glucose phosphate isomerase. Glucose metabolism effects the conversion of Y→M in Mucor rouxii and the cell wall mannan. (Garcia & Villa,1977).

Chitin synthesis is not a decisive factor in dimorphic development as cultures treated with Polyoxin D showed only minor morphological alteration and did not alter the pattern of dimorphic development or the proportion of chitin in the cell wall.

Ultrastructure of the mycelial forms of Paracoddioides, Blastomyces (Carbonell & Rodriguez 1968) and Sporotrichum schenckii (Lane Garrison & Field 1969) was investigated. The mycelium is formed of branching, septate filaments 1.0 to 1.8 μm.

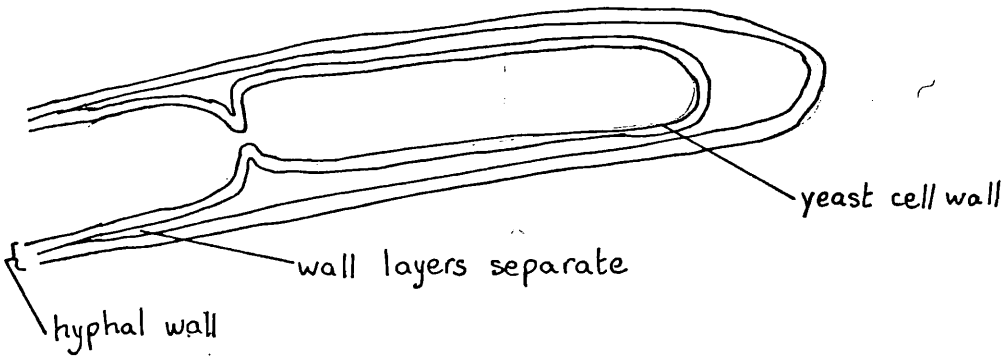
diameter with a cell wall 80 - 140 nm thick. Four Woronin bodies were associated with each septum as were "intra cytoplasmic membrane systems" which may take part in septum formation.

Edwards et al (1959) reported that the yeast phase of Histoplasma had a laminated cell wall but no capsule or slime layer was found. The cells of Blastomyces were multinucleate with nuclei often interconnected by outer limiting membranes. Mitochondria, endoplasmic reticulum and multi vesicular bodies were found in the yeast cells of Blastomyces (Edwards & Edwards 1960). "Myelin figures" of concentric lamellae formed by hydrated phospholipids appear in old yeast cells of Paracoccidioides (Carbonell & Pollak 1962). They appear in electron micrographs as whirls of concentric double membranes, electron dense wavy strands with irregular borders or short OsO_4 stained threads. "Myelin figures" were never related to the cytoplasmic membrane but always close to osmophilic vacuoles (fat drops) presumed to be degenerative cell organelles of spores. (p. 305)

The yeast-like cell of Sporotrichum schenckii is 2.3 µm x 1.7 µm and has a wall 100 - 300 nm thick appearing as a two-layered wall. Multiple storage granules and intracytoplasmic membranes, consisting of circular three-layered electron dense structures which communicate with the cell membrane, were observed in the yeast-like cell.

The mycelium to yeast (M → Y) transformation of Sporotrichum schenckii was investigated by Garrison, Boyd & Mariat (1975). After 48 hours in Y-inducing conditions, the M form produces buds at or near the septa. The Y-cell wall appears to arise from the innermost cell wall layer of the hypha. A possible increase in the numbers of mitochondria was the only cytological change visible in transforming M cells. Thin sections of conidia contained several large lipid bodies. Some hyphae remained M for

·72 - 96 hours and then underwent a process of cytoplasmic condensation to form chains of ordia which separated. Again, the Y wall was contained within the hyphal wall.



The M→Y transformation of Sporotrichum schenckii was compared with that of Ceratocystis stenoceras (Garrison et al 1976). M-cells are similar and transformation occurs by budding and oidia formation. The Y form of Ceratocystis has a thicker wall than the M form and has a layer of microfibrillar material on the outside. It was suggested that conditions necessary to induce Y formation are toxic to M cells as these become disorganized.

In Paracoccidioides the M-Y transformation occurs by an increase in diameter of the hyphae at interseptal spaces. The outer electron dense layer of the wall cracks and these Y cells separate and tend to become rounded. Non-transformed cells are empty or contain membrane remnants, intracytoplasmic membranes possibly and glycogen. (Carbonell 1969b). Nearly all the hyphae are dead within 18 hours.

At the commencement of yeast-mould transformation in Paracoccidioides (Y→M), many intra-hyphal hyphae are seen as live hyphae in dead yeast of hyphal cells. The yeast does not itself become a mycelium but produces an elongated bud with a diameter greater than that of a hypha. This transition cell contains glycogen, ribosomes and many nuclei.

(Carbonell 1969 a & b).

In Blastomyces and Histoplasma a very similar pattern of events occurs (Garrison et al 1970). Between 6 - 8 hours after induction, intracytoplasmic membrane systems were found associated with the plasma membrane in the Y cell. Woronin bodies were observed between 12 - 18 hours at the septum of transitional cell and converting Y cell. Both these cells had increased numbers of mitochondria. After 48 hours, newly formed hyphal cells associated with normal septum formation were observed.

The transformation of Sporotrichum (Y → M) (Lane et al 1969, Lane & Garrison 1970), proceeds like that of Blastomyces and Histoplasma .

Intra cytoplasmic membrane systems are found close to the regions of reorganisation and may be involved in converting one cell type to another (Garrison et al 1970)

Cytoplasm streams into the transition cell which, when mature, the septum is blocked by a Woronin body. Intra hyphal hyphae were observed in cultures converting fron one phase to the other as in Paracoccidioides (Carbonell 1969 a & b). However, Carbonell & Pollak (1962) suggested that they were degenerative membrane organelles and if, in fact, the cells are dying because the altered conditions are toxic, they are unlikely to exert a controlling influence on neighbouring cytoplasm.

Ceratocystis minor isolated from the beetle, shows a Sporotrichum imperfect state. It appears as Y cells in the mycangium (fungal transport pocket of the beetle) along with hyphal-like transition cells. In continuous culture on potato carrot agar, it produced sympodial conidiophores and the resulting conidia resemble Y cells in the mycangium and conidiophores resemble the transition cells.

Table 10

SUMMARY OF M⇄Y TRANSFORMATIONS OF DIMORPHIC FUNGI

M→Y Transformation

Sporothrix schenckii	Budding near septa from inner wall layer Cytoplasmic condensation to form chains of oidia.	Garrison Boyd & Mariat 1975
Ceratocystis stenoceras	" "	Garrison et al 1976
Paracoccidioides brasiliensis	Swelling of intercalary portions, outer wall cracks and Y cells separate and become rounded.	Carbonell 1969b

Y→M Transformation

P. brasiliensis Blastomyces Histoplasma	Elongated bud formed transition cell which contained inner wall layer of Y . Cell organelles increase. ICMS in Ycell	Carbonell 1969a, b Garrison et al 1970
Sporothrix	Cytoplasm streams into transition cell, when mature septum blocked by Woronin body. Intra hyphal hyphae observed.	Lane et al 1969 Lane & Garrison 1970.

ICMS- Intra cytoplasmic membrane systems.

The hypothesis put forward by Kanetsuna et al (1972) for dimorphism in Paracoccidioides assumes that the spherical forms are produced by simultaneous synthesis of the entire cell wall while the cylindrical form by synthesis at the apical region only. The Y-form has α, 1 - 3 glucan in the outer layer of the wall with the inner layer composed of small amounts of β glucan, protein and lipid as well as chitin. They assume that the glucan is in islets in the cell wall as chitin is reported to be in Saccharomyces (Bacon et al 1969), (Cabb & Bowers 1971). Somehow, there is a loss of rigidity around the β glucan and the weakened part is blown out as a bud. β glucanase and protein disulphide reductase may play a role in bud formation. At 37°C, at the bud cell wall, α glucan and chitin are more rapidly synthesized than β glucan, chitin strengthening the spherical form. At 20°C, α glucan synthesis decreases at budding sites and β glucan fibres grow continuously forming apical growth. Proteins may give rigidity to this wall in high levels of S-S bonding and low protein disulphide reductase activity which prevent balloon-like growth of the β glucan. There is much less S-S bonding in the yeast protein. The β glucan cannot be extracted from the M-form unless the chitin is hydrolysed first indicating that the fibrils may be interwoven.

The ultrastructure of Y-M conversion shows the outer α-glucan of the Y cell does not continue into the M form. It is stated that there are generally no distinct layers in the M-form shown by Carbonell (1969) and Carbonell & Rodriguez (1968). The latter paper does, in fact, show layers in the cell wall.

All Y-forms have the capacity to produce M-cells but only a small part of M-form produce Y-form. They postulate that glucan synthetases, protein disulphide reductase may not be evenly distributed throughout the hyphae.

When transferred to 37°C, β-glucan synthesis ceases and the wall may be softened by β glucanase. Simultaneously, synthesis of α-glucan may occur in all parts of the wall resulting in the Y-form.

The requirement of SH groups in the division processes of cells was shown by (Nickerson, 1948). When Candida albicans is grown in glucose-rich medium, sufficient reducing power is generated to maintain the -SH level necessary for budding. When glucose is depleted or a slowly utilizable carbon source is available, the low level of -SH would not support cell division and elongated cells are produced. (Nickerson & Mankowski 1953, Nickerson 1950).

Dimorphism is the interplay between the two fundamental processes of cell growth (elongation) and cell division (budding). When optimally coupled, regular budding of ellipsoidal or spherical cells happens. The selective disruption of cell division would lead to elongation.

Another morphological response to changes in media is shown by fungi which show rhythmic growth. This is demonstrated as concentric zones of alternating high and low branch densities on the surface of cultures on solid media. Other forms of rhythmic growth, e.g. zones of perithecial or conidial development occur but will not be included in this review.

The growth rate of a "rhythmic" colony(but not an individual hypha) remains constant while a paramorph colony shows a reduced growth rate with increased branching. Table II shows some of the conditions in which rhythmic growth occurs but only the effect of media will be discussed in detail.

TABLE II.

Conditions which induce rhythmic growth in some fungi.

Stimulant/Conditions	Organism	Reference.
24 hour light/dark cycle flash white light.	Neurospora crassa	Pittendrigh et al (1959).
Temperature and light	" "	Bianchi (1964)
Complete darkness	periodic colonial and proline less strains of N. crassa.	Sussman et al (1964). Brandt (1953).
Fluctuation of temperature.	Fusarium discolor sulphureum.	Snyder & Hansen (1941).
Media.	Fusarium spp.	Brown (1925) Bisby (1925)
Glucose or temperature.	Sclerotinia sclerotiorum	Humpherson-Jones & Cooke (1977).
12-hours light. Glucose medium in dark.	Leptosphaeria michotii	Jerebzoff & Lacoste (1962).

Berliner & Neurath (1966) reported that rhythmic growth of a "clock" mutant of Neurospora was controlled by sugars. The rhythm was independent of a light-dark cycle. A branching pattern emerged on dextrose, maltose and fructose (0.1 - 2.0%) with a 48-hour period while the radial growth rate remained constant.

A "vague" mutant of Ascobolus immersus showed rhythmic growth with a 34-hour cycle at 26°C. To supress the rhythm, a substrate rich in manganese and cobalt or poor in magnesium and glucose was required. Adenine decreases and aneurine increases rhythmic growth of the mutant (Chevaugeon 1959).

Alternaria tenuis showed marked zonation when the ratios malt/KH_2PO_4 and KNO_3 / KH_2PO_4 were low. (Jerebzoff 1962).

A pH dependent rhythm was reported by Esser (1969) in Podiospora anserina. In the wild type, zonation was not observed between pH6 - pH7 but below pH5, zonation increased while the radial growth rate decreased and a mutant showed zonation between pH 4.6 - pH 7.

Lysek (1972) described a branching zonation in a clock mutant of Podospora anserina. Hyphae elongate by the incorporation of vesicles into the tip (Bartnicki-Garcia 1969). In this mutant, "zonata", this seems to be disorientated and enhanced initiation of branches results (Esser & Minuth 1972). Zonation is only seen on the surface and hyphae inside the medium appear normal, probably because of a limited oxygen supply as no zonation was found to occur under unfavourable conditions. The radial growth rate of the colony is constant while the growth rate of an individual hypha decreases when excessive branching occurs. The growth of these hyphae is eventually stopped by the emergence of hyphae from within the medium.

At the end of the growth cycle of the Neurospora mutant "clock", hyphae were observed to invade dead hyphal segments and

then penetrate the wall to escape. Normal and degenerate cells were separated by a plugged septum. The hyphal wall was 3-layered in the "clock" mutant and 2-layered in the wild type. Intra-hyphal hyphal hyphae were said to be a feature of this mutation but they are also reported to occur in dimorphic fungi during the transition from one form to the other.

In discussing the correlation between carbohydrate metabolism and morphogenesis of Podospora anserina, Lysek and Esser (1971) point out that a single mutation which alters the morphology of a colony like "zonata" cannot effect only a single metabolic pathway. As morphology is related to the structure of the cell wall in Neurospora (de Terra & Tatum 1963), carbohydrate metabolism using the pool sizes of phosphorylated intermediates in Podospora was investigated by Bornefeld & Lysek (1972). After 4 days, when the first growth band had been formed, the following differences between wild type Podospora and the mutant "zonata" were observed after ^{32}P - labelling of the mycelium.

The incorporation of ^{32}P into wild type mycelium in relation to dry weight decreased, while in the mutant it remained constant. This may be because the ratio of the peripheral growth zone : total mycelial mass decreases as the colony gets larger for the wild type (Trinci 1971) whereas in the mutant, the excessive branching which occurs towards the end of the cycle will need increases incorporation compared to the wild type.

In the wild strain, the UDPG content decreased over the four days while the pool size of sugar phosphate increased. In the mutant, the levels of both these are maintained constant at the low level reached by the wild type after four days.

The level of fructose-diphosphate is enhanced in the mutant

probably due to an increased turnover of the phosphofructo kinase reaction which is due to an increased substrate catabolism.

The ATP pool decreased in both mutant and wild type but the ratio of ATP/AMP is larger in the mutant. Phosphofructo kinase ought to be inhibited by a high level of ATP. This points to a disturbed oxidative carbohydrate metabolism previously reported by Lysek & Esser (1971).

The pool size of phosphoryl choline and phosphoryl ethanolamine are increased in the mutant and this may involve the synthesis of phospho lipids which are major components of the membrane.

These observations support the conclusions of Lysek & Esser (1971) that carbohydrate degradation, respiration, anabolic reactions, particularly polysaccharide synthesis, may be involved in the formation of the zoned growth pattern. The very thick hyphal walls found in the mutant (Esser & Minuth 1972) may account for the high levels of wall precursors which are found in the mutant despite its reduced growth and dryweight production.

The "patch" and "clock" mutants of Neurospora crassa show zonation. "Patch" morphology can be induced in the wild type by growing it on equal quantities of sorbose and sucrose. There is a single gene difference between the wild type and "clock" mutants (Sussman et al 1964).

Stadler (1959) investigated the genetics of the "patch" mutant strain of Neurospora crassa used by Pittendrigh et al (1959) and Brandt (1953). When crossed to wild type, growth pattern, proline requirements and mating type segregated with a ratio of 2 : 2. "Patch" morphology and proline requirement were controlled by separate genes and not closely linked, although "patch" was closely linked to mating type. Colonies containing the "patch" gene showed sorbose escape.

Chevaugeon and van Huong (1969) showed that the growth pattern of Ascobolus immersus and Podospora anserina depends on nuclear genes. The process of monopodial continual growth is related to the activity of normal wild type cells, i.e. the three genes must be active and the cytoplasmic sites receiving their messages must be intact. If a gene mutates or its function is hampered by an inhibitor, then disorders occur which give rise to sympodial branching and periodic growth.

Materials.

Organisms used in this study.

1. Ceratocystis adiposa Butl. (Hunt 1956).

Isolate M1 was obtained from the culture collection at Royal Holloway College, Department of Botany.

2. Botrytis fabae. Sand. (Moore 1959).

This was provided by Dr. J.E. Kerr of the Department of Botany, Royal Holloway College.

3. Aspergillus chevalieri (Mangin). Thom & Church. (Raper & Fennell 1965).

The osmotolerant strain M 205 was a gift from Dr. G. Ayerst, Department of Biological Sciences, The Polytechnic, Wolverhampton.

4. Hypocrea pulvinata. Fuckel. (Petch 1938).

This was obtained from the culture collection at Royal Holloway College, Department of Botany.

Standard Synthetic Medium (S S M)

$Na_2H\ PO_4$	0.2 g
$K\ H_2\ PO_4$	1.0 g
$Mg\ SO_4 . 7\ H\ O$	0.5 g
Asparagine H_2O	0.5 g
Glucose	10.0 g
Trace elements	1.0 ml
Water	1000.0 ml

The trace element solution contained :-

$Fe\ (NO_3)_3 .$	$9\ H_2O$	723.5 mgm
$Zn\ So_4 .$	$7\ H_2O$	439.8 mgm
$Mn\ SO_4 .$	$4\ H_2O$	203.0 mgm

These were dissolved in 300 ml distilled water and sufficient H_2SO_4 added to yield a clear solution. This was made up to one litre with distilled water.

Initially, nitrogen was supplied as either asparagine $(NH_4)_2\ SO_4$, KNO_3 or NH_4 tartrate. Asparagine supported the most dense mycelium and was used in all subsequent studies.

Carbon was supplied as asparagine, glucose, galactose, mannose, sucrose, fructose, rhamnose, sorbose or xylose at 1% (w/v) and the plates fixed, stained and photographed after 2 days and 4 days.

Solutions of paramorphogens.

Sorbose, glucose, polyethylene glycol 200 and sodium deoxycholate were dissolved in distilled water to twice the required strength. Standard synthetic medium was made up to double strength and equal volumes of the solutions and media mixed. Sorbose solutions were autoclaved separately at 5 lb/sq.in. for 10 minutes to prevent discolouration and mixed with sterile medium when cool. All other media and solutions were autoclaved at 15 lb/sq.in. for 20 minutes after mixing.

As xylose was the only carbon source which supported less growth than sorbose, a parallel experiment was set up using xylose as a paramorphogen.

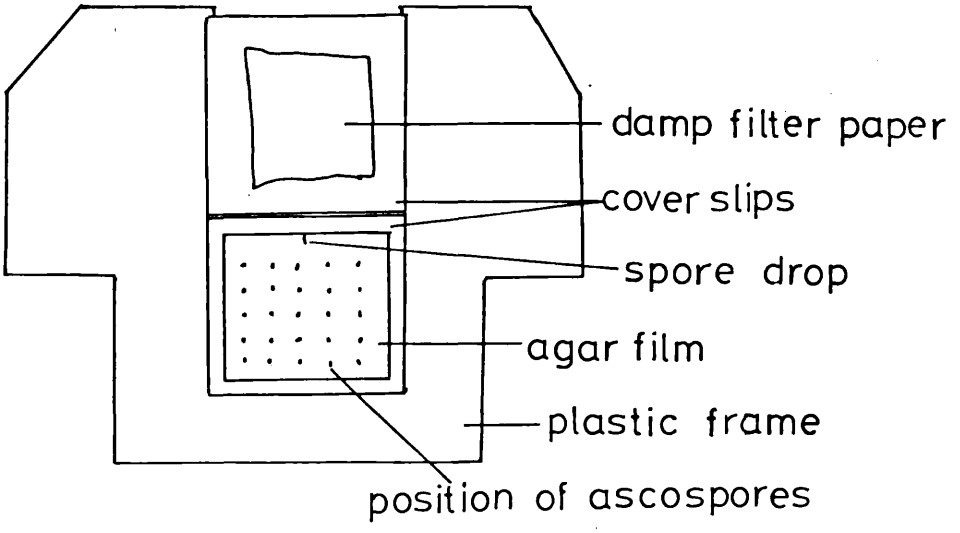
Isolation of single ascospores.

The method used was adapted from that of Dickinson (1926) described by Johnstone (1969) as "Isolation by micromanipulation below the air-gel interface." The moist chamber used was a modification of the chamber described by Dixon (1958). Fig.5

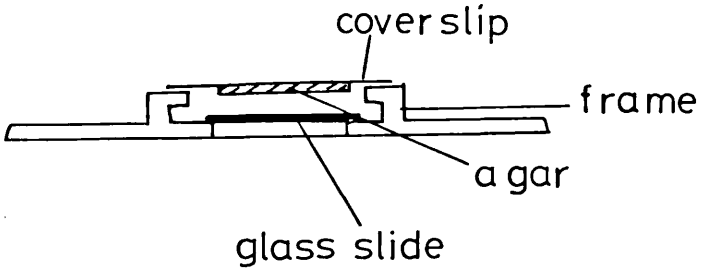
Two thin strips of glass were cut from a No.2 coverslip. They were placed between two other No.2 coverslips on an aluminium plate kept warm over a very low bunsen flame and molten 1.5% water agar drawn between the coverslips. The coverslips and glass strips were sterilized by dripping in absolute alcohol and allowing the alcohol to burn off. The agar was allowed to set by removing the coverslips from the warmed plate. The coverslips were gently drawn apart leaving the agar film attached to one of the coverslips. This coverslip was placed agar-side down on a rectangular plastic frame and secured with drips of nail varnish. The frame was wiped clean with acetone to remove nail varnish and to sterilize it.

Using a sterile glass needle, a spore drop was removed from a perithecium, the glass slide partially withdrawn and the spore drop laid at the edge of the agar film. The slide was replaced to keep the agar surface enclosed in a box and to prevent it drying. The top of the coverslip was marked with rows of small dots in indian ink. Individual spores were removed from the spore mass using a glass needle and placed on the agar film by a mark. This was done under low power magnification (x 100). When the required number of spores had been isolated, the agar film was cut araound each mark into squares. Each spore on a small square of agar was removed and placed on an inverted plate.

Fig. 5
Surface view of apparatus for single spore isolates



View in section



of 2% malt agar and incubated at 25°C. Growth rate (Kr) and morphology of each isolate were observed.

Spore suspensions.

Spore suspensions were prepared from cultures grown in medical flat bottles (200 ml) plugged with cotton wool. Twenty-five ml of 2% malt agar was autoclaved in the bottle and the agar set along one side of the bottle. This was inoculated with 1 ml of a dense spore suspension and incubated at 30°C. At this temperature, perithecial formation is inhibited (Table 12) and fewer large thick-walled conidia are formed. The dry-spore conidia were washed off the mycelium with sterile 0.01% Tween 80. To obtain a uniform suspension, the suspension was vigorously shaken to disperse clumps of spores before serial dilutions, using sterile distilled water, were made and conidia counted using a haemocytometer.

To determine the optimum temperature for growth, conidiation and perithecial formation.

2% malt plates were inoculated with about 7×10^3 conidia spread over the plate and incubated at 20°C, 25°C, 30°C, 35°C.

They were observed at intervals for the development of perithecia and spore drops. Observations were made using a dissecting microscope (x 10).

TABLE 12 (See Table 13 in results).

Effect of temperature on growth of Ceratocystis adiposa.

Temperature	20°C		25°C	30°C	35°C
1 day	individual hyphae visible		dense mycelium	dense mycelium	no growth
2 days	dense mycelium		dense mycelium	dense mycelium	conidia germinated
	developing perithecia		-	-	
	few conidia		many conidia	many conidia	
3 days	mycelium	+ + +			no growth
	perithecia	developed	developed		? germ tubes died
	conidia	+ +	+ + + +	+ + +	
5 days	mycelium	dense dark	dense dark	dense dark	
	perithecia	many with spore drops	few spore drops		
	conidia	+ + + +	+ + + +	+ + + +	

The effect of temperature and paramorphogen on perithecial production.

Method.

Plates containing 20 mls of SSM, SSM + 0.25M glucose, or sorbose, or polyethylene glycol ~~and~~ or 0.25 x 10^{-4} M sodium deoxycholate were inoculated at the centre. The plates were incubated invertedly at 20° C, 25° C and 30° C for 2 weeks. The number of perithecia per consecutive field of view (5 mm diameter) was counted along two perpendicular diameters. The results are shown in Fig. 31

Another set of plates were similarly treated but transferred from 20° - 30° after 3 days or 30° - 20° after 1, 2 or 3 days (Fig 32) and incubated at the latter temperature for a furthur 10 days.

Fixing and staining plates for photography.

Lactophenol cotton blue stain (CMI Phytopathologists handbook). ed. Ainsworth 1968

Phenol (pure crystals)	20g
Lactic acid (S.G. 1.21)	20g
Glycerol	40g
Water	20 ml
Cotton blue	0.05 g

A 1% (v/v) solution of lactophenol cotton blue stain in absolute alcohol was made up as required. The plates were flooded with stain and left for 24 hours. The excess stain was then poured off and the plates rinsed once in distilled water before being stored at 4° C.

Determination of Osmotic Pressure.

The osmotic pressures of the media were determined from the depression of the freezing point. A Knauer Electronic Temperature Measuring Instrument in the Department of Chemistry, Royal Holloway College was used to measure the freezing point depression. Not all solutions were frozen by the machine.

Solutions of polyethylene glycol of molecular weight 1500 or greater would not freeze and concentrations of 0.1M or above of polyethylene glycol 400. A precipitate was formed when sodium deoxycholate was added to SSM and the osmotic pressure was slightly less than that of SSM.

A standard line was obtained using the following concentrations of glucose; 0.01M, 0.05M, 0.10M, 0.25M, 0.50M, 1.00M.

The freezing point was measured using 0.15ml of the solution under test and recorded in arbitrary units on a chart and converted into Pascals (Pa) using the standard curve obtained from glucose. (1 Atmos = 101325 Pa). p 368

Colony growth rate measurement.

The colony diameter of Ceratocystis adiposa decreases with increased concentrations of sorbose and sodium deoxycholate - (Fig. 6).

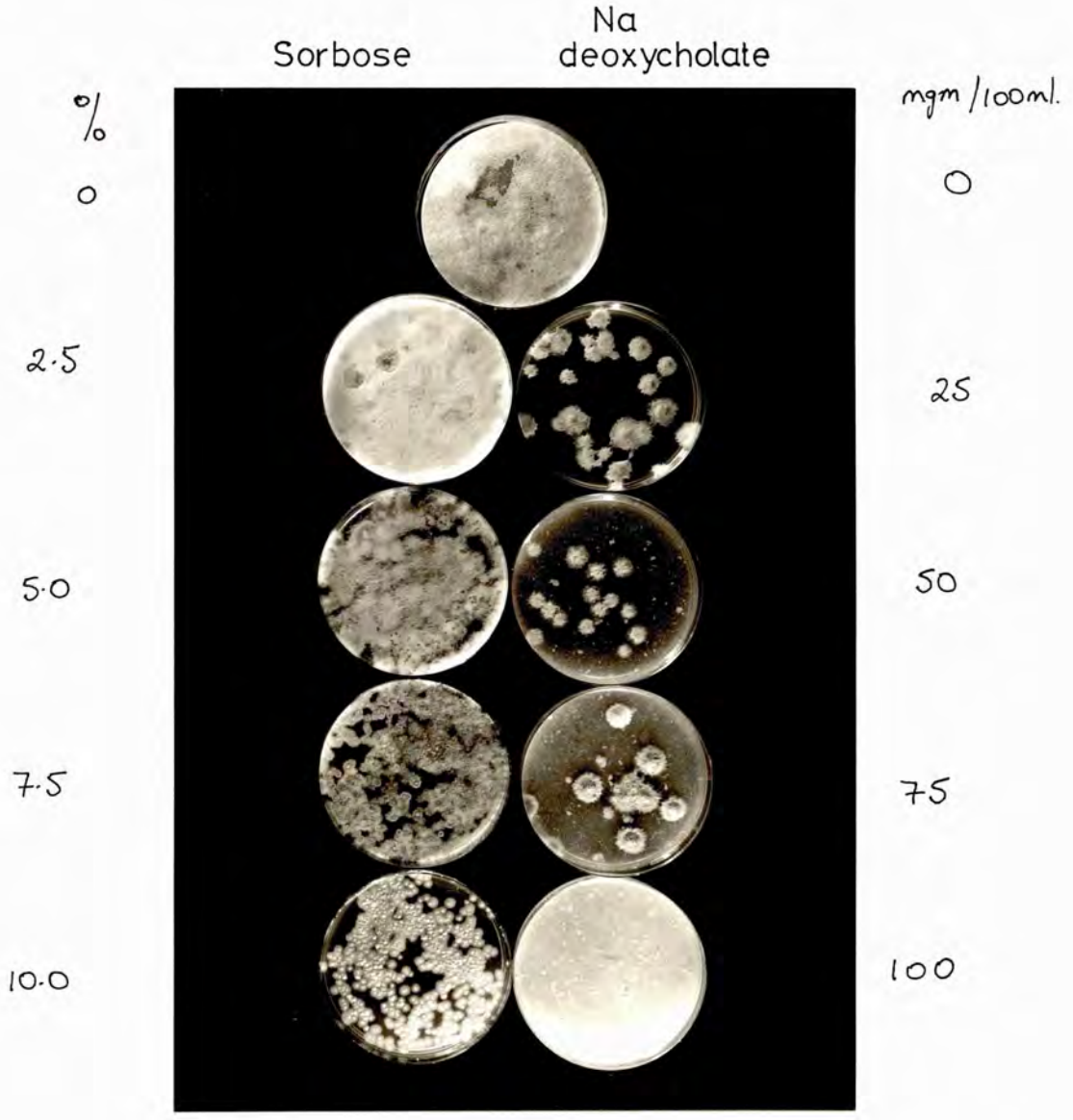
It may be observed that the mycelium on 0.14M (2.5%) sorbose is thicker than that on the control, indicating that the extra carbon provided by sorbose is utilized. The plates were inoculated with about 500 conidia but the few colonies which germinated on the sodium deoxycholate plates indicate that this compound acts as an inhibitor of spore germination. For this reason, 10 point inocula were used to ensure even spacing of colonies, avoid a lag phase and the inhibition which would occur during spore germination on sodium deoxycholate.

Ten point inoculation technique.

Ten pins were set into a 9 cm perspex disc with a holder in the centre. The perspex was wiped in absolute alcohol to sterilize it before commencing any inoculation experiment. Between each inoculation, the pins were stood in a petri dish of alcohol and the alcohol burned off.

Fig. 6

Ceratocystis adiposa on sorbose and sodium deoxycholate media after 7 days at 25°C.



The inoculum was provided by a dense mycelium grown on SSM in 9 cm petri dishes at 25°C. The pins were scraped gently across the plate and then vertically lowered on to the agar of the new plate. The plates were incubated inverted at 25°C.

The growth of the fungi used in this study was observed and measured at intervals. In some cases, photographs were used as these illustrate the density of growth as well as the rate of growth as in the experiment on utilization of various carbon sources.

Radial Growth rate (Kr) was plotted as a percentage of the control in each experiment. The values and standard errors are given in the Appendix. Tables 31 - 37

Dry weights were taken at times previously determined to coincide with the beginning, middle and end of the rapid phase of growth. Table 38

Determination of radial growth rate - Kr.

Twenty Cm^3 of molten agar were pipetted into 9 cm sterile plastic petri dishes to give a constant depth of agar. When set, the plates were inoculated with ten point-inocula and incubated at 25° C in the inverted position. The diameters of 30 random colonies from 5 plates were measured for each treatment (when the diameter was > 7.5 mm). From these the radial growth rate of Ceratocystis adiposa on the various media used could be calculated. The same method was used to determine Kr for Botrytis fabae and Aspergillus chevalieri.

The range of concentrations used were :-

for Ceratocystis adiposa and Botrytis fabae

Sorbose, glucose	0.01 M	-	1.0 M
Polyethylene glycol	0.001 M	-	1.0 M
Sodium deoxycholate	10^{-5} M	-	10^{-3} M.

for Aspergillus chevalieri

Glucose and polyethylene glycol 200	1.0 M	-	5.0 M.

Determination of the specific growth rate -

The specific growth rate was determined in S.S.M. and at concentrations of sorbose, glucose, polyethylene glycol and sodium deoxycholate which reduced the radial growth rate to 50% (i.e. Kr_{50}).

The concentrations used were as follows :-

	Ceratocystis adiposa	Botrytis fabae
Glucose	0.25M	
Sorbose	0.25M	0.01M
Polyethylene glycol	0.25M	
Sodium deoxycholate	0.25×10^{-4} M	0.5×10^{-4} M

Conical flasks (100 ml) containing 25 ml liquid medium were inoculated with about 10^6 conidia. The flasks were incubated on an orbital shaker at 25°C and 150 r.p.m. Five flasks were removed for each treatment at a time and the contents filtered through a dry, weighed sintered glass crucible. The mycelium was washed with distilled water and dried at 110°C. After cooling in a dessicator, the crucibles were weighed. The mean dry weight ± standard error for each set of replicate flasks was calculated. The regressions $y = a + bx$ of dry weight (y) on time (x) were calculated and compared with that obtained for a control in each experiment using a modified t test (Bailey)(1959)

$$\text{degrees of freedom } f = \frac{1/u^2}{n_1 - 2} + \frac{(1-u)^2}{n_2 - 2}$$

$$t = \frac{b_1 - b_2}{\sqrt{S_1^2/\Sigma_1(x-\bar{x})^2 + S_2^2/\Sigma(x-\bar{x})^2}} \qquad u = \frac{S_1^2/\Sigma_1(x-\bar{x})^2}{S_1^2/\Sigma_1(x-\bar{x})^2 + S_2^2/\Sigma_2(x-\bar{x})^2}$$

Determination of hyphal growth unit - HGU.

By using a projection head on a microscope, it was possible to make accurate scale drawings of individual branching hyphae at the edge of colonies, care being taken to sample hyphae in the immediate upper layer of the agar thus avoiding the effects of reduced oxygen tension in increasing depths of agar. From these drawings the HGU was obtained as a measure of branching.

$$\text{HGU} = \frac{\Sigma \text{ lengths of hyphae}}{\text{number of tips}} \qquad \text{(Trinci 1973)}$$

The peripheral growth zone (Trinci 1971a) was not determined because there was insufficient difference between the colony margin in the area where the peripheral growth zone had been cut and that which had not.

Nitrogen source.

Nitrogen sources used were KNO_3, $(NH_4)_2SO_4$, ammonium tartrate and asparagine. All nitrogen sources supported growth but the densest mycelium, with a normal growth rate, was produced on asparagine which was therefore used as the nitrogen source in all subsequent experiments.

Carbon source.

The growth of Ceratocystis on SSM in which the carbon source was varied is shown in Fig. 7 . Ceratocystis adiposa showed growth on all carbon sources after 2 days. Fructose, glucose, sucrose, galactose and mannose supported more growth than asparagine or rhamnose. Sorbose and xylose supported very little growth. (Fig. 7) After 4 days, (Fig. 7) sorbose-grown cultures showed more growth than rhamnose, xylose or asparagine-grown cultures and after 14 days, the plates were completely covered by a dense mycelium.

Botrytis fabae showed a different response to the various carbon sources. Fructose, glucose, mannose, sucrose and galactose supported the most growth. The mycelium on the asparagine plate covered the plate but with a very sparse mycelium after 7 days. Xylose and Rhamnose supported very little growth and sorbose supported no growth at all. Fig.8

Temperature.

The effects of temperature on growth, conidiation and fruiting of Ceratocystis adiposa are given in Table 12+13 At 35°C, conidia appeared to germinate but a day later, the germ tubes had died and no further growth occurred. No perithecia were formed at 30°C. As many thin-walled conidia were formed at this temperature and fewer thick-walled conidia than at lower

Fig. 7

Carbon sources for C. adiposa.

a)

Galactose	Glucose	Asparagine
Fructose	Sucrose	Mannose
Xylose	Sorbose	Rhamnose

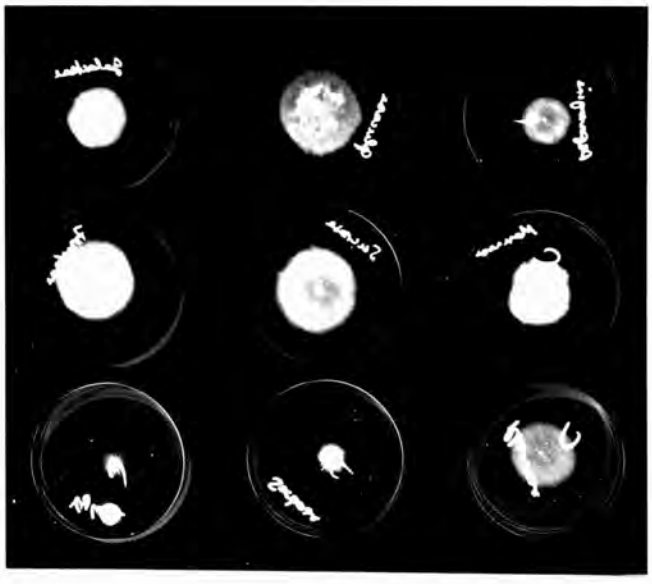
b)

Asparagine	Mannose	Rhamnose
Glucose	Sucrose	Sorbose
Galactose	Fructose	Xylose

Fig. 7

Ceratocystis adiposa grown on various carbon sources (1%) at 25° C.

(a) for 2 days. (b) for 4 days.



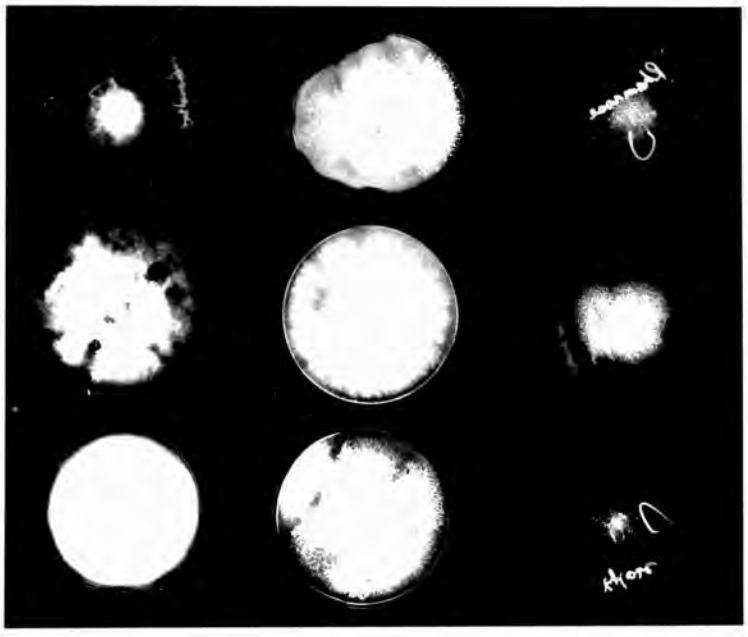


Fig. 7a

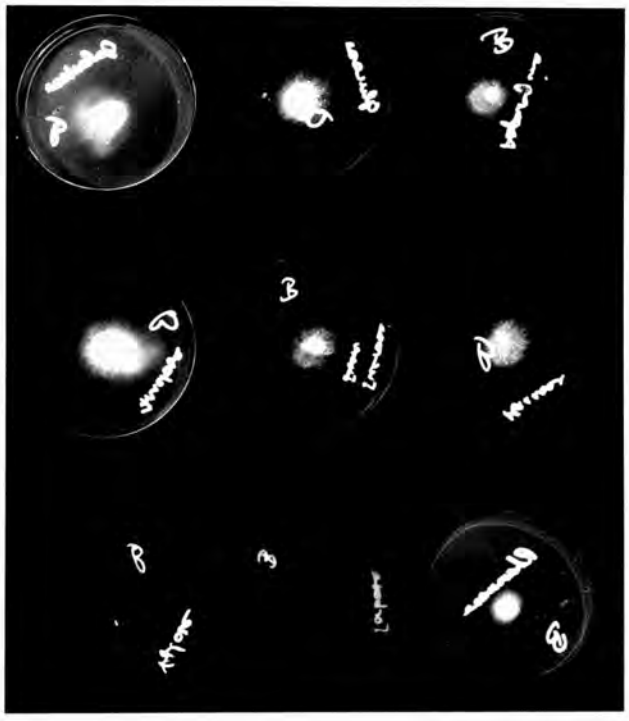
Carbon sources for *B. fabae*.

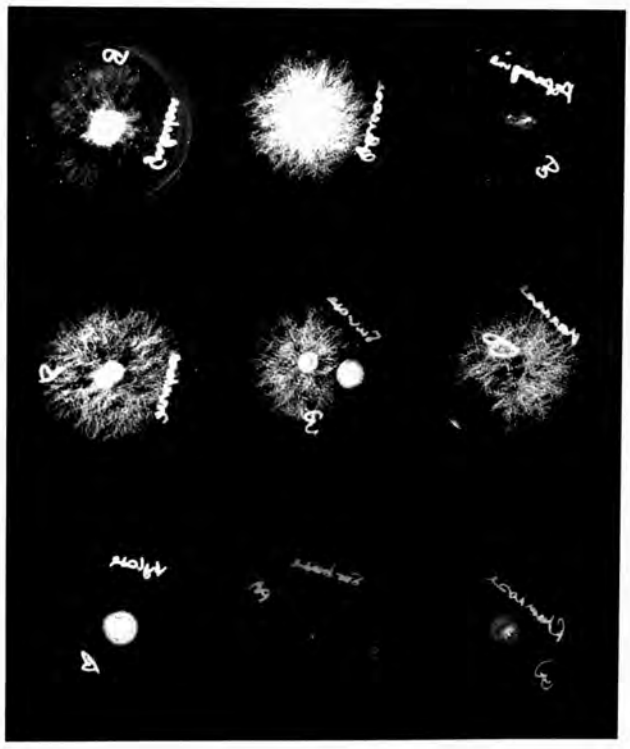
Galactose	Glucose	Asparagine
Fructose	Sucrose	Mannose
Xylose	Sorbose	Rhamnose

Fig. 7a

Botrytis fabae grown on various carbon sources (1%) at 20°C.

(a) for 4 days. (b) for 7 days.





temperatures, cultures grown for spore suspensions were incubated at 30° C. Although perithecia were always formed at 25° C, the production of spore drops was not consistent. Incubation of cultures at 20° C gave consistent ascospore production with mature spore drops.

TABLE 13

To show the effect of temperature on growth and perithecial development of Ceratocystis adiposa.

Temperature.	20°C	25°C	30°C	35°C
Age 1 day	sparse mycelium individual hyphae still visible	dense mycelium	dense mycelium	no growth
2 days	dense mycelium few conidia developing perithecia	dense mycelium many conidia no perithecia	dense mycelium many conidia no perithecia	conidia germinated.
3 days	dense grey fewer conidia developed perithecia	mycelium most conidia	many conidia no perithecia	no growth
5 days	dense dark mycelium with many conidia. many perithecia with spore drops.	many perithecia but few spore drops. *	no perithecia	no growth

* Spore drops were formed on some occasions but not others. This may be due to fluctuating temperature in the incubator.

The development of plates spread with a dense conidial suspension and those colonies developed from a point inoculum are described in Table 14 . Perithecia were not produced on spread plates other than 10^{-4} M - 10^{-5} M sodium deoxycholate.

Mycelial development on plates containing up to 0.25M of the other paramorphogens developed in the following manner. There was a mat of white mycelium after 20 hours which covered the plate. After 45 hours, the mycelium became grey. Aerial hyphae developed on control, .05M glucose and sorbose and all sodium deoxycholate plates. Conidia were present on all plates except 1.0 M glucose, sorbose and polyethylene glycol. The 1.0 M plates had very small compact, highly-domed colonies in which hyphae were not distinguishable. The colonies remained white with a brownish centre for two weeks. After this time, other mycelia were black and heavily conidiated.

The effects of the paramorphogen concentration on radial growth rate - Kr and specific growth rate - α s of Ceratocystis adiposa, Botrytis fabae and Aspergillus chevalieri.

Radial growth rate was reduced at concentrations of sorbose above 0.03 M. These higher concentrations of sorbose impart an increasing osmotic pressure effect and a parallel series of plates of corresponding glucose concentrations was set up. The values obtained for Kr at each concentration of glucose and sorbose are shown in Fig. 8+9 . Table 31.

Polyethylene glycol was introduced into the experiment as a non-metabolised compound of molecular weight 200 comparable to glucose and sorbose (180). The same molar concentrations would give similar osmotic pressures at the start of the experiment.

Fig. 8

Ceratocystis adiposa grown on various concentrations (M) of glucose sorbose and polyethylene glycol 200 at 25°C for (a) 1 day (b) 3 days. in SSM.

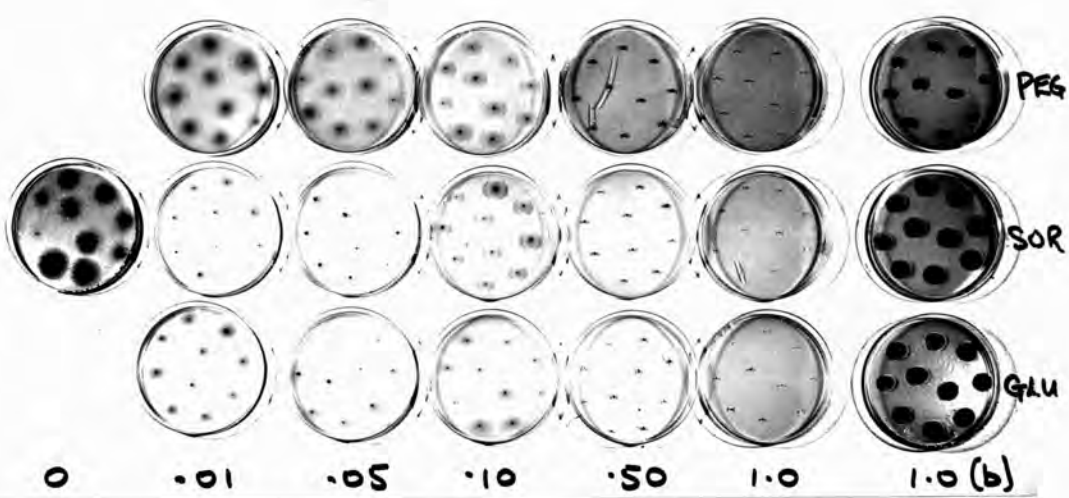


Fig. 9.

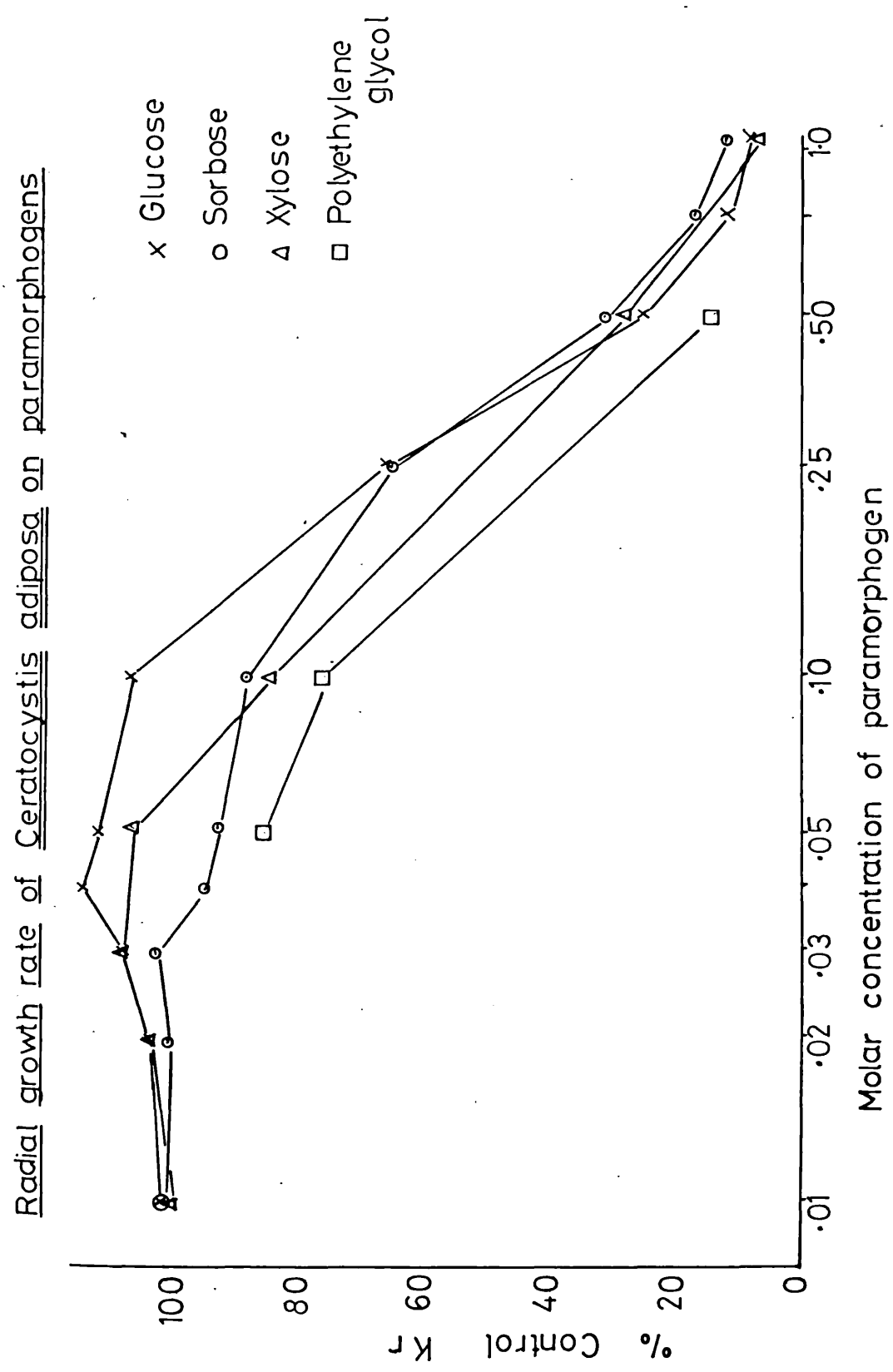

Radial growth rate of Ceratocystis adiposa on paramorphogens
× Glucose
o Sorbose
Δ Xylose
□ Polyethylene glycol
% Control Kr
100
80
60
40
20
0
·01
·02
·03
·05
·10
·25
·50
1·0
Molar concentration of paramorphogen

TABLE 14

To show the effect of age and the paramorphogens

Medium \ Age	20 hours *	45 hours *
CONTROL	Thin mat of mycelium covered plate and no restriction apparent /20	Grey mycelium with much aerial growth and many conidia /47
.05M GLUCOSE	/19	/47
.05M SORBOSE	/19	/48
.05M POLY ETHYLENE GLYCOL	hint of restriction when viewed with naked eye. /19	as above but very little aerial growth /45
.25M GLUCOSE	restriction visible with naked eye /11	mycelium beginning to grey /23
.25M SORBOSE	but mycelium covered plate /10	many conidia /24
.25M POLY ETHYLENE GLYCOL	restriction more marked /12	no aerial growth /26
1.0M GLUCOSE	colonies very dense and /2	colonies domed with brown centres /5
1.0M SORBOSE	restricted /2	no conidia /4
1.0M POLY ETHYLENE GLYCOL	very little growth / 2	very little growth / 2
5×10^{-5} M SODIUM DEOXYCHOLATE	slightly sparser cover than control /16	Grey mycelium aerial growth conidia /29
10^{-4} M "	but restriction not apparent /9	as above more aerial growth /19
10^{-3} M "	very restricted colonies / 2	white mycelium few conidia aerial hyphae /18

* Plates spread with 8×10^{5} conidia and incubated invertedly at 25 C in darkness. Figures give diameter (mm) from point inoculum plates.

There was no perithecial development on these plates.

TABLE 14

on Ceratocystis adiposa at 25° C.

2 Weeks	Perithecia 4 days Ø	Spore drops 4 days Ø	Perithecia 2 Weeks Ø	Spore drops 2 Weeks Ø
Black mycelium Many conidia especially on aerial hyphae.	+		+	+
very black thick walled conidia on aerial hyphae	−		+	+
	+		+ +	+ +
	+		+	+
very black as above	−		(+)	−
less black	−		+ +	+ +
least black	−		+ + +	+ + +
colonies discrete domed with brown centres	−		−	−
	−		−	−
very compact	−		−	−
black mycelium colonies no longer discrete	+ +		+ + +	+ + +
"	+		+ + +	+ + +
Perithecia appeared after 3 weeks on 10^{-4} M - 10^{-5} M	+ +		+ + +	+ + +

Ø Point inoculum at centre of plate.

Xylose supported about the same amount of growth of Ceratocystis adiposa as sorbose initially and so Kr was measured on a series of concentrations of xylose.

The effects of various concentrations of all these paramorphogens on Kr of Ceratocystis adiposa are shown in Fig. 9 Table 31 The reduction in Kr obtained from the sugars and polyethylene glycol shows a similar trend. At concentrations up to 0.03 M, the results are not significantly different but at 0.03 M those for glucose and sorbose diverge while those for xylose and polyethylene glycol continue at the same level. At 0.04 M there was a significant difference (P. 0.05) between the values of Kr on glucose and sorbose. Ceratocystis adiposa shows an enhanced growth rate on a medium with a slightly higher osmotic pressure than SSM as values for Kr at concentrations below 0.05 M are greater than control values.

The graph for sodium deoxycholate shows no initial plateau but Kr is reduced to 87% at 10^{-5} M. Between 6 - 8 x 10^{-5} M there is an increase in Kr from 40 - 47 % and this increase was confirmed in two further experiments. Fig. 10 + 11 Tables 31-34

At concentrations greater than 0.1 M, the Kr on polyethylene glycol is consistently lower than on the sugars.

The effect of the same paramorphogens on Botrytis fabae, a fungus known to be sensitive to sorbose, was observed for a comparison with Ceratocystis adiposa. The results are shown in Fig. 12 Table 34 Sorbose reduces the Kr of Botrytis fabae by about 70% at 0.01 M. Xylose causes an increase in Kr at 0.01 M while glucose has no significant effect up to 0.1 M at which concentration Kr is increased. Polyethylene glycol causes an increase in Kr at 0.01 M but has no effect up to 0.1 M. Sodium deoxycholate causes a steady decrease in Kr with a possible fluctuation in the curve between 7.5 x 10^{-5} - 10^{-4} M. Fig 10 Tables 31-34

Fig 10

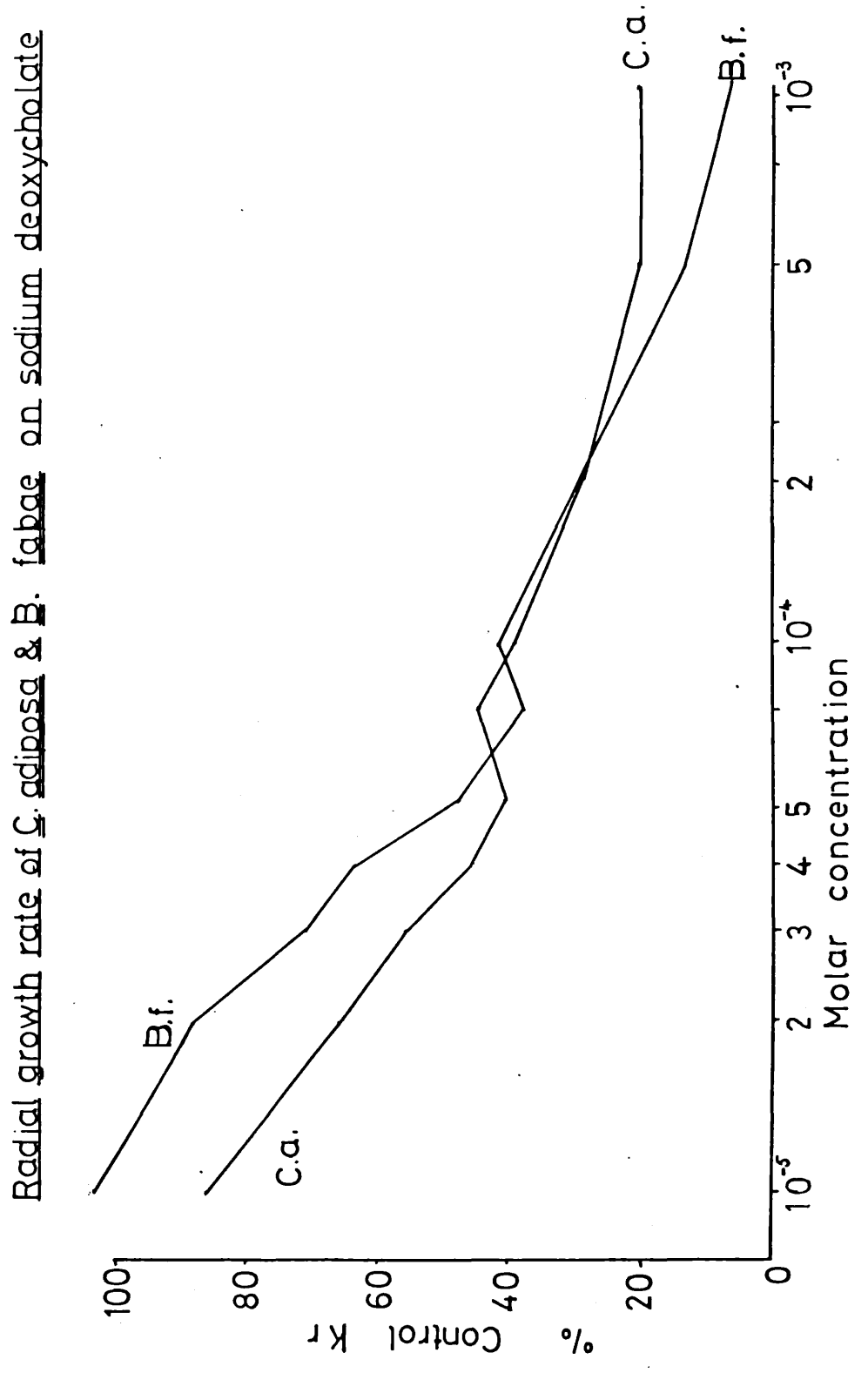

Radial growth rate of C. adiposa & B. fabae on sodium deoxycholate
% Control Kr
100
80
60
40
20
0
B.f.
C.a.
C.a.
B.f.
10^{-5}
2
3
4
5
10^{-4}
2
5
10^{-3}
Molar concentration

Fig. 11

Ceratocystis adi osa grown on various concentrations in SSM ($x10^{-5}$) of sodium deoxycholate at 25°C for

(a) 1 day (b) 3 days

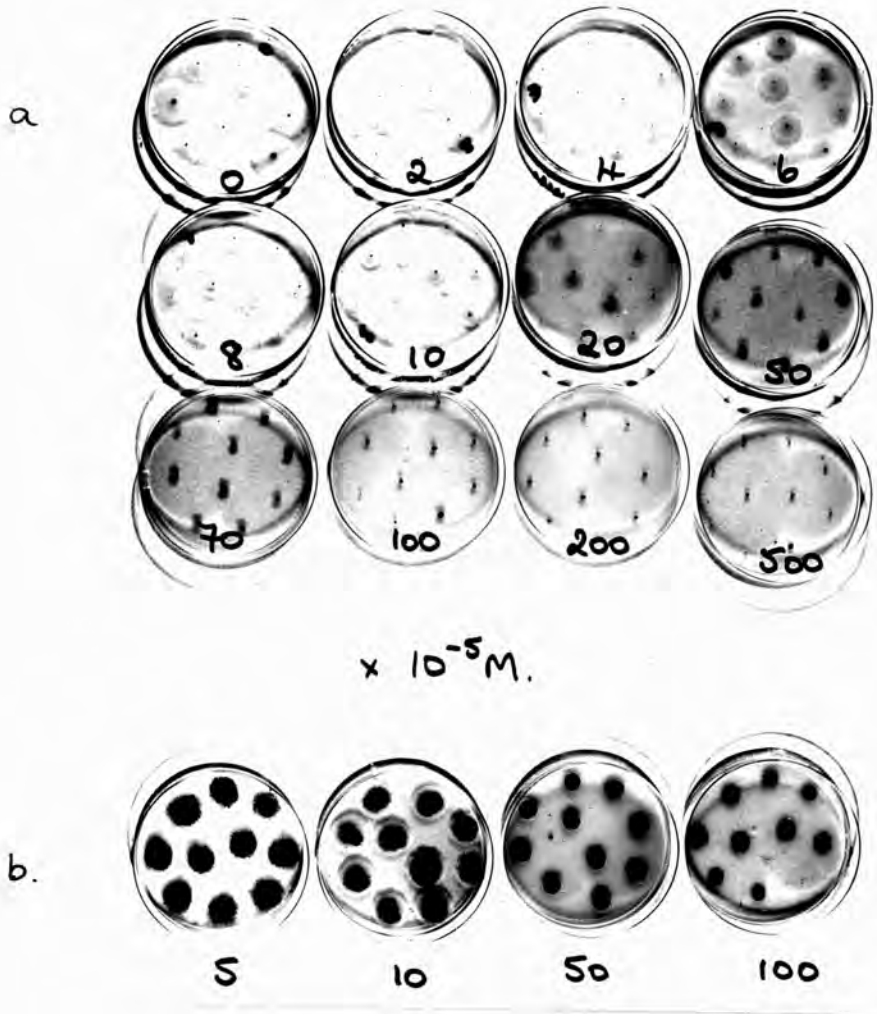


Fig 12

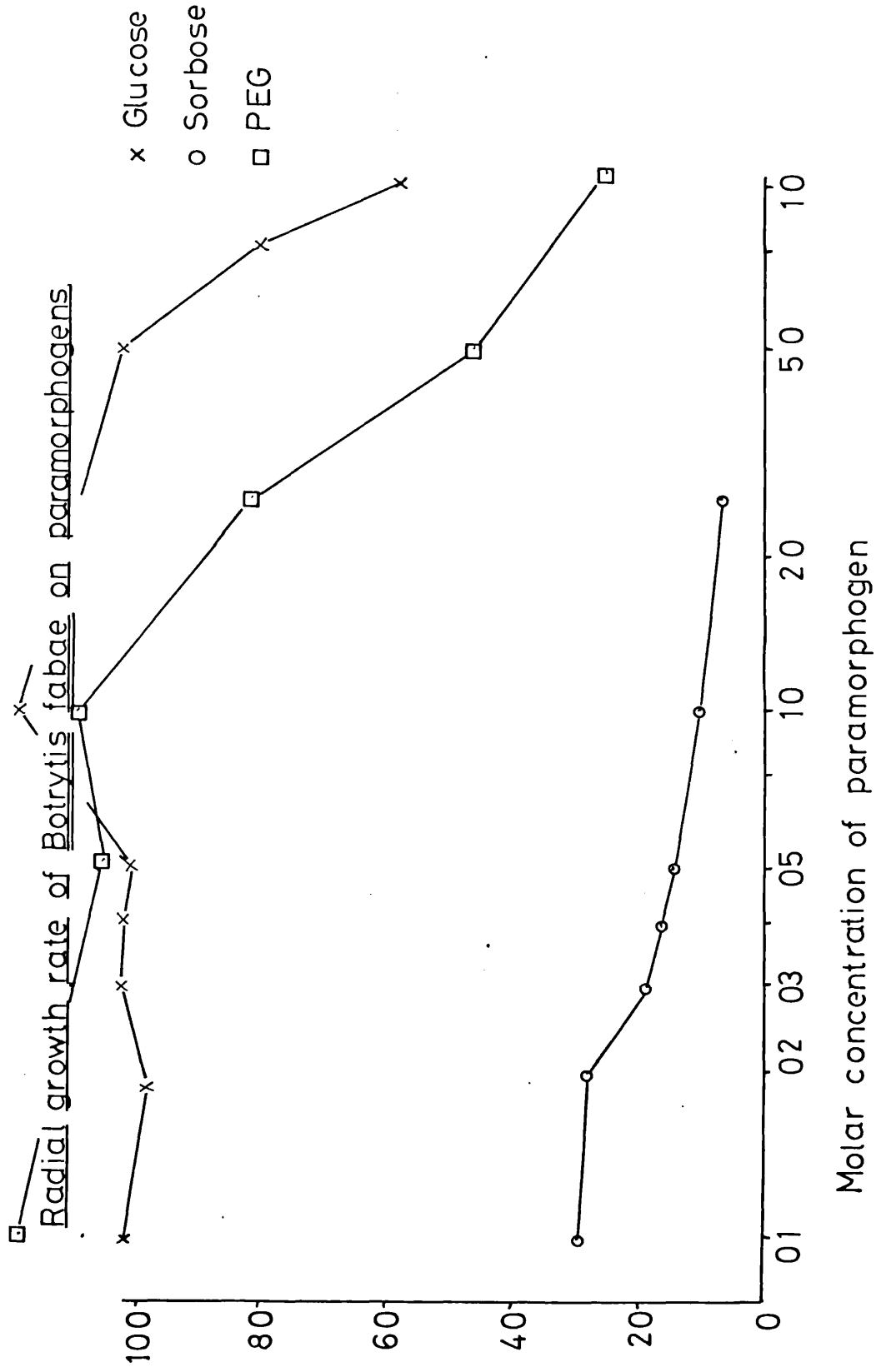
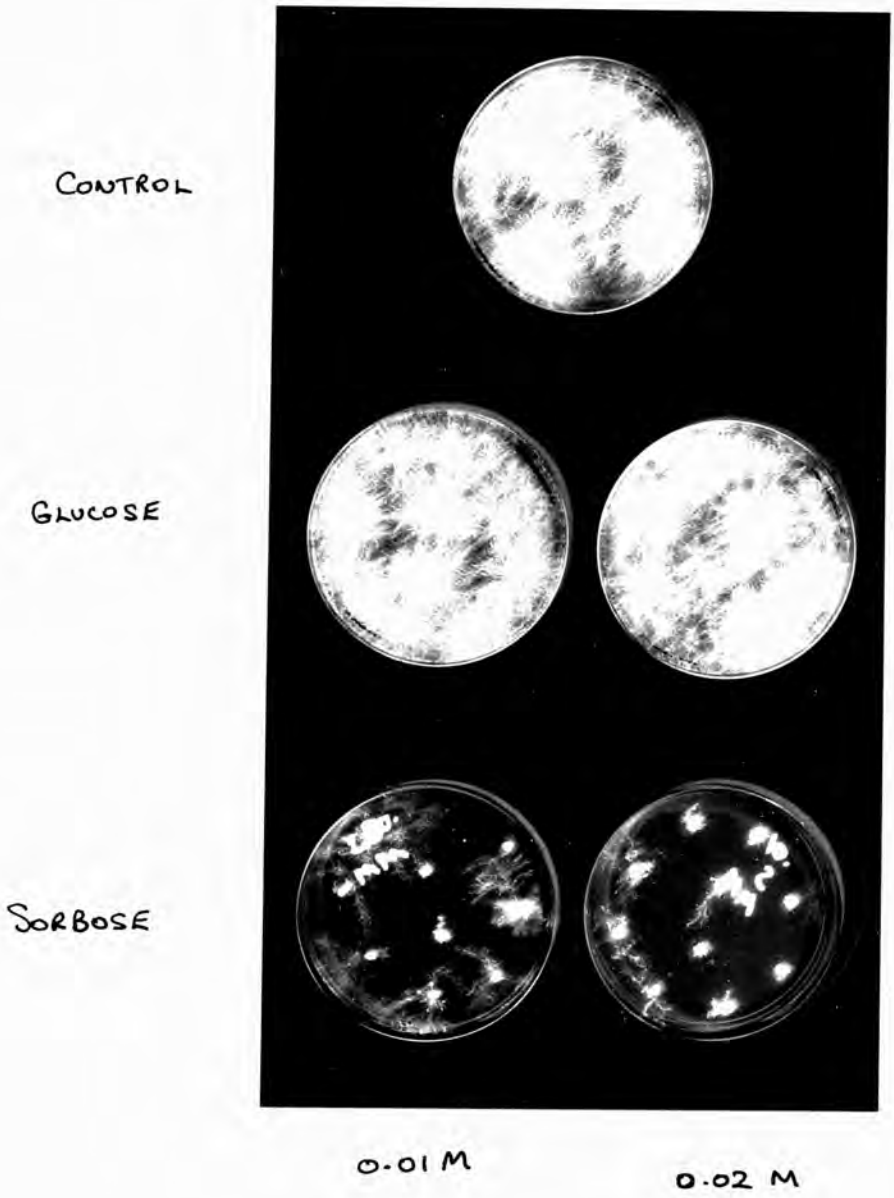

Radial growth rate of Botrytis fabae on paramorphogens
× Glucose
o Sorbose
□ PEG
100
80
60
40
20
0
01
02
03
05
10
20
50
10
Molar concentration of paramorphogen

Fig.13

Botrytis fabae on sorbose and glucose media after 4 days at 20°C.



The growth of Botrytis fabae on glucose and sorbose is shown in Fig. 13 It was observed that the colony margin on sorbose is very irregular. This is because resistant hyphae emerge from the main colony and grow at a faster rate than those hyphae inhibited by sorbose (personal communication - Dr. J.E. Kerr).

As the effect of the sugars on Ceratocystis adiposa was partly due to increased osmotic pressure of the medium, it was decided to compare the reaction of Ceratocystis adiposa with a quick-growing filamentous fungus which was also osmotolerant. Aspergillus chevalieri was obtained for this purpose. Aspergillus chevalieri produced many dry yellow conidia which were easily dispersed when the plates were moved and consequetly many secondary colonies developed. There was a difference between the responses of Aspergillus chevalieri to glucose and polyethylene glycol (Fig. 14-17. Table 36-37). Polyethylene glycol inhibits growth at the concentration used (1.0 M) and growth was completely inhibited at 3.0 M whereas glucose radial growth rate is maximal at 2.5 M and occurred on plates containing 5 M glucose after 7 days.

It may be observed from Fig. 15-16 that the lag before growth occurred increased with increasing concentration of glucose and polyethylene glycol.

The growth rates on 2.0 M, 2.5 M and 3.0 M glucose are the highest, other concentrations supporting a slower growth rate. (See Table 37 and Fig. 14).

Fig 14. Radial growth rates of Aspergillus chevalieri on glucose and PEG 200

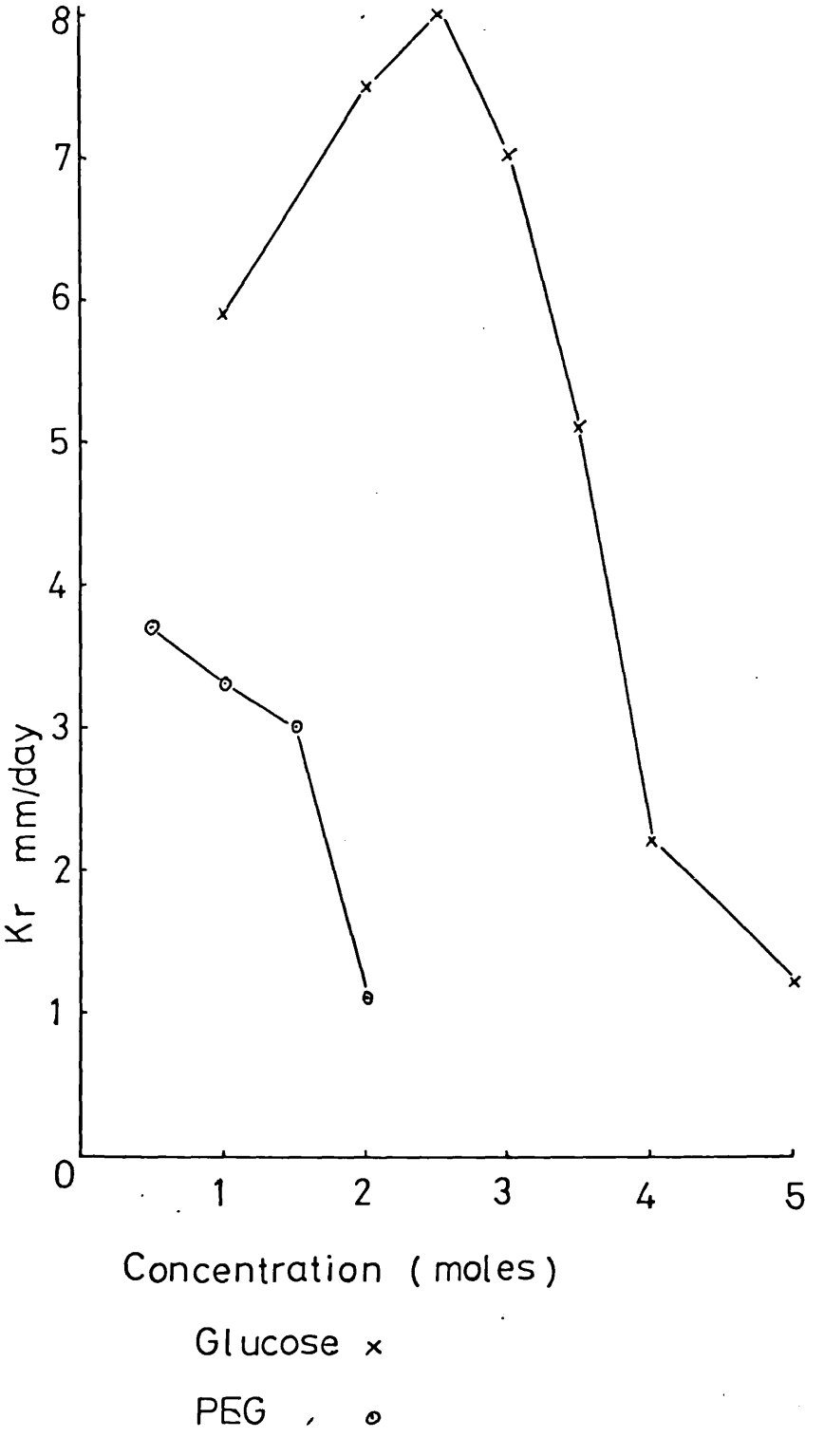


Fig. 15

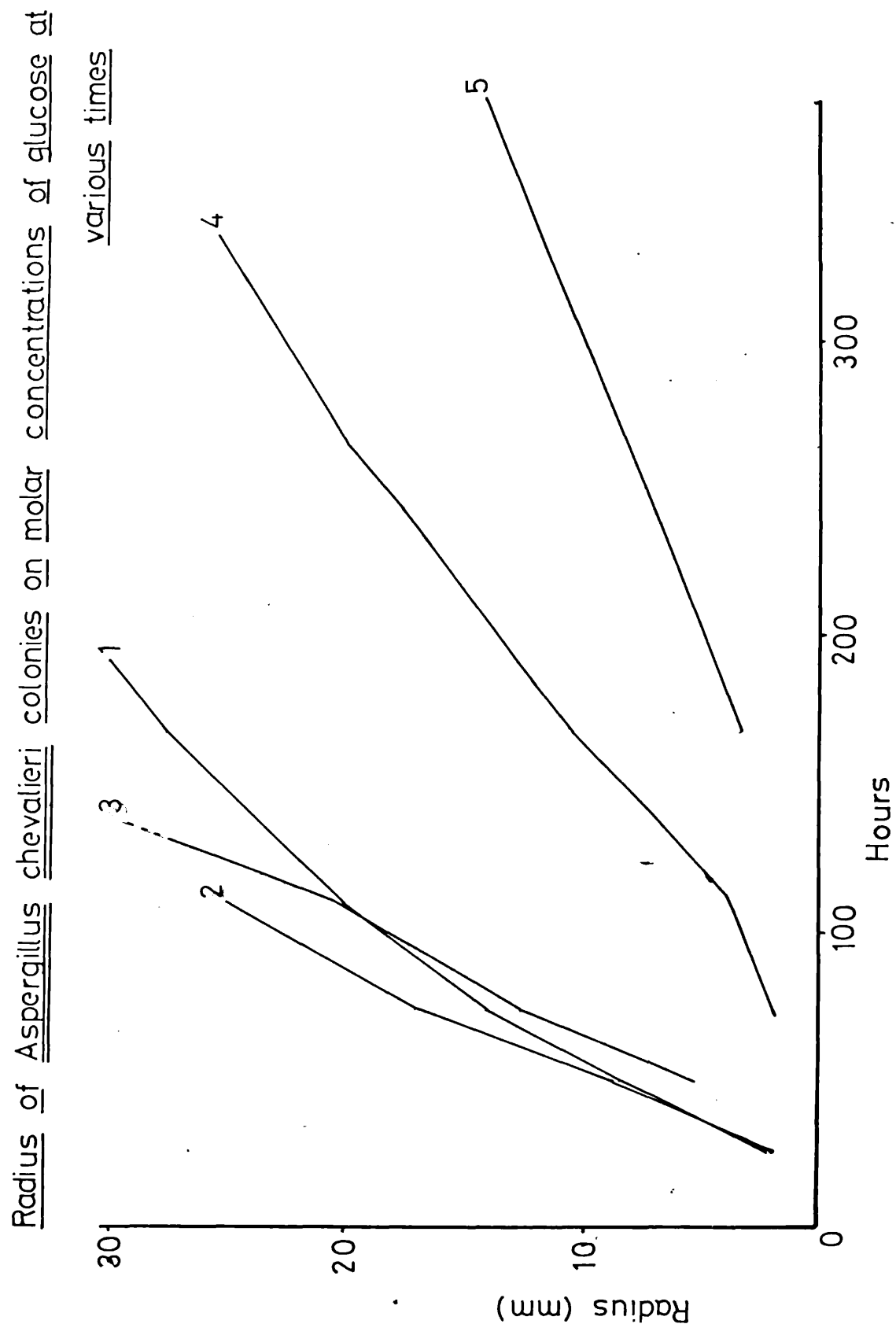

Radius of Aspergillus chevalieri colonies on molar concentrations of glucose at various times
Radius (mm)
30
20
10
0
100
200
300
Hours
1
2
3
4
5

Fig.16.

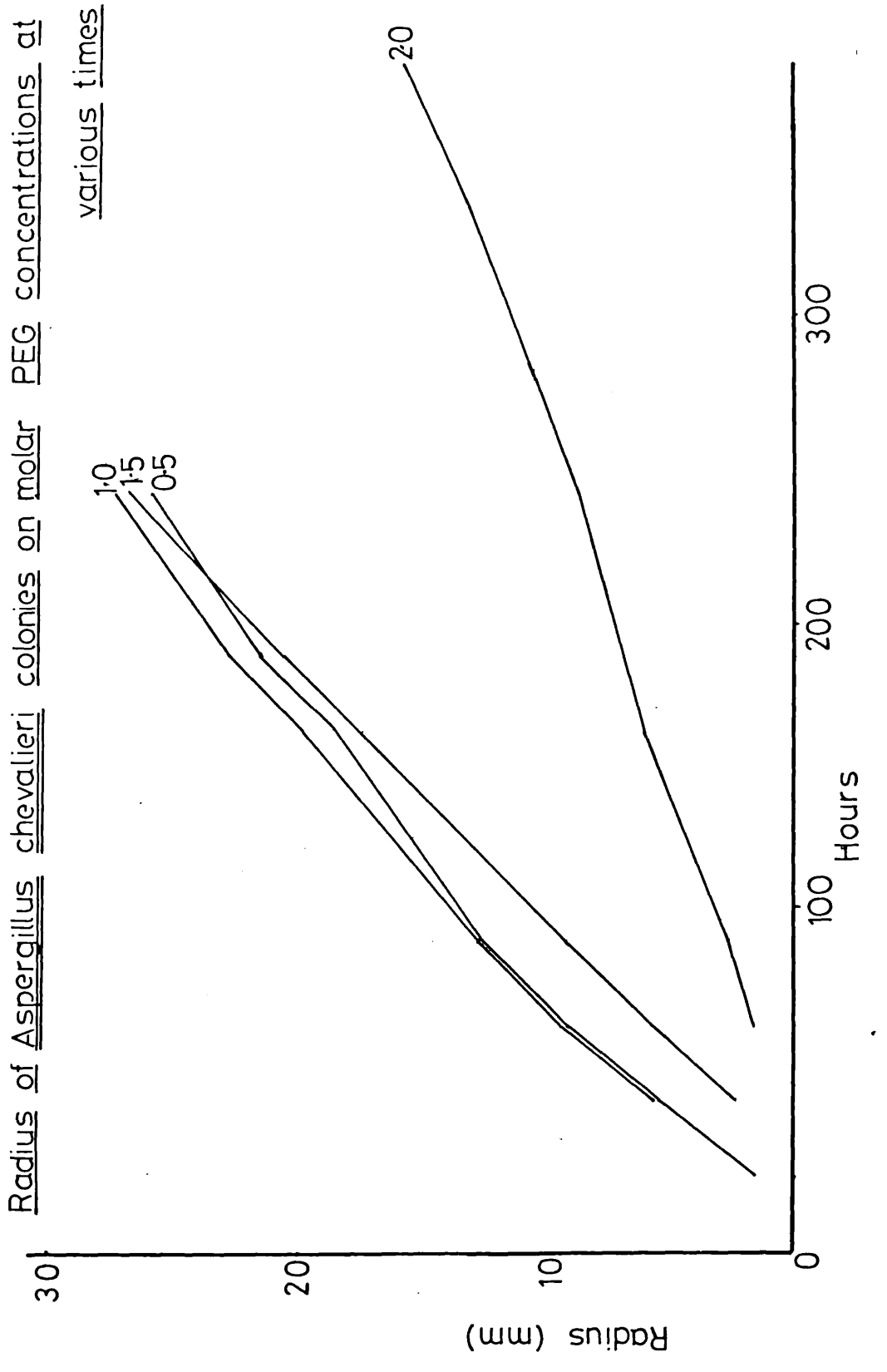
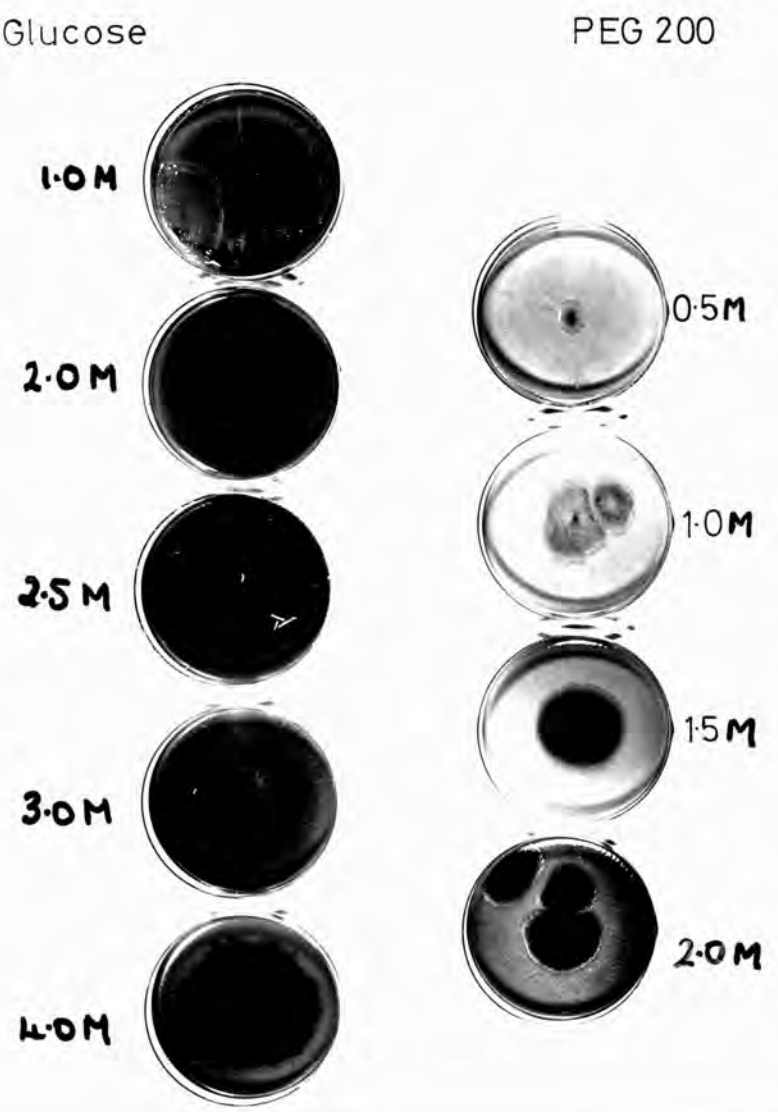

Radius of Aspergillus chevalieri colonies on molar PEG concentrations at various times
1·0
1·5
0·5
2·0
30
20
10
0
100
200
300
Hours
Radius (mm)

Fig. 17

Aspergillus chevalieri on glucose and polyethylene glycol 200 media after 10 days at 25°C (15 days for 4M glucose and 2M polyethylene glycol 200)



Ceratocystis adiposa colony margin. The margin of a colony grown on SSM shows the hyphae growing fairly straight and with sparse branching. When grown on 0.1 M glucose, sorbose or polyethylene glycol 200, the hyphae appear narrower and branch more frequently. They also appear tortuous especially on polyethylene glycol 200. (Fig.18+19). At 0.5M concentrations, the hyphae are narrow and tortuous on all the media. Ceratocystis adiposa will not grow on plates supplemented with 1.0M polyethylene glycol but on 1.0M glucose and sorbose, the colonies are very compacted with highly branched narrow hyphae. (Fig.20)

Sodium deoxycholate at 5×10^{-4}M concentration does not effect hyphal branching very much. Resistant hyphae at 10^{-3} M, Fig 20 are similar in size and branching to those on 5×10^{-4}M but the colony margins appear different due to different hyphal densities. At 10^{-3}M, the margin appears similar to that on 1.0M glucose or sorbose

Fig 18 Hyphae of C. adiposa from surface colonies grown on different media

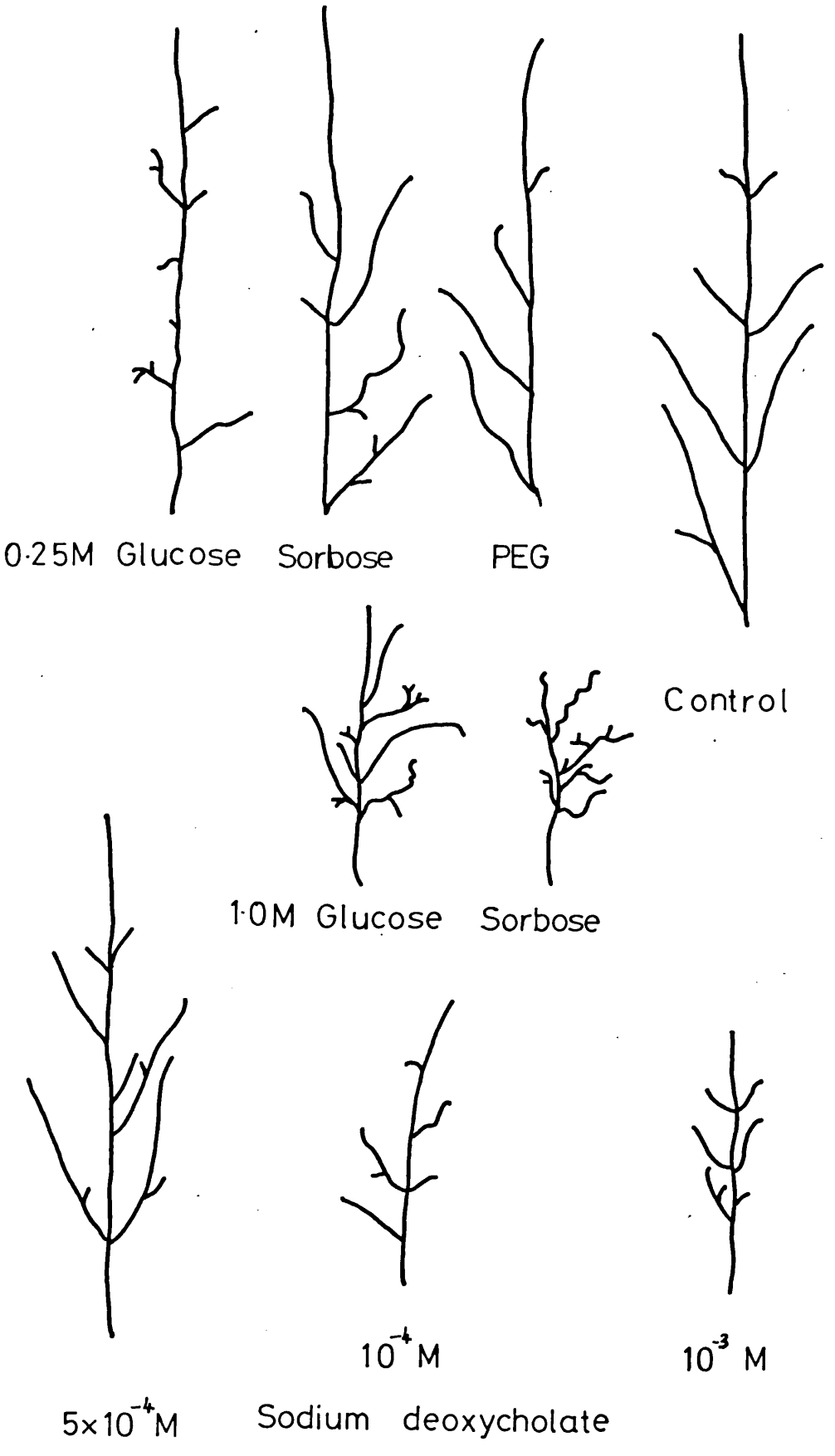


Fig. 19

Colony margin of Ceratocystis adiposa grown on various media.

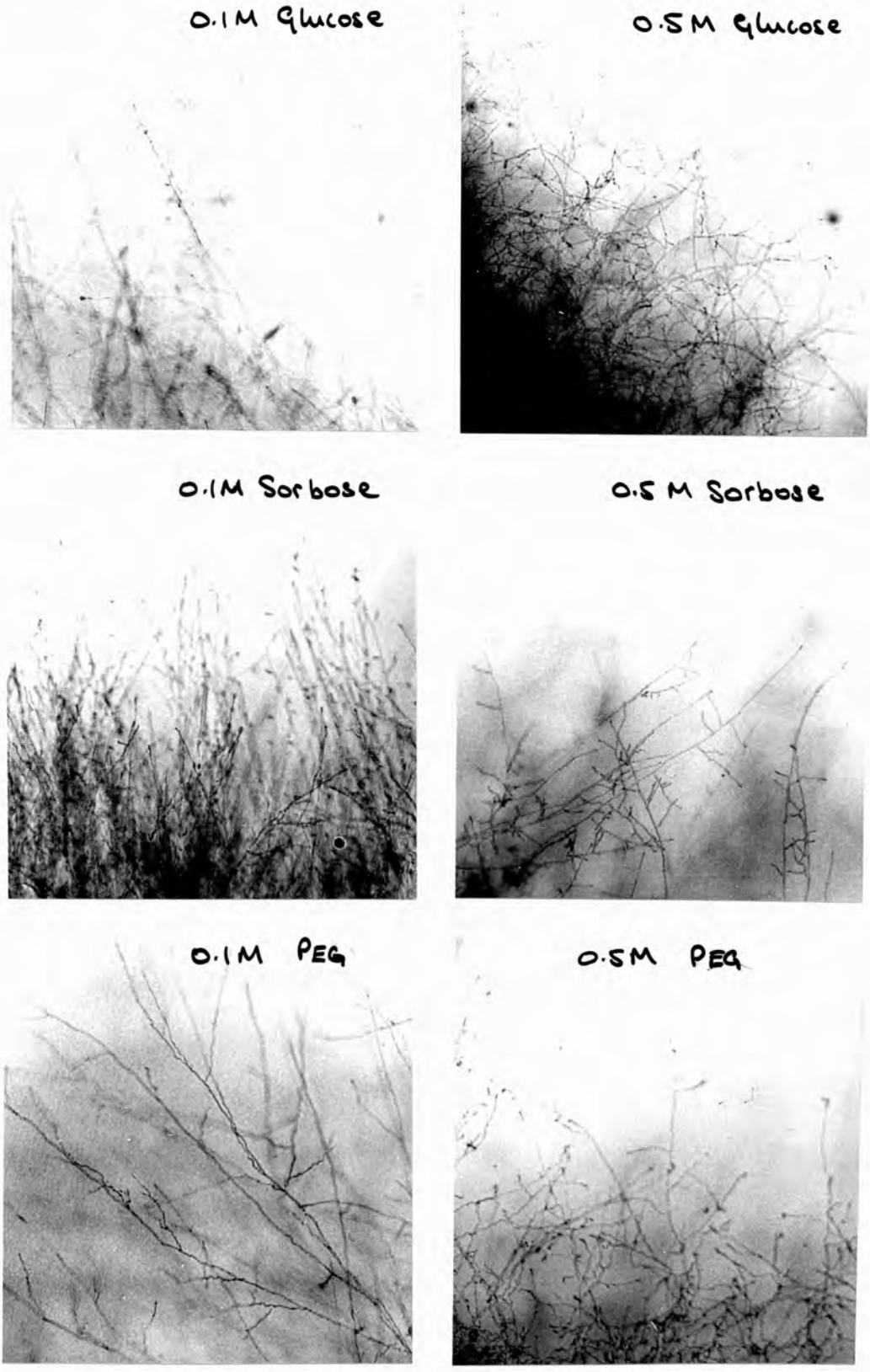
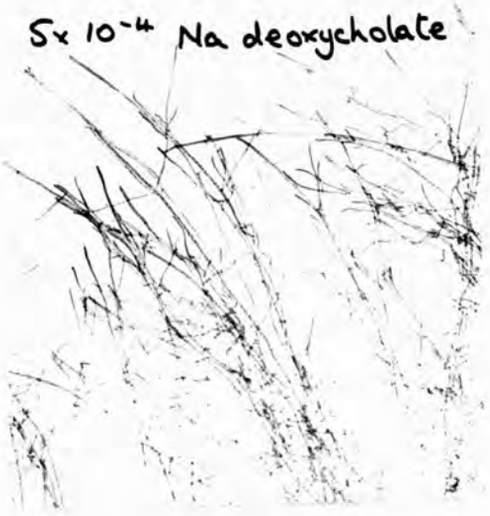
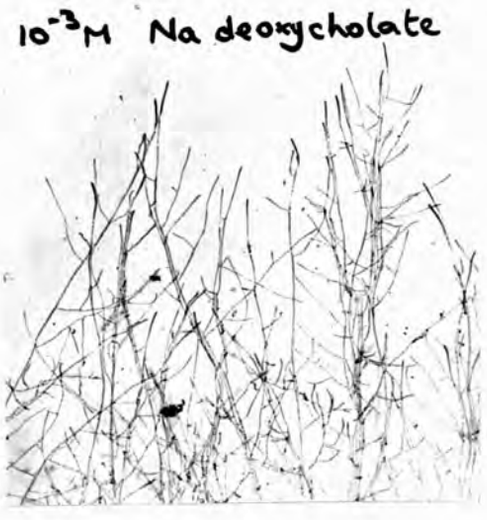


Fig.20

Colony margin of Ceratocystis adiposa grown on various media.





HYPHAL GROWTH UNITS. Table 15

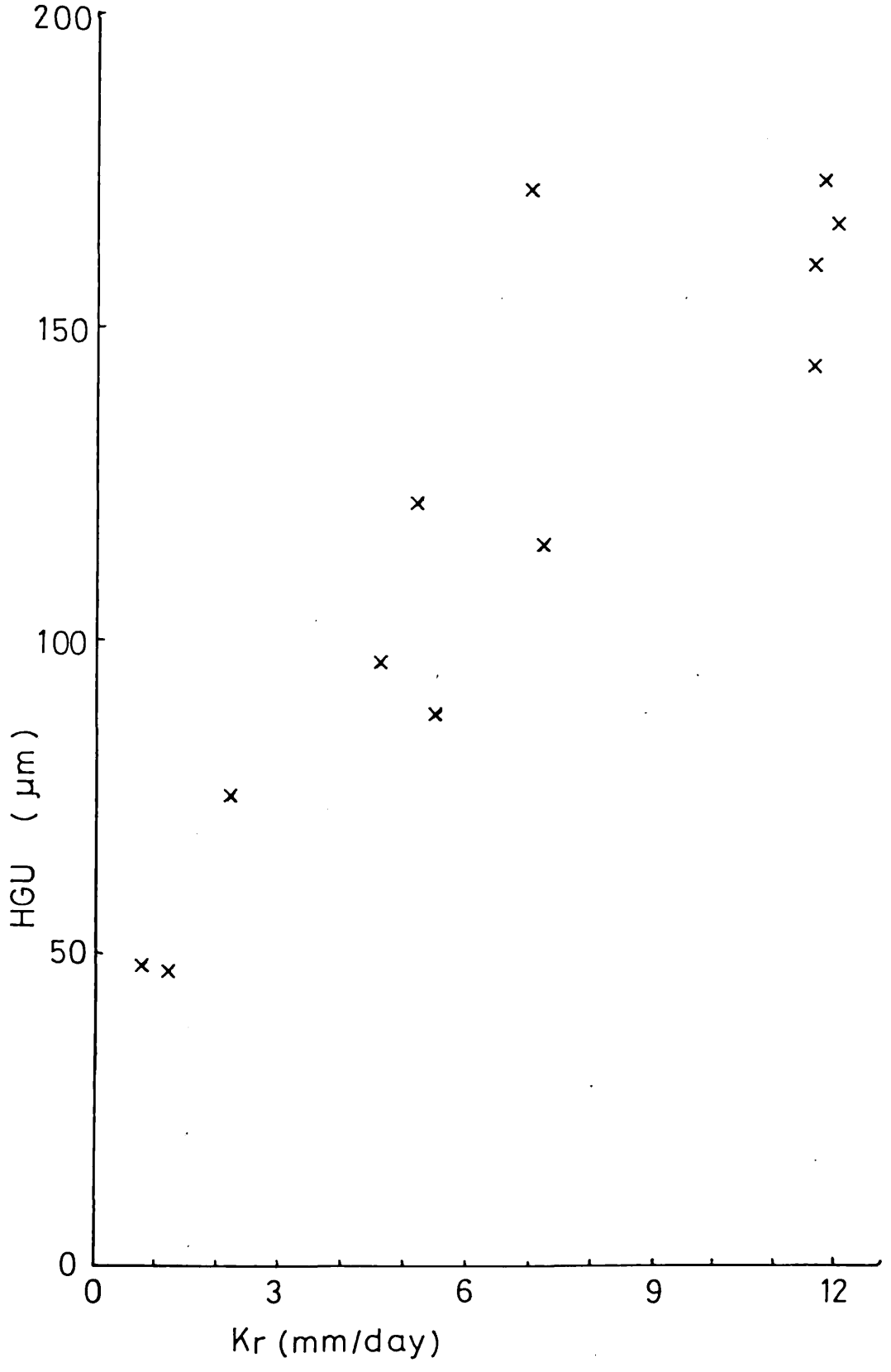
These were made from drawings done using a projection head to the microscope (Fig.18) mean ± standard error for 10 hyphal tips

Treatment.	HGU (μm).	Kr(mm/day).
Control.	160.5 ± 18.6	11.6
0.05M Glucose	144.5 ± 18.6	11.6
0.05M Sorbose	174.4 ± 32.5	11.8
0.05M Polyethylene glycol	167.5 ± 15.2	12.0
0.25M Glucose	88.2 ± 9.0	5.5
0.25M Sorbose	121.8 ± 18.0	5.2
0.25M Polyethylene glycol	172.2 ± 24.2	7.0
1.0 M Glucose	48.0 ± 6.7	0.8
1.0 M Sorbose	47.0 ± 9.4	1.2
5×10^{-5} M Sodium deoxycholate	114.6 ± 12.2	7.2
10^{-4} M Sodium deoxycholate	96.5 ± 10.1	4.6
10^{-3} M Sodium deoxycholate	75.0 ± 9.0	2.2

As the concentration of the paramorphogen increased, except PEG the value of HGU decreased. The correlation coefficient between HGU and Kr was calculated to be 0.9064. Fig 21

Generally, Ceratocystis showed a significant association between increased branching and reduced Kr in response to higher paramorphogen concentrations.

Fig 21 Relationship between Kr and HGU for C. adiposa r = ·9064



Unlike Ceratocystis,

Aspergillus chevalieri is more branched on 1.0M glucose and 0.5M polyethylene glycol than on 5.0M glucose and 2.0M polyethylene glycol. Hyphal growth units are given in Table 15a, values increasing with increased concentration. When concentration HGU and Kr are plotted together, Fig 25 it is shown that for values of Kr between 6 and 8 mm/day there are two values of HGU depending on whether the fungus was grown above or below its optimum concentration of glucose.

The range of HGU obtained in this experiment is similar for glucose and polyethylene glycol but it is achieved with a much smaller range of polyethylene glycol concentrations than of glucose concentrations. Fig. 22 - 25

Table 15a The effect of concentration of glucose and polyethylene glycol on branching in Aspergillus chevalieri.

Concentration. M	Hyphal growth unit. HGU in μm ± Standard error.	
Glucose		
0.5	83.76	14.05
1.0	86.70	24.18
1.5	94.06	15.53
2.0	95.41	36.18
2.5	109.35	24.06
3.0	129.23	18.94
3.5	134.70	36.82
4.0	140.65	32.24
5.0	188.59	24.71
Polyethylene glycol.		
0.5	74.23	2.94
1.0	93.88	17.64
1.5	125.23	19.18
2.0	212.17	39.17

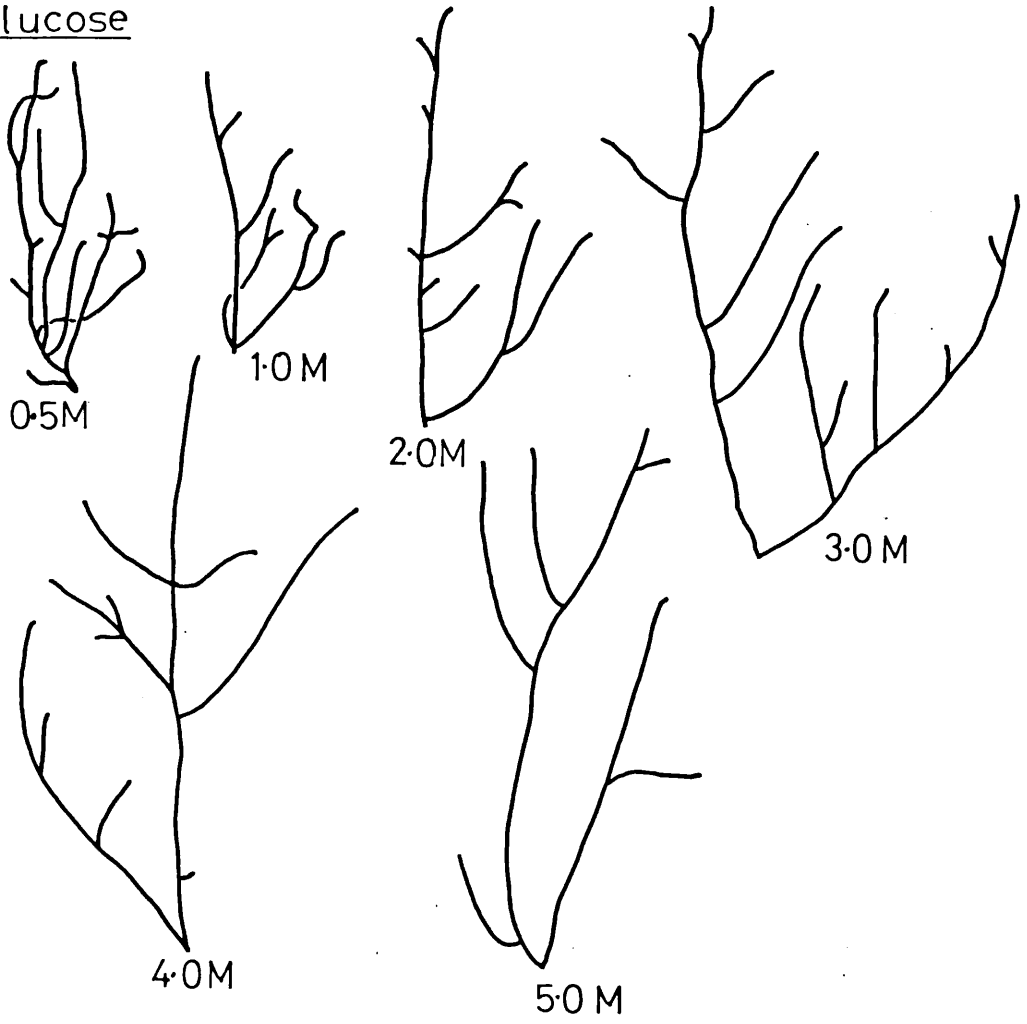
The correlation coefficient calculated for Concentration and HGU is -

0.9626 for glucose

0.9432 for polyethylene glycol.

Fig 22
Hyphae of A. chevalieri from surface colonies

Glucose



PEG 200

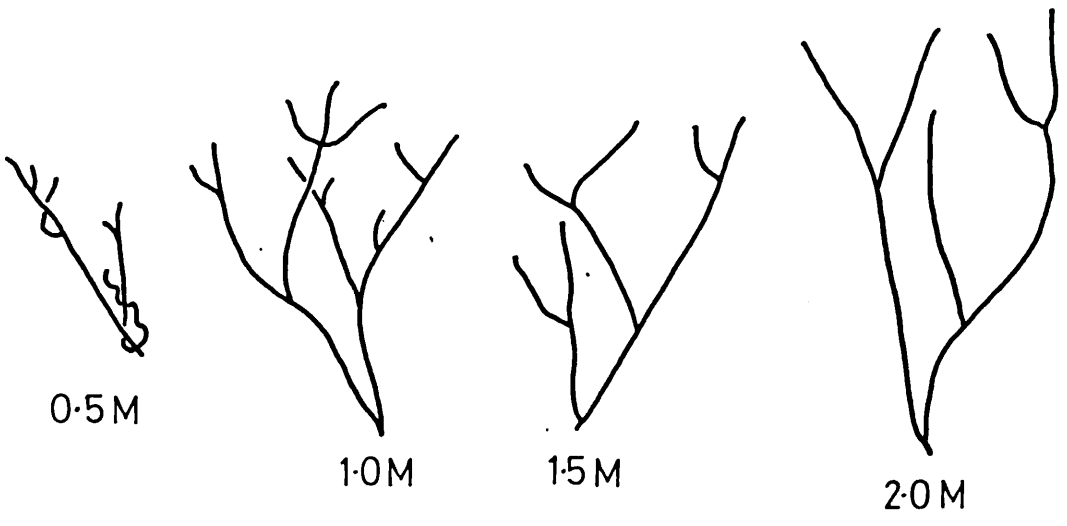


Fig.23

Colony margin of Aspergillus chevalieri grown on glucose media.

Fig.24

Colony margin of Aspergillus chevalieri grown on polyethylene glycol 200 media.

Fig 25

The Relationship between Kr, HGU and Molar concentrations of Aspergillus chevalieri on glucose.

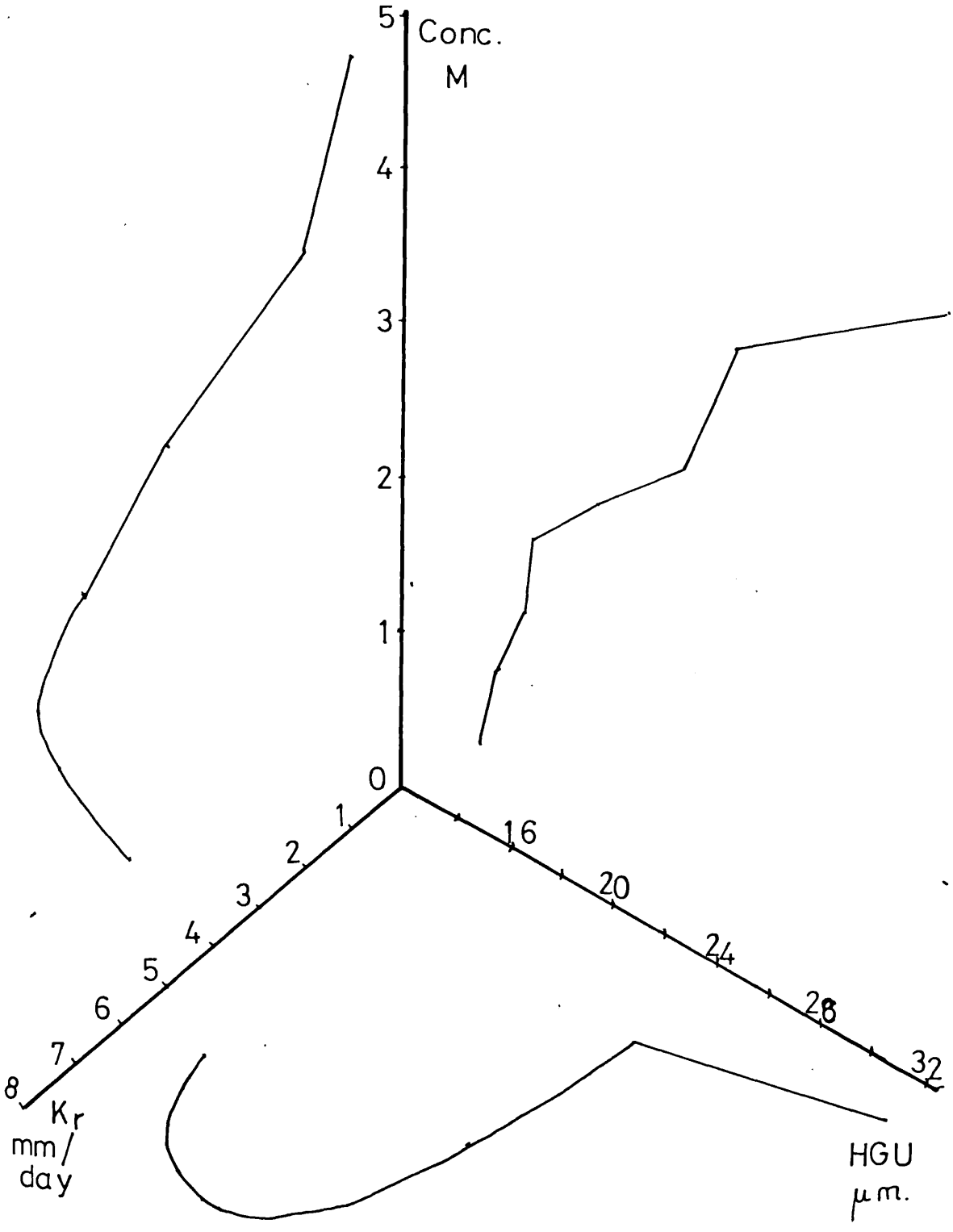
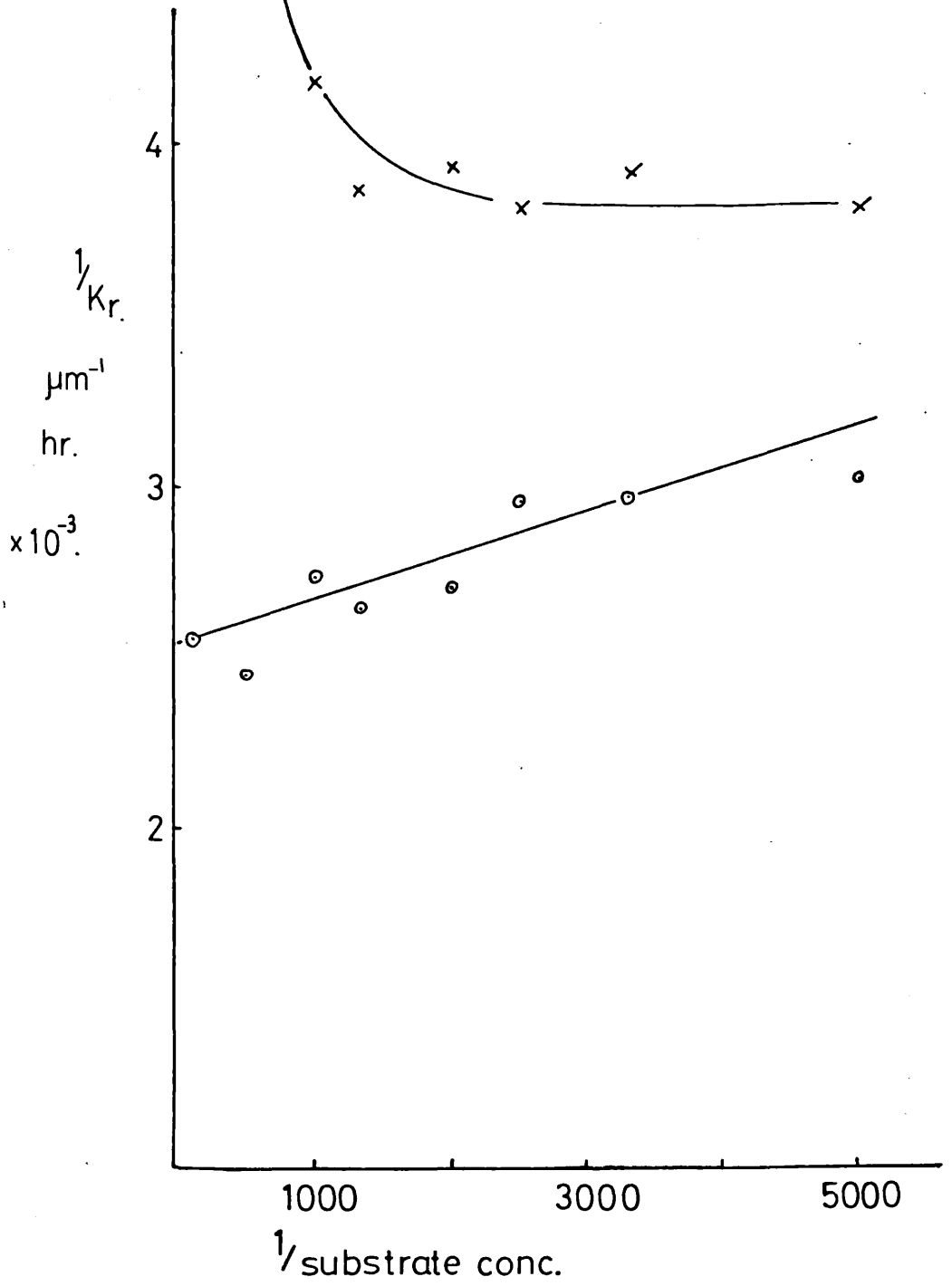


Fig 16 (p 371)

Kr of C. adiposa at low substrate concentration to estimate K_s



Using values obtained for Kr on a series of low glucose and sorbose concentrations, a value for Ks was obtained for glucose. 5.117×10^{-5} M (Fig.26). It was not possible to obtain a value for sorbose by this method because the reciprocal of growth rates did not give a straight line when plotted against the reciprocal of time.

The values for Kr were in mm/hour and for substrate concentration in moles.

b = 0.00013014

a = 2.5433

Therefore $\frac{1}{Kr}$ max. = 2.5433.

Kr Max = 0.3932 mm/hour = 9.4366 mm/day.

from the equation $y = bx + a$

$$Kr = b[S] + a$$

when Kr = 0, $b(S) = -a$

$$-\frac{1}{Ks} = \frac{-a}{b} = -19542.8001$$

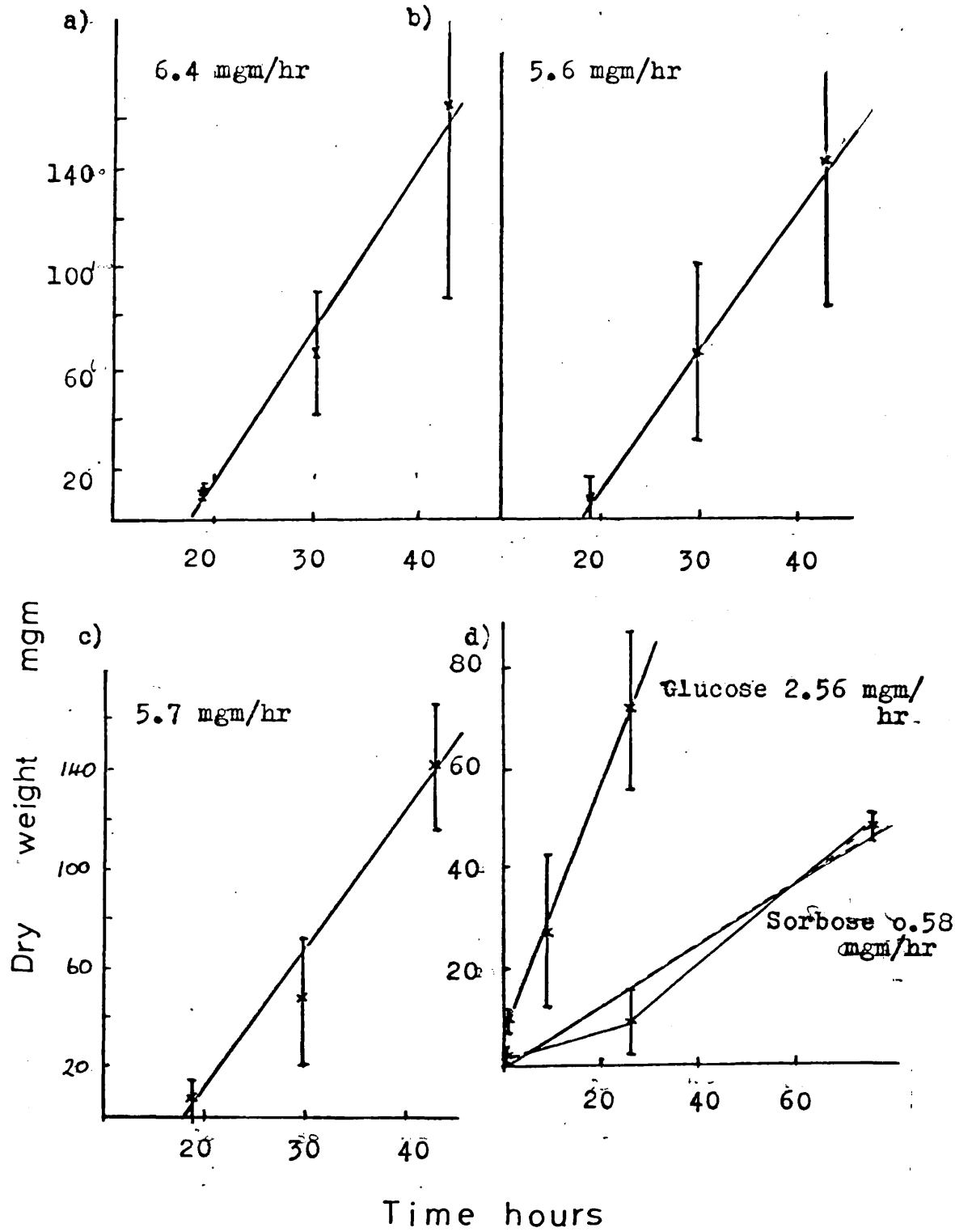
$$= 51.17 \times 10^{-6} \text{ M.}$$

αs. SPECIFIC GROWTH RATE.

Values for αs were obtained from regression lines calculated on all points (dry weight v. time). The lines (Table 16) from cube root, logarithmic and arithmetic plots for Ceratocystis adiposa grown in glucose, sorbose and the control were compared.

The arithmetic plots appeared to give the best straight line values for the correlation coefficient were calculated for each treatment and are given in Table 16. Except when Ceratocystis adiposa is grown in sorbose containing medium, the data showed the highest correlation coefficients for arithmetic plots. The

Fig.27 Specific growth rates of Ceratocystis adiposa.
a) SSM as Control; b) Glucose, c) Sorbose added at 0.25M as paramorphogens. d) Comparison of Glucose and Sorbose (1%) as sole carbon sources in SSM mineral solution.



values given for specific growth rates are, therefore, taken from the regressions calculated on dry weight against time. These values are given in Table 16a

There may be a longer lag phase when sorbose is the sole carbon source. Fig 27

Pellet size was reduced in shake culture by the paramorphogens. From Table 16a it can be seen that sorbose and glucose do not have a significant effect on αs at the concentration which halves Kr. However, polyethylene glycol and sodium deoxycholate do reduce the αs and may be slightly toxic. Sorbose and sodium deoxycholate do not cause a significant reduction in αs of Botrytis fabae.

TABLE 16.

CORRELATION COEFFICIENTS CALCULATED FOR PLOTS OF DRY WEIGHT AGAINST TIME.

CERATOCYSTIS ADIPOSA.

Treatment	Arithmetic	Logarithmic	Cube root
Control	.9946	.9700	.9918
Glucose	.9994	.9532	.9807
Sorbose	.9843	.9798	.9978
Control	.9948	.9056	.9464
Polyethylene glycol	.9949	.9194	.9539
Sodium deoxycholate	.9897	.8998	.9381
BOTRYTIS FABAE.			
Control	.9852	.9994	.9970
Sorbose	.9647	.9649	.9654
Sodium deoxycholate	.9959	.9716	.9821

TABLE. 16a

THE EFFECT ON α_s OF THE PARAMORPHOGENS ON CERATOCYSTIS ADIPOSA AND BOTRYTIS FABAE.

	$\alpha_s \pm S\bar{x}$ mgm/hr.	Probability that the treatment is the same as control.
CERATOCYSTIS ADIPOSA.		
Control (SSM)	6.4 ± 0.96	
Glucose (0.25M)	5.6 ± 0.81	P > 0.1
Sorbose (0.25M)	5.7 ± 0.53	P > 0.1
Control	4.5 ± 0.18	
Polyethylene glycol (0.25M)	2.9 ± 0.22	P < 0.001
Sodium deoxycholate (0.25×10^{-4} M)	3.1 ± 0.15	P < 0.001
BOTRYTIS FABAE.		
Control	0.52 ± 0.08	
Sorbose (0.01M)	0.38 ± 0.07	0.1 > P > .05
Sodium deoxycholate (0.5×10^{-4} M)	0.28 ± 0.09	P > 0.1

To compare glucose and sorbose as carbon sources

Glucose (1%) 2.57 ± 0.56

Sorbose (1%) 0.59 ± 0.19

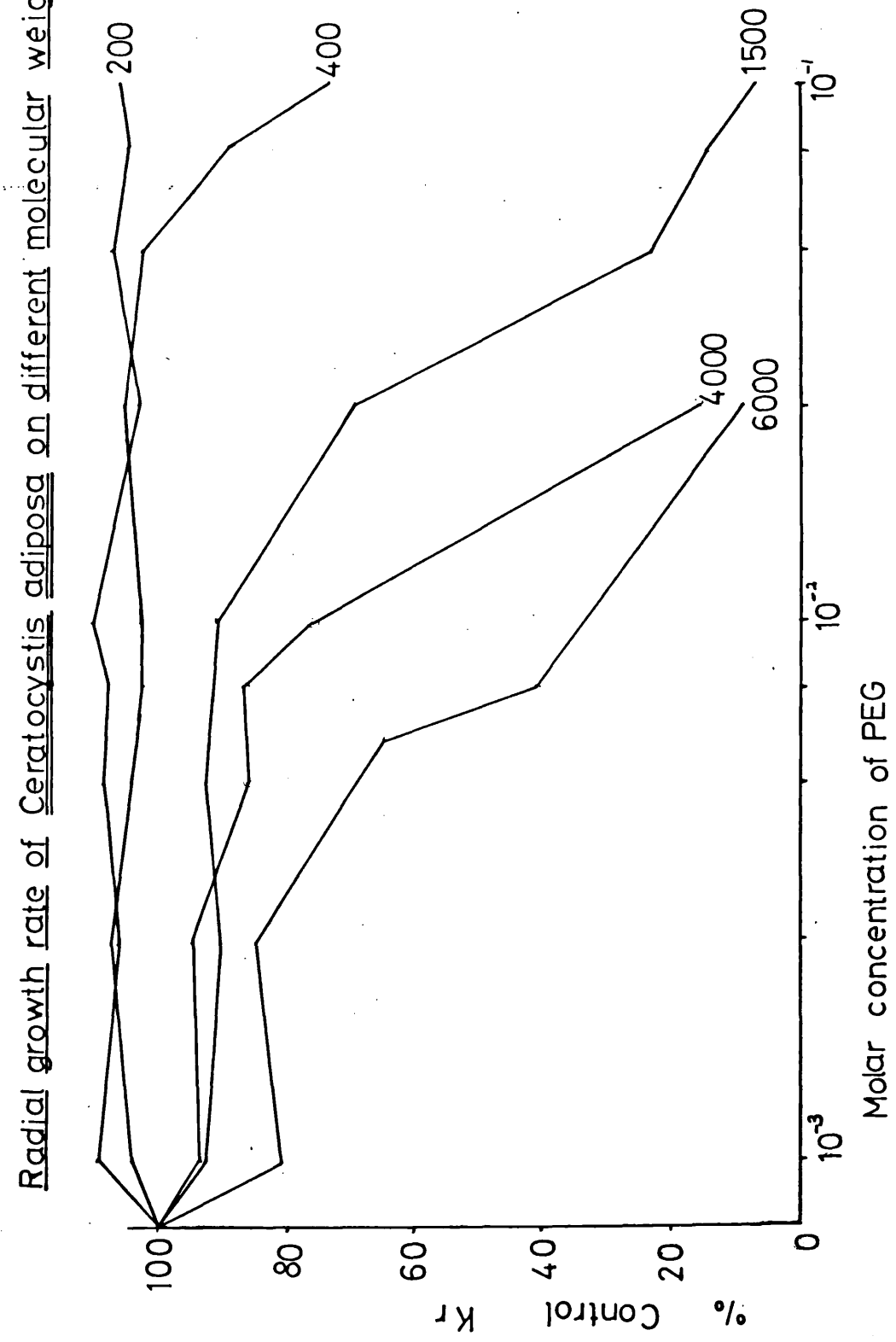
(See Fig. 27)

EFFECT OF POLYETHYLENE GLYCOL ON CERATOCYSTIS ADIPOSA.

The values were obtained for Kr of Ceratocystis adiposa grown on a series of concentrations of polyethylene glycol of different molecular weights. Increasing concentrations and increasing molecular weights of polyethylene glycol reduce Kr. (Fig.28). This observations was confirmed by an analysis of variance ($P < .01$). (See p. 160)

Values for the osmotic pressures of these media were obtained when possible from freezing point depression data.

Fig 28


Radial growth rate of Ceratocystis adiposa on different molecular weight PEG
200
400
1500
4000
6000
100
80
60
40
20
0
% Control Kr
10^{-3}
10^{-2}
10^{-1}
Molar concentration of PEG

When osmotic pressure was used to compare values of Kr, (Fig.29) molecular weight seemed to influence Kr as well as the concentration. Similar differences in Kr were also obtained when % (w/v) polyethylene glycol was used (Fig.30).

The effect of various molecular weights of polyethylene glycol on Ceratocystis adiposa.

ANOVAR using Kr as % of control.

MOLECULAR WEIGHT.

Concentration. M	200	400	1500	4000	6000	Σ	Σ^2/N
.001	109	105	93	93	81	481	46272.2
.0025	106	107	90	95	85	483	46657.8
.0050	108	104	93	86		391	38220.3
.0075	108	103	69	87	41	408	33292.8
.01	110	103	91	75		379	35910.3
.025	103	105	69	15	9	301	18120.2
.05	107	102	22	0	0	231	10672.2
.075	104	88	14	0	0	206	8487.2
.100	105	73	7	0	0	185	6845
Σ	960	890	548	451	216	3065	
Σ^2/N	102400	88011.1	33367.1	22600.1	6665.1		

G^2/N = $9394225/_{43}$ = 218470.35

SS(MW) = 253043.47 - G^2/N = 34573.12

TOTAL = 289421.00 - G^2/N = 70951.35

SS (concentration). = 244475.9 - G^2/N = 26005.55

Source	SS	df	MS	F
MW	34573	4	8643	25 **
Concentration.	26005	8	3251	9.4 **
Error	10373	30	346	
Total	70951	42		

Both concentration in moles and the molecular weight of polyethylene glycol have a significant effect ($P < .01$) on the radial growth rate of Ceratocystis adiposa.

Fig 29

The effect of osmotic pressure induced by glucose, PEG 200 & PEG 1500. on Ceratocystis adiposa.

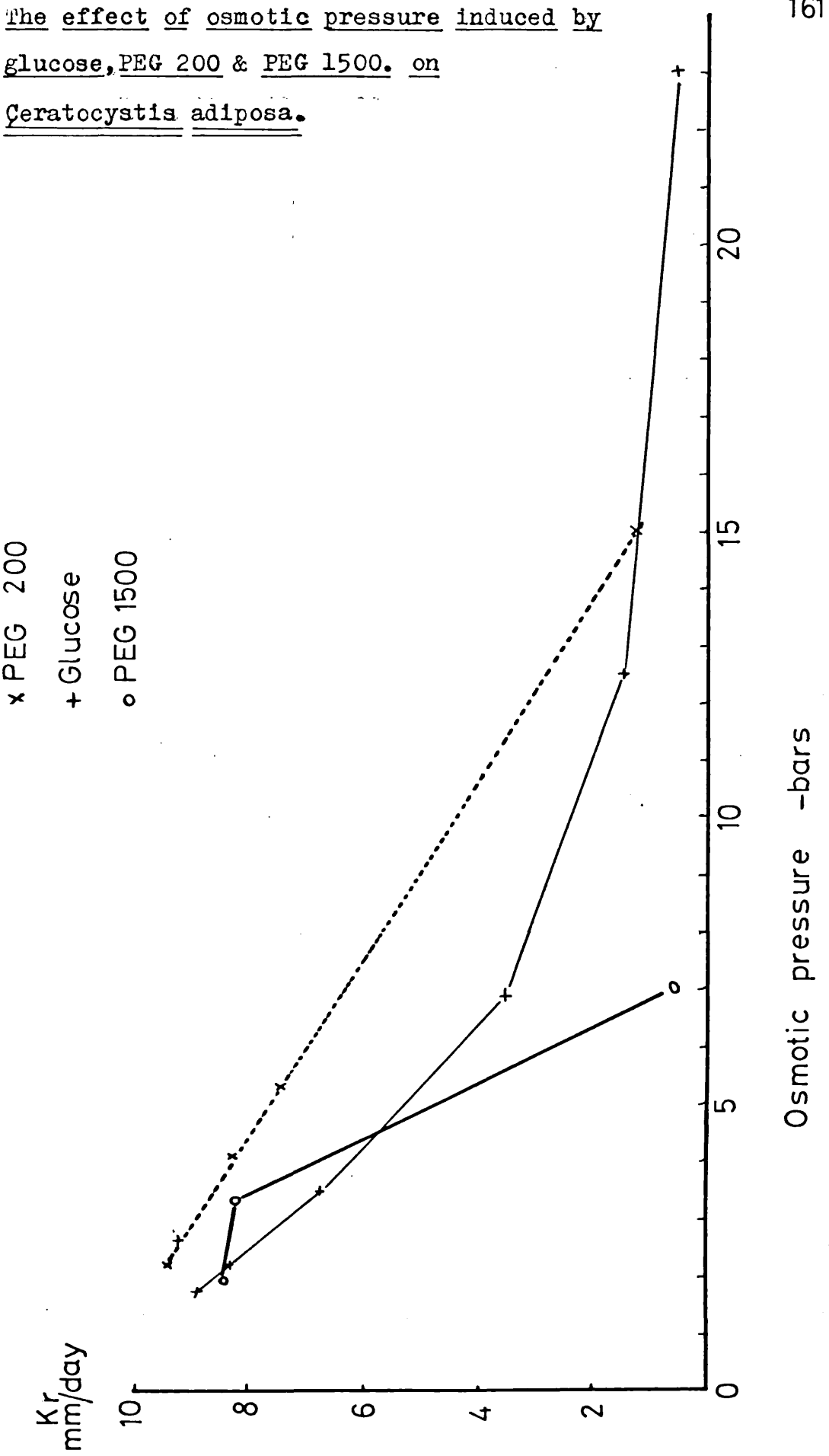
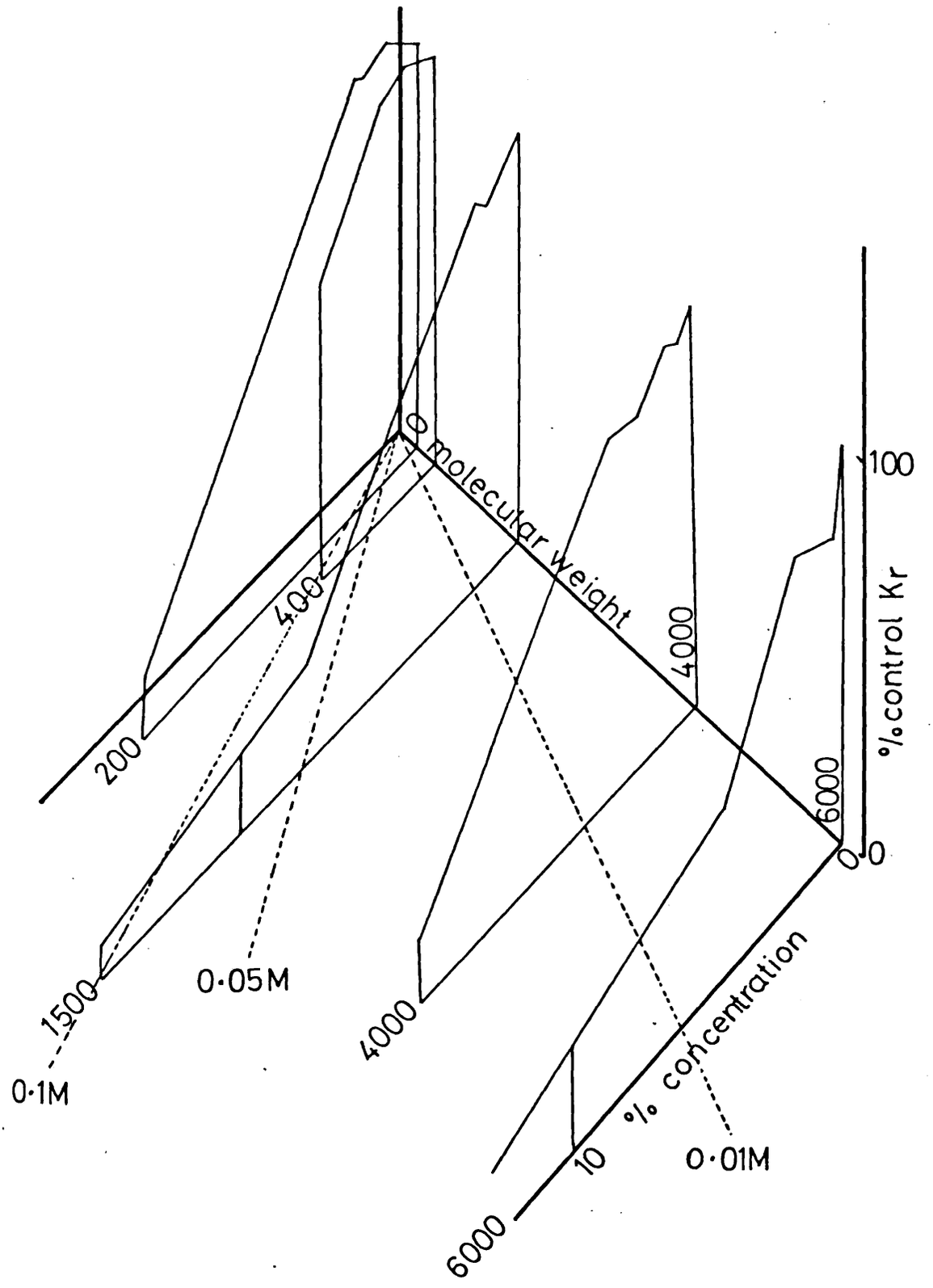


Fig 30

To show the relationship between % concentration, %Kr and molecular weight PEG for C. adiposa



THE EFFECT OF TEMPERATURE AND PARAMORPHOGEN ON PERITHECIAL PRODUCTION.

A. The numbers of perithecia in four fields of view on the various media at the temperatures stated are shown in Fig.31 No perithecia were formed at 30° C on any medium. At 25°C, no perithecia were formed on glucose, sorbose or polyethylene glycol media. On control and sodium deoxycholate media, fewer perithecia were formed at 25°C than at 20°C. More perithecia were formed over a larger area of the mycelium on sodium deoxycholate than on control at 25°C but at 20°C, this is reversed. Most perithecia were produced on the control and polyethylene media at 20°C. Very few were formed on glucose medium, probably because the carbon in the medium was very high and took longer to deplete. Sorbose medium supported a few more but still considerably less than the control again because the carbon content is increased.

B. 20°C - 30°C after 3 days.

On these plates, perithecia were only observed on the mycelium produced during the first day of growth. This indicates that the mycelium requires three days at a suitable temperature for perithecia to become initiated and then, when moved to an inhibitory temperature, they still develop. There were no perithecia on the mycelium which had been incubated at 20°C for less than 3 days. Fig 32

C. Cultures incubated initially at 30°C and then moved to 20° C have more perithecia than those started at 20°C and moved to 30°C. There is a very densely fruiting area at the centre of all cultrues except those on polyethylene glycol medium which were transferred after 2 or 3 days. On control medium this peak of perithecial production declines to almost the edge of the plate. There is a secondary peak on mycelium produced on the

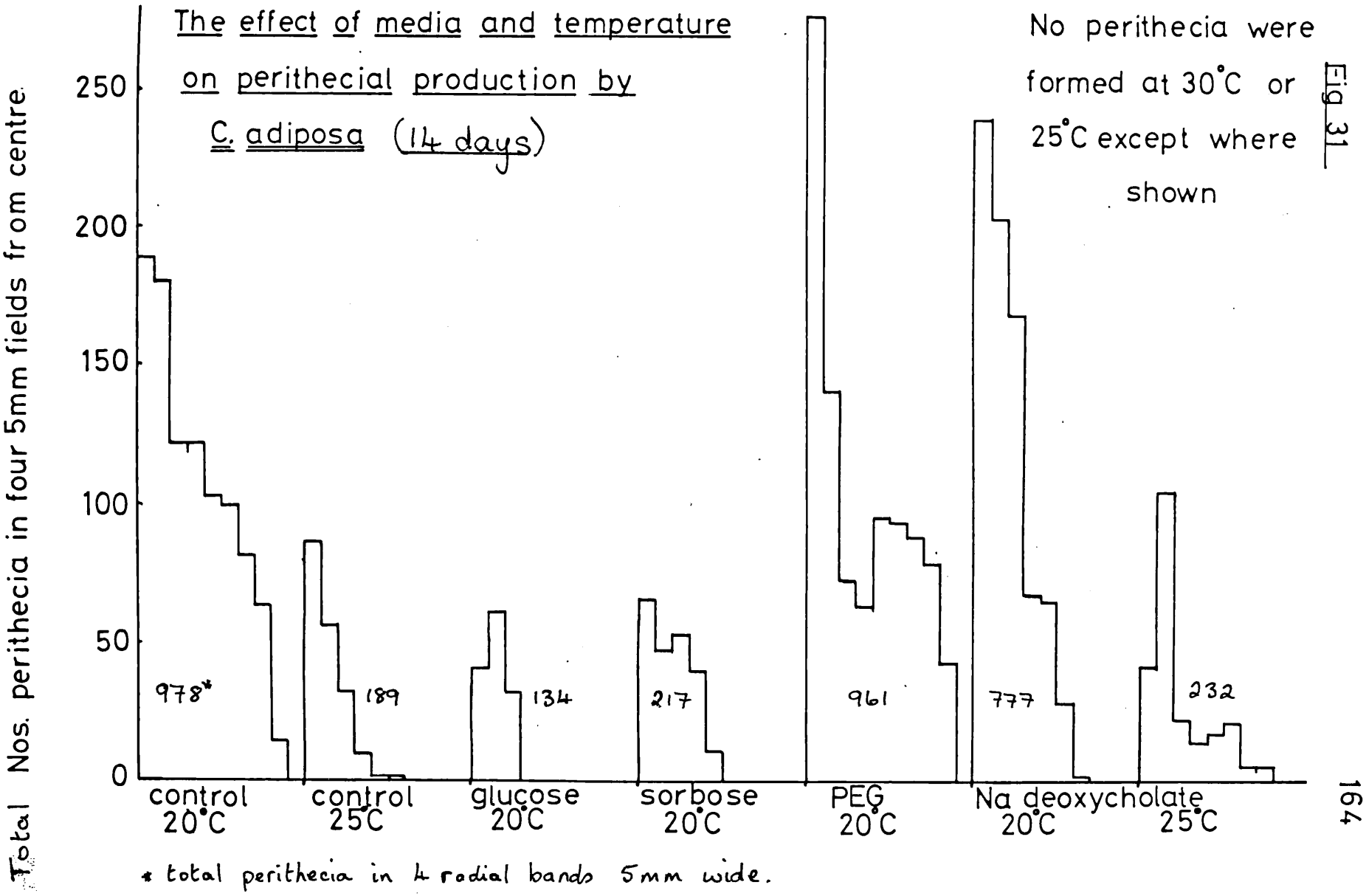
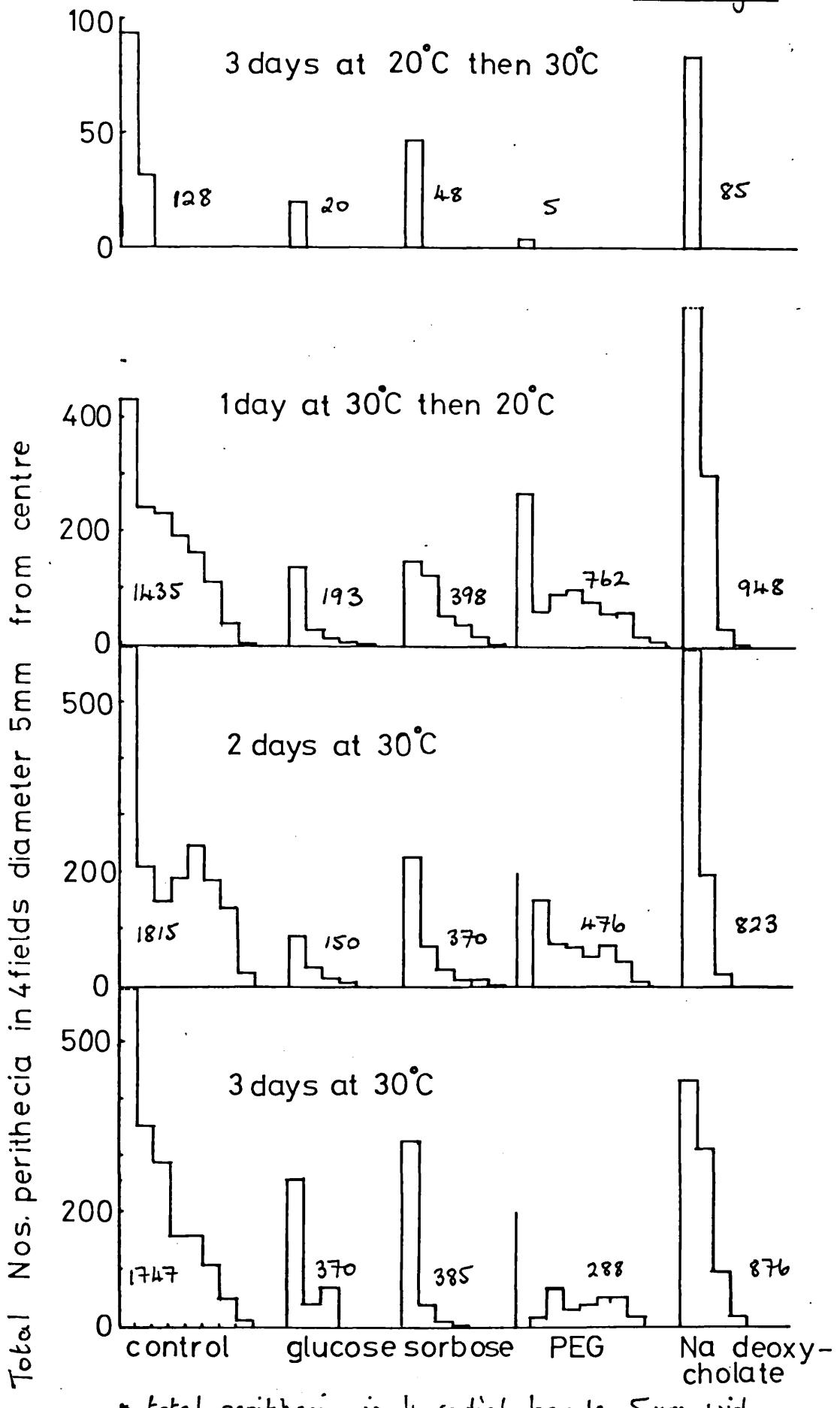

The effect of media and temperature on perithecial production by C. adiposa (14 days)
No perithecia were formed at 30°C or 25°C except where shown
Total Nos. perithecia in four 5mm fields from centre
250
200
150
100
50
0
978*
189
134
217
961
777
232
control 20°C
control 25°C
glucose 20°C
sorbose 20°C
PEG 20°C
Na deoxycholate 20°C
25°C
* total perithecia in 4 radial bands 5mm wide.

Fig 31

Fig 32 The effect of media and changes in temperature on perithecial production by C. adiposa (14 days)



* total perithecia in 4 radial bands 5mm wide.

third day of growth on the culture which was moved after 2 days at 30°C. On glucose medium again, perithecial production is reduced. The slight increase on the culture transferred after 3 days may be because vigorous growth at 30°C for 3 days had depleted the glucose supply more than on the other plates before transferring to 20°C where growth is slightly slower.

On sorbose medium again, total perithecial production is reduced but the slightly better production is on mycelium which had been at 30°C for less time unlike the glucose cultures.

The change from 30° - 20°C is clearly reflected in the polyethylene glycol grown cultures. After only one day at 30°C inhibition of perithecial development is not obvious. After 2 days at 30°C, there are very few perithecia on the first days growth and after 3 days at 30°C, the reduction covers the first 2 days growth. With increasing time at 30°C, there is a decrease in the total number of perithecia produced.

There is a decrease in the perithecial density with time at 30°C on sodium deoxycholate although the total numbers do not decrease very much. On sorbose, glucose and control media, there appears to be an increase in perithecial density at the centre. The maximum density found was 150 perithecia/field of view = 2 perithecia / mm^2 . It was impossible to be able to count accurately numbers of perithecia greater than 150/field because of overcrowding.

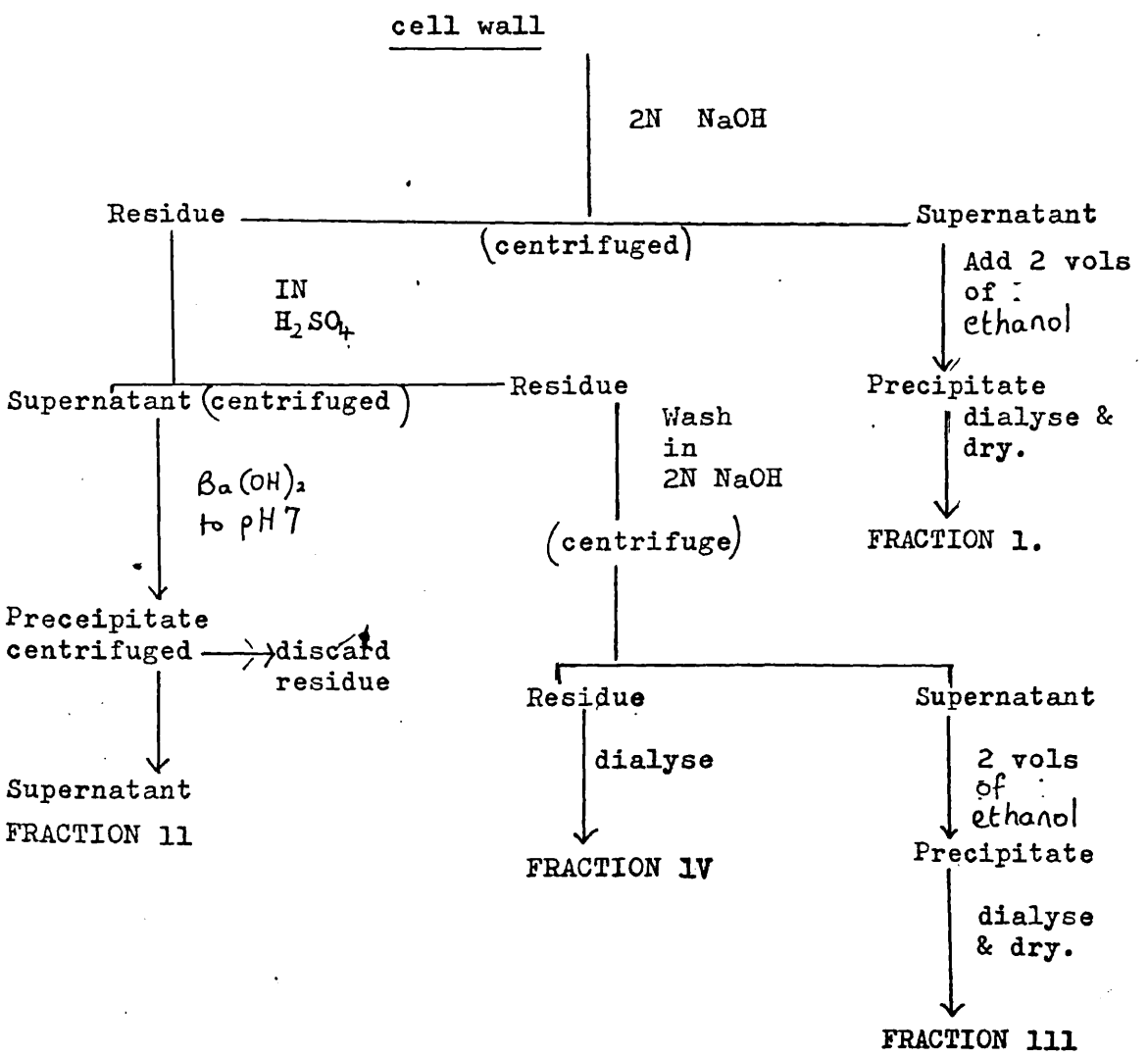
CHEMICAL ANALYSIS.

In some electron micrographs (Figs. 38, 40, 44, 47), there appears to be a layer of loosely aggregated material on the outside of the cell wall. Hyphae from cultures grown in liquid media were stained with aqueous 0.01% Ruthenium red (Gurr 1965) but the staining reaction was faint and irregular. The medium, from which the hyphae had been removed, was concentrated by rotary evaporation and two volumes of alcohol added. The precipitate obtained took up Ruthenium red stain indicating that it is a polymer of galacturonic acid (pectin). (Bonner 1950). It was assumed that the bulk of this external layer was removed during the shaking in culture and the cell wall cleaning procedure as all water soluble material would be removed from the external layer.

To avoid the inhibition of chemical degradation of the fungal cell wall by large amounts of melanin present in older hyphae (Ellis & Griffiths 1975), the cell wall for analysis was obtained from 24 hour cultures grown in 250 ml flasks. 100 ml media were inoculated with 10^6 conidia, incubated at 25°C and shaken at 150 rpm. The flask contents were filtered through a double layer of muslin to collect the mycelium. Cells were disrupted using an X-Press (type X-25). (Make AB Biox, Nacka, Sweden).

CHEMICAL FRACTIONATION OF CELL WALL.

(from Mahadevan and Tatum 1965.)



The X-Press was cooled to between -25°C and -30°C in a dry ice and acetone bath. The mycelial yields from 10 - 14 flasks were compounded to provide about 25 ml of mycelium. This was placed in a hydraulic press and the frozen mycelium forced through the shearing aperture at a pressure of 10 ton / sq. in.

The resulting paste was shaken with 1% sodium dodecyl sulphate overnight at 10°C. Autolytic degradation of the cell wall material was assumed to be negligible at this temperature. (de Terra & Tatum 1963).

The cell wall material was repeatedly washed with distilled water and centrifuged until the supernatant gave a negative reaction with ninhydrin. The final material was then dehydrated through a graded alcohol series, filtered onto a membrane filter and dried at 37°C.

Fractionation of the cell wall (Mahadevan & Tatum, 1965).

A known weight (~1g) of cell wall was suspended in 100 ml 2N NaOH and shaken overnight at room temperature. The residue was centrifuged and washed with 2N NaOH and then water. The supernatants were pooled and twice the volume of ethanol added. The resultant precipitate was allowed to settle, most of the clear supernatant was poured off and the remaining suspension centrifuged. The preceipitate was dialyzed for 24 hours against running tap water and then distilled water to removed Na OH and alcohol.

The precipitate, FRACTION 1, was dried in a dessicator at room temperature.

The remaining cell wall residue was suspended in 100 ml N H_2SO_4 at 96°C overnight, centrifuged and washed twice with distilled water. The supernatant, FRACTION 11, was treated with Ba $(OH)_2$ to pH 7 and the precipitated $BaSO_4$ centrifuged off.

The remaining cell wall residue was washed with 2N NaOH for 30 minutes, centrifuged and two volumes of ethanol added to the supernatant. This was repeated until no precipitate was formed. The supernatants were pooled and the precipitate allowed to settle. FRACTION 111 was further treated as in FRACTION 1.

The remaining cell wall was dialysed against distilled water to removed the NaOH and dried in a dessicator at room temperature. This was FRACTION 1V.

The dry weights of FRACTIONS 1, 111 & 1V were obtained and the amount of carbohydrate in FRACTION 11 determined by the method of Dubois et al (1956) using glucose as a standard.

The cleaned cell wall appeared grey and FRACTION 1V was dark brown in colour. This indicates that melanin was formed during the first 24 hours of the development of the fungus. The chemical analysis was repeated on cell wall obtained from 3-day old cultures.

To compare the % cell wall in mycelium.

Mycelium was grown from a dense inoculum in 100 ml medium in 250 ml flasks for 3 days at 25°C and shaken at 200 rpm. Six flasks from each treatment were removed and the contents filtered, washed and dried in weighed sintered glass crucibles. Cell wall was extracted from the remianing flasks and cleaned as previously described and the total weight of cell wall from 10 - 14 flasks obtained.

Hydrolysis of the cell wall fractions.

To determine the optimum conditions for hydrolysis, 0.025g laminarin (β-1,3 glucan containing some mannose; Chesters & Bull, 1963b) was hydrolyzed with 5 ml of either 30% HCl or N H_2SO_4 or 98% formic acid in the following ways:

a) the laminarin was dissolved in the minimum amount of 2N NaOH and then the acid added.

b) the acid was added directly to the solid laminarin.

Hydrolysis was carried out in a boiling water bath overnight. Undissolved laminarin hydrolyzed in 30% HCL was found to give glucose and a small amount of mannose. There was very little degradation of the glucose and no slow moving spots corresponding to oligosaccharides on the chromatogram. (Plate 34).

The hydrolysis was not complete after 16 hours with N H_2SO_4 or formic acid.

To explore the rate of hydrolysis, a second experiment was set up with 0.2g laminarin in 40 ml of 30% HCL and samples removed at hourly intervals. Hydrolysis was complete after 4 hours therefore all the cell wall fractions were hydrolyzed in 5 ml of 30% HCL for 4 hours. The hydrolyzed samples were rotary evaporated to dryness at 100°C over KOH, washed with distilled water and evaporated twice to removed the HCL. The dry sample was dissolved in 1 ml water and about 5 µl spotted onto descending paper chromatograms. (Whatmans No.1 paper 46 x 57 cms).

Standard sugar solutions used were 1% solutions of glucose, galactose, mannose and trehalose. The solvent phase used was ethyl acetate: acetic acid: formic acid: water in the volume ration 18 : 3 : 1 : 4.

After 24 hours the papers were dried and the sugar spots were developed in the following three solutions. (Trevelyan et al 1950)

A) 5 ml saturated aqueous Ag NO_3 made up to 1 litre with acetone and distilled water added dropwise until the suspension clears.

B) 40 ml saturated aqueous NaOH made up to 1 litre with absolute alcohol.

C) 10% aqueous sodium thiosulphate to clear the background.

The mean dry weight of mycelium per flask was -

Control	285.8	±	18.1 mgm
Glucose	169.7	±	18.95 mgm
Sorbose	345.4	±	46.5 mgm
Polyethylene Glycol	186.3	±	24.1 mgm
Na Deoxycholate	292.7	±	14.5 mgm

TABLE. 17

Comparison of mycelial and cell wall yield from treatments of Ceratocystis adiposa.

Treatment	Mean dry weight mycelium per flask mgm	Mean dry weight cell wall per flask mgm.	Cell wall as % mycelium.
Control	285.8	161.15	56.4
Glucose	169.7	57.61	33.9
Sorbose	345.4	122.22	35.4
Poly ethylene glycol	186.3	69.22	37.5
Na Deoxy-cholate	292.7	193.81	66.2

Mycelium grown in solutions of increased osmotic pressure shows a reduction in the amount of cell wall produced while that grown in Na De medium shows an increased amount of cell wall. (Table 17).

In the experiment to compare 24 hour and 72 hour cell wall, the amounts of cell wall retrieved after the extraction procedure were 85% and 88% respectively. (Table 18). There was an increase with age of 2.2% in FRACTION 1 and 7.7% in FRACTION 1V. FRACTION 111 was decreased by 0.7% and FRACTION 11 by 5.3%. During the extraction, the cell wall remaining after each step became darker brown due to melanin. The % recovery of 24 hour cell wall was higher than that from 72 hour wall. (Table 19). Comparisons within each experiment are valid as the % recovery for two control samples were 85% and 88%. However, comparisons between the different age walls are more difficult to make. The results were ranked so that consistently high and low values could be more easily seen.

The returns of over 100% obtained in the first experiment are probably due to the chemical estimation of FRACTION 11. When equal percentage concentrations of glucose and laminarin are analysed by Dubois method, the laminarin gives a much darker colour so any polysaccharides present in FRACTION 11 would give a false high reading when used with a glucose standard.

TABLE 18

Comparison of a 24-hour and 72-hour cell wall from control media.

Age of culture	% cell wall in each fraction			
	1	11	111	1V
24-hour	8.88	33.61	29.82	27.69
72-hour	10.97	26.13	27.71	35.18

TABLE 19

% recovered in the four fractions from original weight of cell wall.

24-hour		72-hour	
Control	105.9	Control	88.4
Glucose	107.6	Glucose	90.5
Sorbose	80.0	Sorbose	91.7
Polyethylene Glycol	102.4	Polyethylene Glycol	46.7
Na De	102.0	Na De	86.8

TABLE 20

% cell wall in each fraction and rank *

		I		II		III		IV
24-hour		*		*		*		*
C	5.9	2	51.7	4	5.2	1	37.2	4
G	13.0	5	32.5	1	15.4	3	39.1	5
S	9.6	3	46.0	2	19.8	4	24.6	3
P	11.0	4	70.9	5	12.1	2	6.0	1
N	4.5	1	48.4	3	25.1	5	22.0	2
72-hour								
C	11.0	4	26.1	4	27.7	4	35.2	2
G	12.6	5	29.4	5	31.0	5	27.0	1
S	6.0	1	16.4	1	17.4	1	60.0	5
P	7.0	2	24.9	3	19.6	2	48.5	4
N	9.0	3	23.8	2	21.1	3	46.1	3

TABLE 21

Sum of ranks for each treatment and fraction of cell wall.

Treatment	Fraction			
	I	II	III	IV
C	6	8	5	6
G	10	6	8	6
S	4	3	5	8
P	6	8	4	5
N	4	5	8	5

In the 24-hour mycelium, sorbose, polyethylene glycol and sodium deoxycholate appear to have decreased the FRACTION 1V content of the cell wall. Glucose appears to have increased FRACTION 1, polyethylene glycol increased FRACTION 11 and FRACTION 111 increased by all the paramorphogens.

The 72-hour wall analysis shows two distinct groups: (Table 20)

a) Control and Glucose grown wall which has a high content of FRACTION 1 - 111 and a low FRACTION 1V.

b) Sorbose, polyethylene glycol and sodium deoxycholate where the glucose containing fractions are low and FRACTION 1V is high.

The extreme values are found in glucose and sorbose cell walls.

On the ranked date, the highest $\leq$ rank is for glucose FRACTION 1 and the lowest for sorbose FRACTION 11. Table 21.

Chromatographic analysis of hydrolyzed wall fractions.

The developed chromatograms are shown in Plates .

FRACTION 1 contains glucose and mannose.

FRACTION 11 contains an unknown sugar or possibly glucose which has reacted with HCL and so forms a clear area and mannose (possibly left from FRACTION 1).

FRACTION 111 glucose and an unidentified series of oligosaccharides.

FRACTION 1V little glucose, an unidentified substance x probably a disaccharide and a faster migrating substance y.

The composition of the growth medium had no effect on the composition of any fraction.

Standards

glu glucose

gal galactose

man mannose

tre trehalose

Samples

1-4

Treatments

C Control SSM

G Glucose

S Sorbose

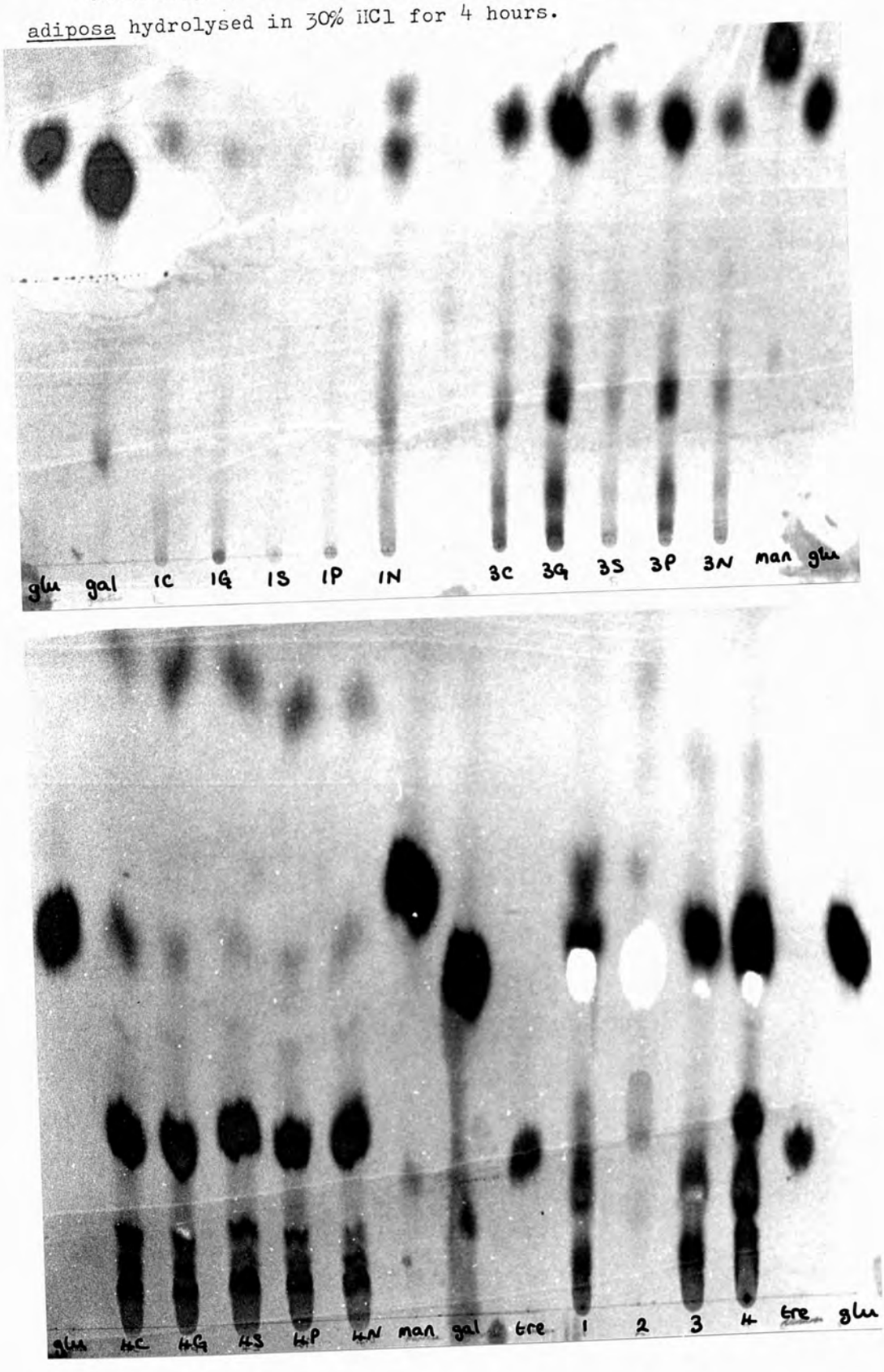
P Polyethylene glycol

N Na deoxycholate

Eg. 1C Fraction 1 from Control cell wall

Fig.34

Chromatogram of cell wall fractions of Ceratocystis adiposa hydrolysed in 30% HCl for 4 hours.



1. Enzymes.

The enzymes used were commercial (Koch-Light) pronase, cellulase and chitinase at 1 μg/ml in 0.05M acetate buffer pH5.0.

Cellulase ex Aspergillus niger.

Chitinase - fungal

Pronase ex Streptomyces griseus.

Glucanase was obtained from Hypocrea pulvinata (Hp4).

Hypocrea was grown in 250 ml flasks in 100 ml of SSM + 0.5 g/l yeast extract for two weeks. The mycelium was filtered out using muslin and the spores removed by centrifugation. The medium was then freeze dried and stored. When required, the solid was dissolved in as little water as possible and .25 g/ml sucrose added. This was introduced into the top of a G 100 Sephadex column (98 x 1.2 cms) and run with 0.05M acetate buffer pH5.0 at 12 ml/hour. 3 ml fractions were collected and the 3 fractions corresponding to the peak of the UV scan pooled and used.

2. Enzyme hydrolysis of the cell wall. (Vessey & Pegg 1973).

The enzyme reaction mixture contained 20 mgm cell wall, 0.2 ml enzyme and 0.8 ml, 0.1M acetate buffer, pH 5.2 and was incubated at 37°C in the dark for 48 hours. The following mixtures of enzymes were used : Chitinase, Pronase, Cellulase, Chitinase + Pronase, Chitinase + Cellulase, Pronase + Cellulase, Chitinase + Pronase + Cellulase, Glucanase, Chitinase + Glucanase, Pronase + Glucanase, Cellulase + Glucanase, Chitinase + Pronase + Cellulase + Glucanase.

3. Glucose estimation. (Nelson,1944 & Somogyi, 1952).

Reagent A.

12g Na_2Co_3 + 12g Rochelle salt + 10g $NaHCO_3$ + 100g Na_2SO_4 dissolved in 400 ml H_2O and made up to 500 ml.

Reagent B.

15% $CuSO_4 \cdot 5H_2O$ aqueous solution + 2 drops conc. H_2SO_4 per 100ml.

Colour Reagent.

12.5g of NH_4molybdate in 225 ml H_2O. Add 10.5ml conc. H_2SO_4. Dissolve 1.5g Na_2 H AsO_4 $7H_2O$ in 12.5ml water and add to the above solution. Incubate at 37°C for 48 hours and store in a glass-stoppered bottle.

Solutions A & B were mixed in the ratio of 24 + 1. 1 ml of A + B was added to 1 ml of enzyme reaction mixture and heated in a boiling water bath for 5 minutes. When cool, it was diluted to 25ml with water and read in a Spectrophotometer at 660 nm.

4. Protein estimation.

Reagent A.

4% Na_2CO_3 filtered.

2% Cu SO_4

4% Na K tartrate.

The solutions were mixed in the ratio 100 + 1 + 1 by volume.

Folin Ciocalten's phenol reagent (BDH).

This was diluted with an equal volume of water.

5 ml of reagent A was added to the enzyme reaction mixture and allowed to stand for 10 minutes. 0.5ml of Folin reagent was added and the tubes read at 660 nm.

5. Ehrlich reagent for N Acetyl glucosamine (Vessey & Pegg 1973)

0.3ml saturated $Na_2B_4O_7 \cdot 10H_2O$ was added to the enzyme reaction mixture and heated at 100°C for 7 minutes. 8.7ml glacial acetic acid and 1 ml Ehrlich reagent (2g p-dimethyl-amino benzaldehyde in 100 ml glacial acetic acid + 5 ml concentrated hydrochloric acid) were added and the colour allowed to develop for 30 minutes. The tubes were read at 540 nm.

Molecular Weight determination.

A G 100 Sephadex column 98cm x 1.2cm was calibrated with 0.05M acetate buffer pH5.0 and a gravity flow rate of 8 - 12 ml per hour. The standards of known molecular weight were blue dextran (2×10^6), Yellow dextran (2×10^4) and vitamin B (1357).

Substrate specificity.

Enzyme reaction mixtures were made up as for enzyme activity determination but using a range of substrates.

Substrate	linkages.
laminarin	β - 1, 3.
nigeran	α - 1, 3. α - 1, 4.
starch	α - 1, 4.
cellobiose	β - 1, 4.
maltose	α - 1, 4.
chitin	β - 1, 4 - N - acetyl glucosamine.

Controls for each substrate were made with boiled enzyme and a drop of each reaction mixture spotted on to a paper chromatogram. The chromatogram was run in 18 : 3 : 1 : 4 solvent (see p. 171) and developed with silver nitrate reagent (see p. 171) with glucose as a standard.

Enzyme activity.

Glucose determination.

A 0.1% laminarin suspension was made in acetate buffer. 0.5 ml of substrate suspension and the enzyme solution were mixed in a test tube and incubated at 30°C for 30 mins. 2 ml of glucose oxidase reagent was then added and the mixture incubated for a further 30 mins at 37°C. Then 2 ml conc. H_2SO_4 were added and the mixture left for an hour. Before reading, 5 ml water

were added. The solution was poured into 10 ml cuvettes and read using 604 filter on an EEL colourimeter.

Protein determination.

1 ml of enzyme solution was mixed with 5 ml of Solution A and allowed to stand for 10 mins. 0.5 ml of half strength Folin reagent was added and readings made on an EEL colourimeter using a 609 filter.

Standard solutions using glucose and bovine serum albumen were made up at .01, .05, and 1 mgm/ml.

Glucanase from Hypocrea Pulvinata.

Methods.

Hypocrea pulvinata was grown as previously described and the mycelium filtered from the medium which was freeze dried. The crude enzyme mixture was dissolved in water and dialysed to remove components of the medium especially glucose. The enzyme solution was made up to 20 mls with distilled water and $(NH_4)_2$ So_4 added to concentrations of 20%, 40%, 60% and 80% saturation. The precipitate obtained in each fraction was centrifuged and dialysed. Molecular weight, enzyme activity and protein content of each fraction was estimated as follows.

Results.

When cell wall was incubated with water and analysed, small amounts of glucose, N-acetyl glucosamine and protein were released. (0.5 mgm, 0.03 mgm and 0.3 mgm respectively). After incubation with enzymes, much larger quantities were released (Fig.35). N-acetyl glucosamine was released when chitinase was present in the reaction mixture. 0.2 mgm were released when chitinase was the sole enzyme and less released by mixtures of enzymes because they contained less chitinase.

Glucose was released by all enzymes and mixtures.

Cellulase released more glucose than glucanase but the specific activities of the enaymes were not determined. Chitinase released more glucose than any other single enzyme.

Protein is released by all enzymes.

Results.

Molecular weight.

Elution volumes (Ve) were 18 ml for Blue dextran (MW 2 x 10^6) 28 ml for Yellow dextran (MW 2 x 10^4), 48 ml for Vitamin B (MW 1357) and 22 ml and 23 ml for two samples of glucanase. The void volume (Vo) is 18 ml.

$\frac{Ve}{Vo}$	log MW
1. 0	6.3010
1.61	4.3010
2.66	3.1320

The molecular weight of the glucanase was found to be 5.62 x 10^4 and 5.02 x 10^4 in two experiments.

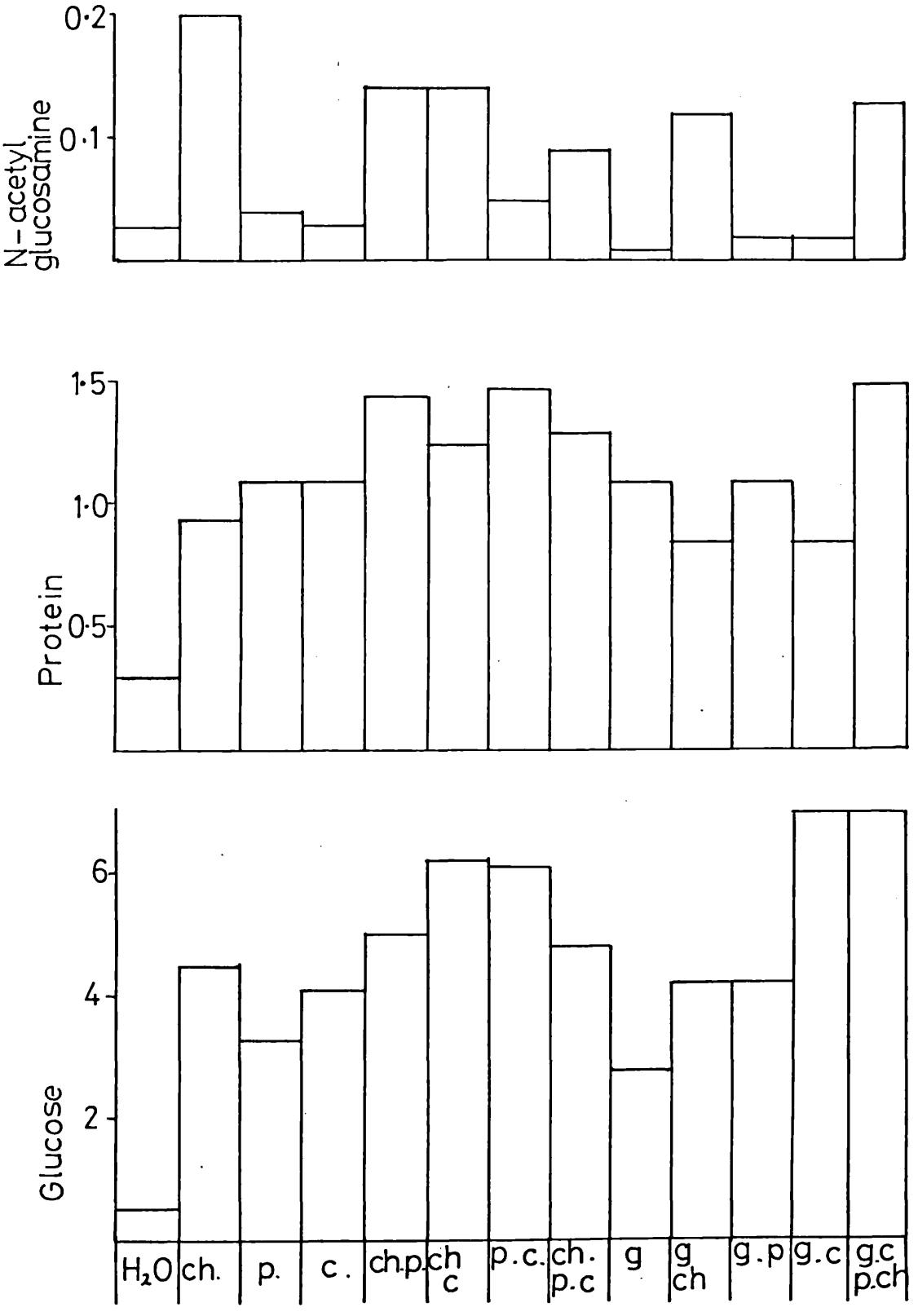
Specific activity.

The specific activity of each of the $(NH_4)_2\ SO_4$ precipitated fractions was found.

Fig 35

Enzyme degradation of Ceratocystis adiposa cell wall

Yield(mgm) from 20mgm wall (p 370)



ch-chitinase p-pronase g-glucanase c-cellulase

ENZYMES

% saturation with $(NH_4)_2SO_4$.	mgm glucose released by enzyme.	mgm protein	specific activity.
20	0.3	0.28	1.07
40	0.1	0.10	1.00
60	0.13	0.03	4.30
80	0.2	0.03	6.66

The fraction precipitated by 80% saturation with $(NH_4)_2So_4$ had the highest specific activity of 6.66 mgm glucose released per mgm protein.

Substrate specificity.

The presence or absence of a glucose spot from the various substrates when incubated with glucanase is recorded in Table 22.

Table 22.

Release of glucose from various substrates by glucanase.

Substrate	enzyme	control
laminarin	+	-
nigeran	+	+
starch	-	-
cellobiose	-	-
maltose	-	-
chitin	-	-

Nigeran tends to be unstable as the same sized spot occurred for both enzyme and control treatments. Laminarin is hydrolyzed by the enzyme but none of the other substrates are hydrolyzed. Therefore, the enzyme is specific in hydrolyzing β - 1, 3 glucan as no other linkages are hydrolyzed.

Interaction between sorbose and glucose uptake.

Methods.

Standard synthetic medium without sugar was made up and after it had been autoclaved, sterile solutions of glucose and sorbose were added to give a final sugar concentration of 2%. The ratios of the two sugars in different flasks were

glucose : sorbose 3 : 0, 2 : 1, 1 : 2 and 0 : 3.

50 ml medium in 100 ml conical flasks were inoculated with 2 ml of a fast growing culture and 1 μCi of ^{14}C glucose or ^{14}C sorbose added to each flask (Table 23). The flasks were sealed and sterile air bubbled through the medium and CO_2 evolved was collected in lime water. The cultures were incubated in a shaking water bath at 25°C.

At intervals (see Table 23), 5 ml samples of the culture were removed and filtered onto glass fibre filter papers, washed with 30 ml of distilled water and dried overnight at 40°C. When dry, the filter papers were put into stoppered glass vials with 10 ml scintillation fluid and counted in a Beckman scintillation counter L 230 for 5 minutes. The scintillation fluid contained 4g of 2, 5- Diphenyloxazole (PPO) and 0.2g of 1, 4 - Di - 2 - (5 - phenyloxazolyl) - benzene in 1 ℓ toluene. (Patterson & Greene 1965).

The lime water was filtered and the precipitated $Ca^{14}CO_3$ dried and counted as before. However, a thick deposit of $Ca^{14}CO_3$ accumulated on the sides of the tubes and once the lime water had been saturated with CO_2 then counts would be lost in the escaping CO_2 . Therefore, the results for CO_2 evolved are probably too low.

The amount of ^{14}C glucose and sorbose taken up by the mycelium is shown in Table 23 . Carbon dioxide from all cultures contains some ^{14}C, the highest values being obtained from 24 hour cultures containing 2% glucose and 1⅓% glucose and the 48-hour culture from sorbose medium.

The uptake of glucose appears to be stimulated by 1⅓% sorbose but inhibited by ⅔% sorbose at all times. After 2 hours the uptake of sorbose follows the same pattern, i.e., stimulation by a higher concentration of glucose and inhibition by a lower concentration although at 6 and 24 hours, any concentration of glucose inhibits sorbose uptake.

When the ^{14}C label is in the lower concentration sugar of the 2 = 1 mixtures, the uptake of glucose after 24 hours is 10 x that of sorbose and when the higher concentration is labelled, glucose is taken up about 7 x as much as sorbose. Uptake from glucose medium is 5 x that from sorbose medium, reflecting the differences in specific growth rates in shake culture which were found to be 2.56 mgm/hr in glucose and 0.58 mgm/hr in sorbose. 7g 27d.

TABLE. 23

Distribution of ^{14}C from glucose and sorbose in the mycelium and CO_2 from Ceratocystis adiposa, grown in different media.

Counts rounded up to nearest whole number per minute.

Media	Label	Time Hours			
		1	2	6	24
Glucose mycelium	G.	189	340		9460
$CaCO_3$			181		1059
Glucose:Sorbose M	G.	143	278		8616
2 = 1 $CaCO_3$			178		1807
	S.		491	434	1489
			45	55	48
Glucose:Sorbose M.	G.	224	637		15106
1 = 2 $CaCO_3$			56		88
	S.		372	401	1176
			57	372	199
Sorbose mycelium	S.		542	602	1957
$CaCO_3$			62	62	205

Time	30hrs	48hrs
Sorbose Mycelium	4948	15429
$CaCO_3$	669	1508

A. Fixatives.

1. $KMnO_4$. Freshly prepared as a 4% aqueous solution.

2. Glutaraldehyde. Freshly prepared as a 2% - 3% solution in 0.1M cacodylate buffer pH 7.3 from a 25% aqueous solution.

3. OsO_4. A 1% solution in cacodylate buffer pH7.3 was made and stored in a refrigerator overnight to allow the OsO_4 to dissolve completely.

B. Resin. (Taab embedding resin kit, Taab labs. Reading Eng.)

Taab embedding resin	20 g
Dodecenyl succinic anhydride	10 g
Methyl nadic anhydride	10 g
Benzyl di methylamine	0.8 ml

Each component was added and mixed thoroughly before the next one was added.

C. Stains.

Uranyl acetate. A saturated solution in 50% alcohol was adjusted to between pH4 and pH5 with NaOH solution. The stain was stored in a brown glass bottle in the dark.

Lead citrate.

1.33 g $Pb(NO_3)_2$ + 1.76 g $Na_3(C_6H_5O_7)\ 2H_2O$ were added to 30 ml water and shaken occasionally for 30 minutes. 8 ml 1N Na OH were added and the suspension diluted to 50 ml and allowed to stand until clear. The stain was stored in a plastic bottle which was kept in a larger wide necked plastic jar containing NaOH pellets.

Preparation of material for the electron microscope.

Ceratocystis adiposa conidia were inocculated into flasks containing :-

SSM

SSM + 0.25M glucose, sorbose and polyethylene glycol 200.

SSM + 0.25×10^{-4} M Sodium deoxycholate.

The flasks were incubated overnight at 25°C on an orbital shaker at 150 rpm. The small pellets were centrifuged to compact them and a small volume of 5% agar added. The agar was allowed to set and the block cut into cubes (< 1.5 mm across).

Conidia were also observed after being treated for 6 hours as follows. Only in SSM were germ tubes formed.

SSM
SSM + 1.0M glucose sorbose and polyethylene glycol 200
SSM + 10^{-3} M sodium deoxycholate.

The conidia were centrifuged and fixed in glutaraldehyde before being embedded in agar.

$KMnO_4$ fixation.

The agar blocks containing the fungal material were fixed in 4% aqueous $KMnO_4$ for 30 minutes at room temperature and washed with distilled water until the washings remained clear.

Glutaraldehyde/Osmium fixation.

0.1M cacodylate buffer pH 7.3 was used throughout this treatment being compatible with both glutaraldehyde and OsO_4 (Juniper etal 1970) The material was fixed for one hour in buffered 2% glutaraldehyde at 4°C overnight and washed four times in buffer. Post fixation was carried out in 1% buffered OsO_4 for 1½ hours and again washed four times in buffer.

Dehydration.

The fixed blocks were dehydrated by being passed through a graded alcohol series (25%, 50%, 75% and 100%) with 30 minutes in each grade.

Embedding.

The dehydrated blocks were placed in a 1 : 1 mixture of absolute alcohol : propylene oxide (epoxy propane) for 30 minutes and propylene oxide for 30 minutes. The blocks were transferred to 10% resin in propylene oxide overnight to allow the resin to penetrate. During the interval, the propylene oxide evaporated slowly.

The blocks were transferred into two changes of fresh resin for 2 hours and finally embedded in freshly made resin in round dishes (33 mm diameter) or capsules. These were placed in an oven at 60°C under a vacuum of 400 mm mercury for the first 20 minutes and left for 48 hours to polymerize.

Sectioning.

The resin blocks were cut with a hacksaw and trimmed to the correct size with a glass knife on the ultramicrotome. Glass knives were cut at 45° on an LKB 7801B Knife-maker and mounted in the LKB Ultrotome III. Sections were cut and collected in the water-bath behind the knife edge. Gold coloured sections, i.e., those between 90 - 150 nm in thickness were flattened using dichloro-ethylene vapour, picked up on formvar and carbon-coated grids and stored on fluffless tissue paper in plastic petri dishes.

Post-section staining.

$KMnO_4$ fixed material did not require furthur staining. Glutar-aldehyde/Osmium fixed material was stained in uranyl acetate and lead citrate.

Drops of uranyl acetate stain were placed on wax sheets in a petri dish and the grids placed in the stain for one hour after which they were washed with distilled water. A sheet of wax was placed in a petri dish with a few pellets of NaOH to reduce CO_2 contamination. Drops of the lead citrate stain were placed on the wax and the copper grids placed in the stain immediately after being washed from the uranyl acetate stain. The sections were stained for 30 minutes and then washed with 0.02N Na OH and then distilled water.

Preparation of Copper Grids.

Copper grids, (100 micron) were obtained from Taab laboratories. Clean dust-free glass slides were dipped into a 0.2% - 0.5% solution of formvar in chloroform and allowed to drain. When dry, the edges of the slide were scored with a razor blade. The slide was then care-

fully lowered at an angle into a dish of membrane filtered distilled water and the film of formvar floated away from the slide. Copper grids were placed face down on the film and the film collected on another slide. This was allowed to dry and then coated with a thin film of carbon using an Edwards carbon evaporator.

Microscopy.

The Sections were examined in the electron microscope (Japan Electron Optics Laboratory Co., Ltd., Type JEMT 6S) using a 60KV current. The beam intensity was increased slowly to prevent the sections burning. Photographs were taken on Ilford plates and developed in Contrast FF developer.

FIGURE 36 .

A transverse section of a hypha grown in SSM shows the cell wall to have two light layers separated by a narrow dark band. A darker layer of material like mucilage lies to the outside of the cell wall and becomes dispersed into the medium. The cytoplasm is dense and granular with three vacuoles.

The cell wall is 200 nm thick.

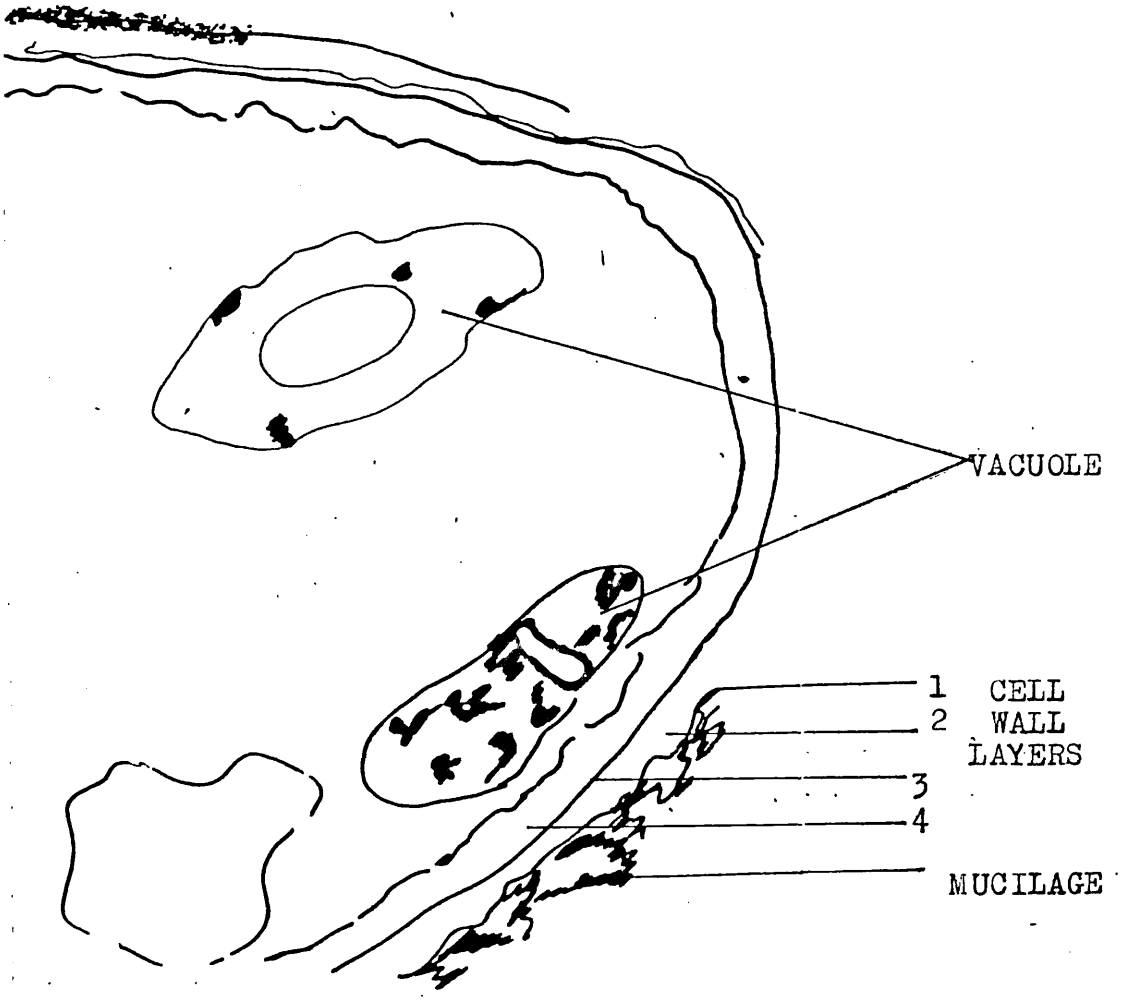


Fig. 36

Transverse section of a hypha of Ceratocystis adiposa grown in standard synthetic medium.

x 70000. glutaraldehyde/osmium fixed.

FIGURE 37.

A transverse section of a hypha of Ceratocystis adiposa grown in 0.25 M glucose medium shows the wall structure to be very similar to that of the control but there is much less mucilage. A dark outer layer surrounds the wall which has two inner layers. These are separated by a very faint dark line. The layer nearer the cell membrane is lighter than the outer layer. Dense darkly-stained cytoplasm fills the cell and a vacuole and nucleus are visible. The cell wall is 100 nm thick.

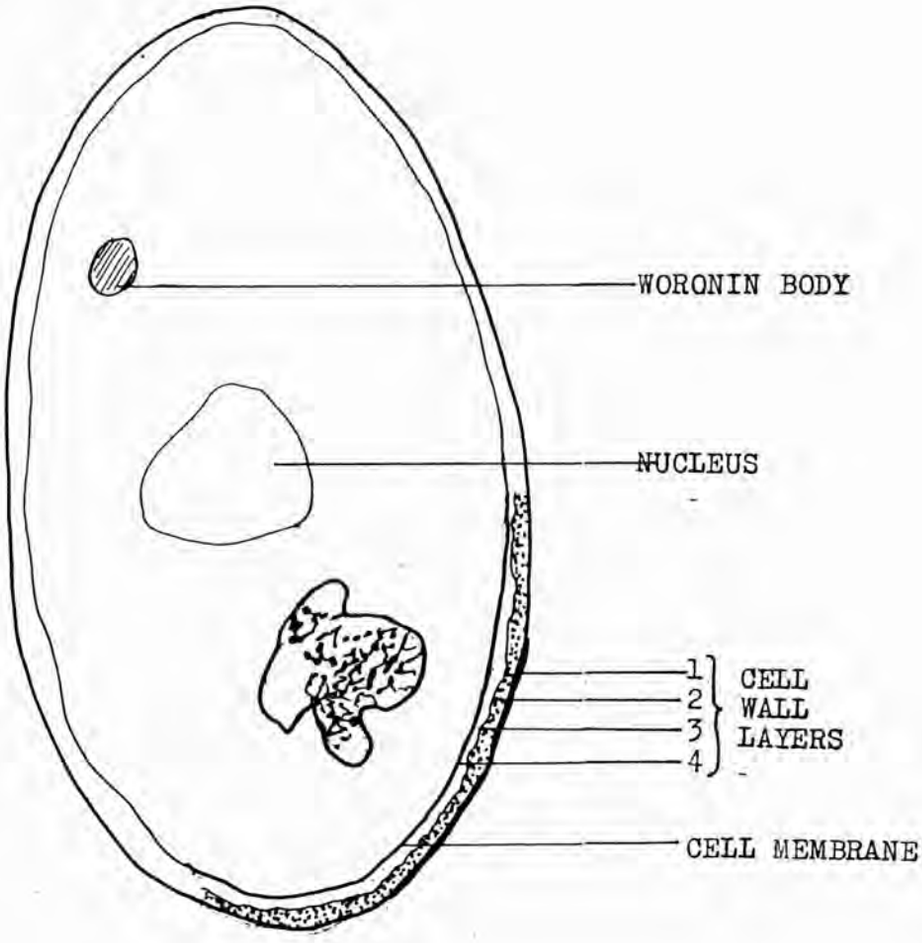


Fig. 37

Transverse section of a hypha of Ceratocystis adiposa grown in glucose (0.25M) medium.

x 40000. glutaraldehyde/osmium fixed.

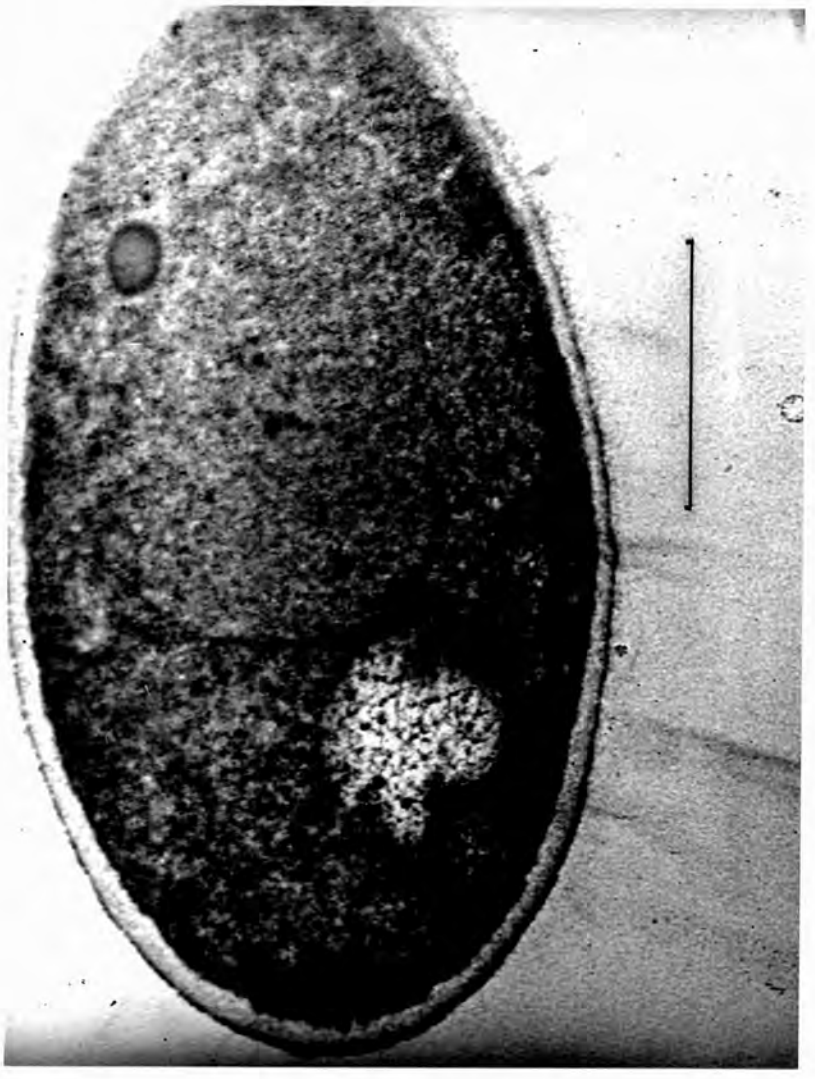


FIGURE. 38

A transverse section of a hypha of Ceratocystis adiposa grown in o.25M Sorbose medium and an ungerminated conidium.

In the hypha, the cytoplasm is very vacuolated indicating a physiologically older part of a hypha than in the previous cases. Although there is no thick layer of mucilage around the cell wall, it appears to have been dispersed into the liquid medium. The cell wall is 200 nm thick and appears to have three layers surrounded by a very dark narrow layer, possibly containing melanin. This four-layered wall structure is shown where septa have been formed (see Fig. 42) and may be characteristic of an older cell wall.

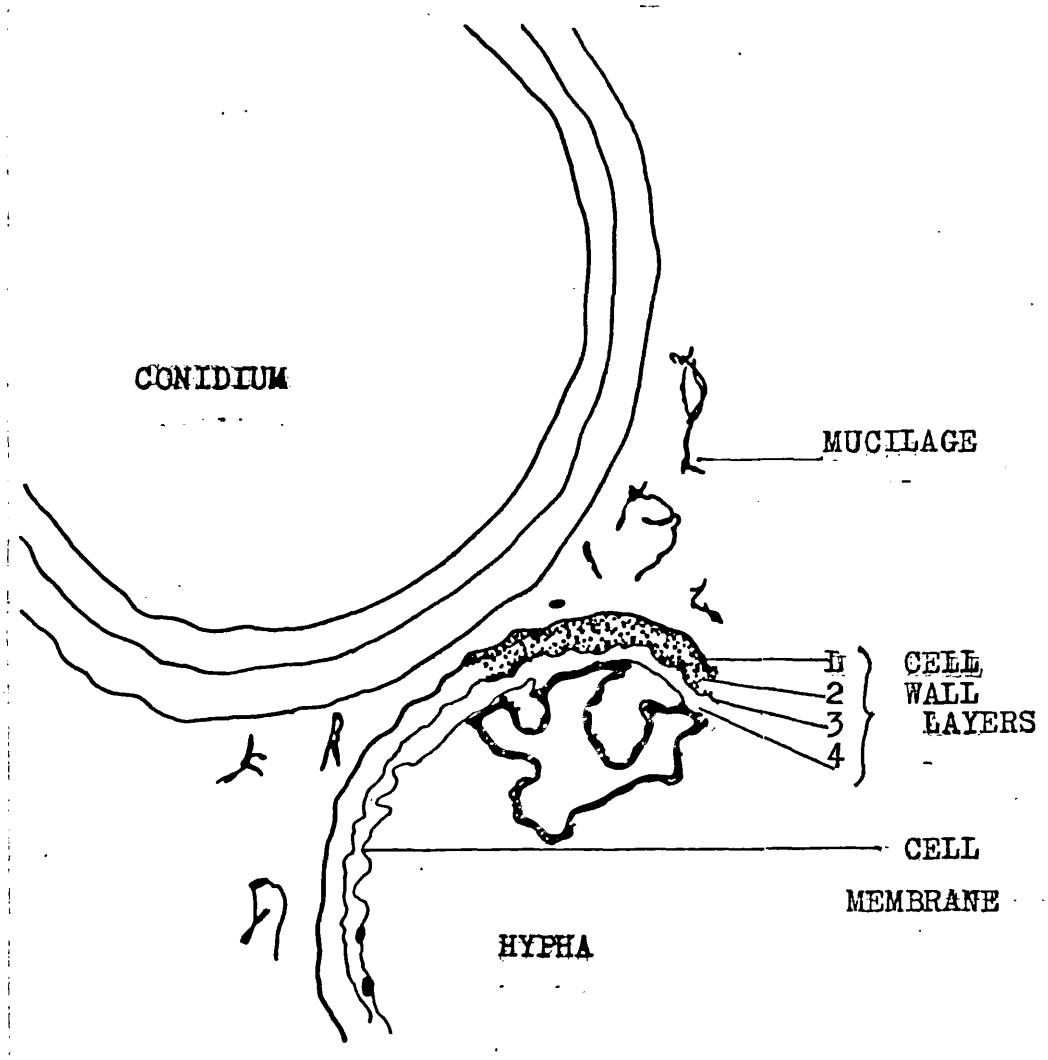


Fig. 38

Transverse section of a conidium and a hypha of Ceratocystis adiposa grown in sorbose (0.25M) medium.

x 21000. glutaraldehyde/osmium fixed.

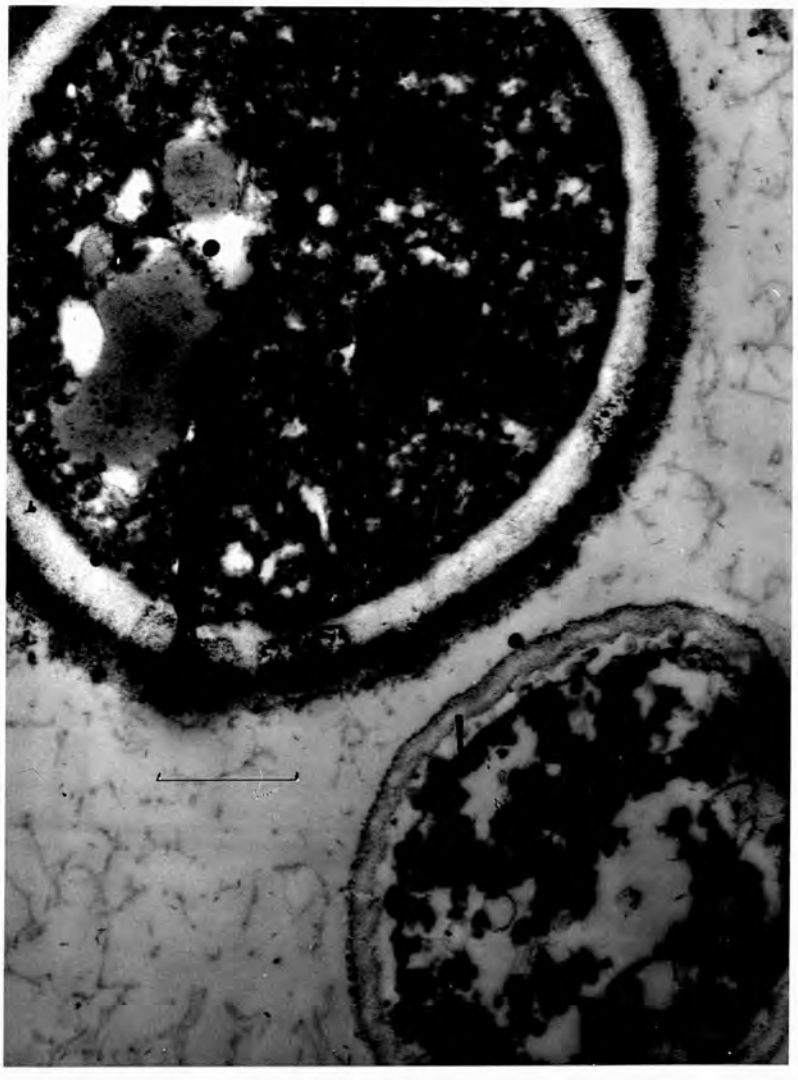
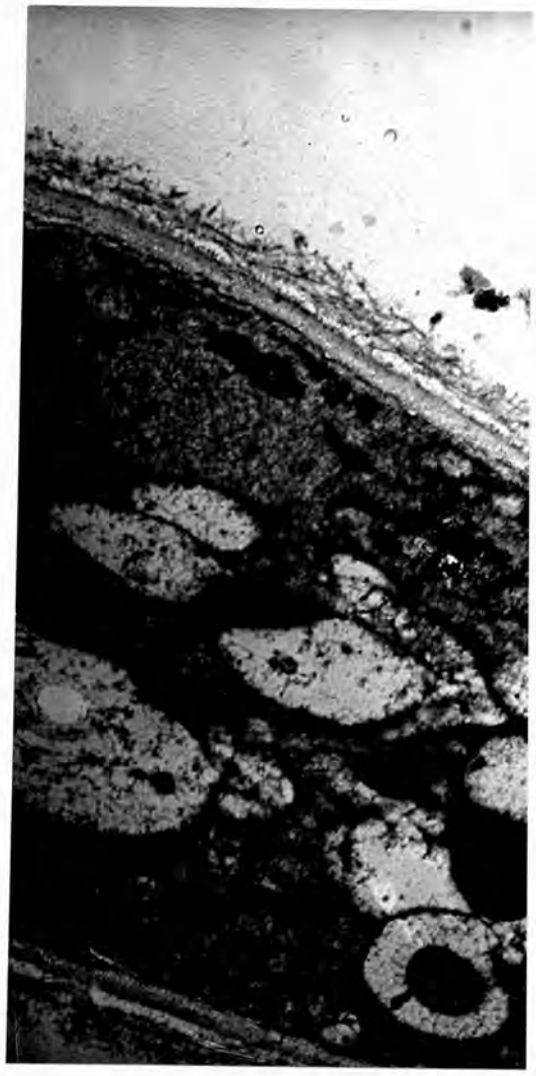


Fig. 3

Oblique section of a hypha of Ceratc
in polyethylene glycol 200 (0.25M) medi
x 24000. glutaraldehyde/osmium fix



.s adiposa grown in
ıg to the cell wall in
ɜ cell wall which is
ɜd and there are also
ibly indicating a

Fig. 39

Oblique section of a hypha of Ceratocystis adiposa grown in polyethylene glycol 200 (0.25M) medium.

x 24000. glutaraldehyde/osmium fixed.

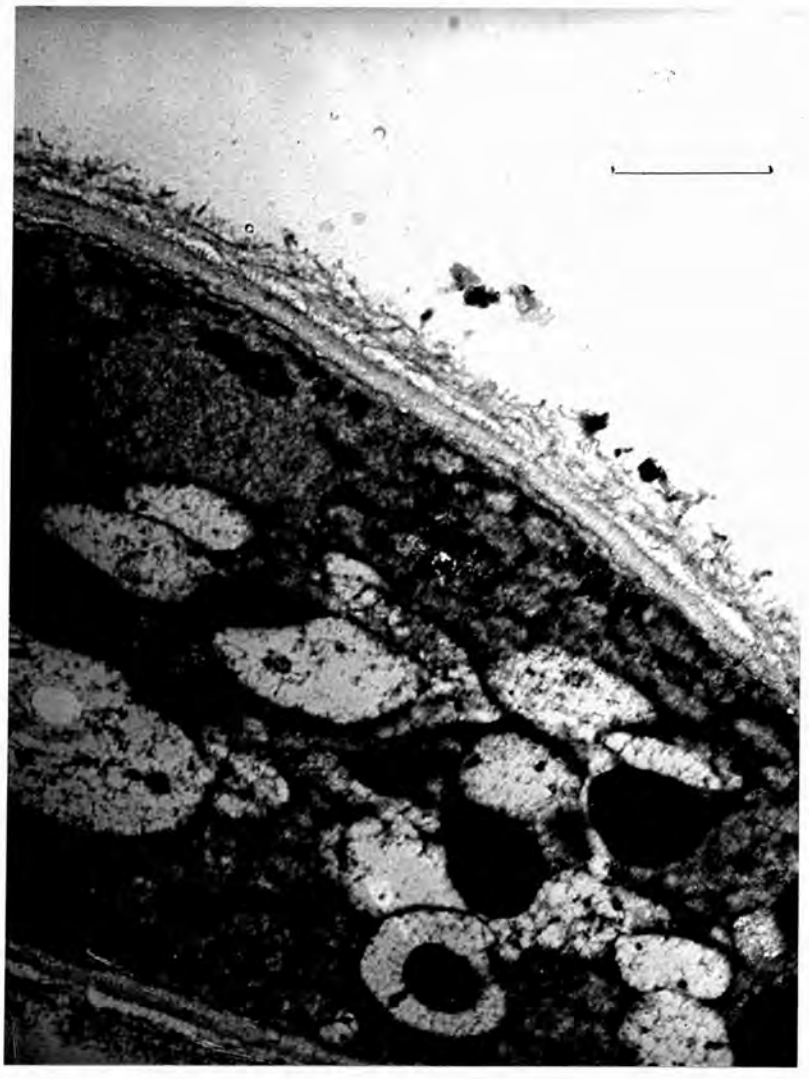


FIGURE 40.

The transverse sections of hyphae grown in sodium deoxycholate show that the cytoplasm in these hyphae appears granular and vacuolated. The mucilage is dispersed in the medium and has been precipitated by the treatment. The wall (100 nm) is surrounded by a narrow dark layer and two wider layers, separated by a dark line. The cell membrane (8 nm) is visible as a three-layered structure in places.

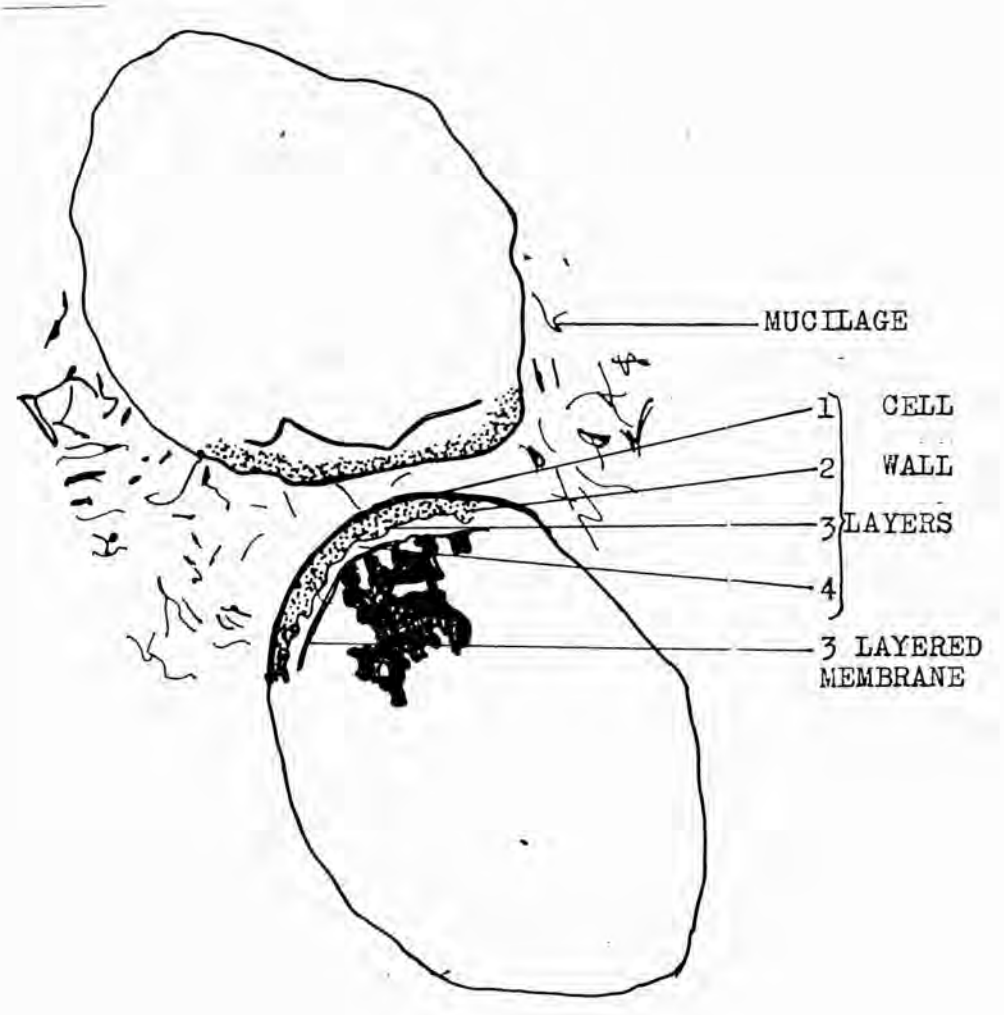
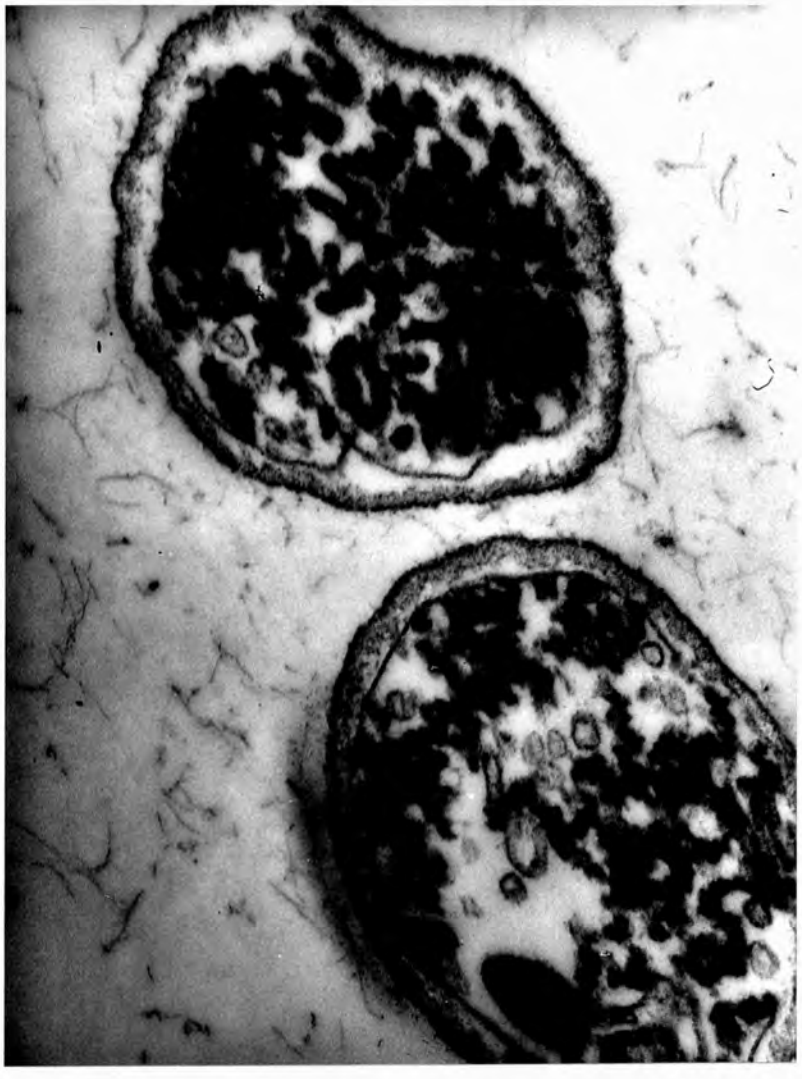


Fig. 40

Transverse sections of hyphae of Ceratocystis adiposa grown in sodium deoxycholate (0.25 x 10 M) medium.
x 60000. glutaraldehyde/osmium fixed.



To summarize, the evidence indicates that the cell wall has four layers. The wall is 100-200 nm wide and may have a diffuse layer of mucilage to the outside of the wall. The outermost dark layer may be melanin and is very thin. There are then two lighter layers separated by a dark line, the outer one being slightly darker than the inner one. When sections pass through a septum, another darker layer occurs to the inside of the cell wall but is not always seen in these sections. The slight differences between the cell walls in these photographs are probably not very significant because there is considerable variation between different preparations of the same material.

SEPTUM STRUCTURE.

The structure of the septal region of Ceratocystis adiposa is shown in Fig.41-44. There is a pore shown in the centre of the septum (Fig.41) with three darkly-stained bodies in the vicinity which may be Woronin bodies. The hypha is slightly swollen at the point where the septum is formed. The swelling is more obvious (Fig. 42) and the wall structure clearer. There is a dark narrow outer layer and three wider layers, a light layer between two darker layers. Again, three Woronin bodies can be seen near the septum. Directly to each side of the pore are two other smaller darkly-stained bodies and there appear to be strands of material passing through the pore (see Fig.42 also). Another interesting feature of this section is membrane body surrounded by many vesicles.

Fig. 43 shows the septal pore to be blocked by a large, very dark body possibly formed from the Woronin bodies. The wall is four layered and bulges where the septum is formed. The contents of the right-hand cell are much darker than those in the left-hand cell and this may be the limit of the peripheral growth zone of this hypha.

The septum may have grown round the plug and so completely separate the two cells.

The thick layer of mucilage adhered to the cell walls is shown in Fig. 44 but the layers of the wall are not clearly distinguished. A nucleus with its nucleolus can be seen in each cell and mitochondria are present in the dense cytoplasm. The septal pore appears to be almost blocked by a dark Woronin body.

Fig. 41

Longitudinal section through the septum of a hypha of *Ceratocystis adiposa*.

x 70000. glutaraldehyde/osmium fixed.

Fig. 42

Longitudinal section through the septum of a hypha of Ceratocystis adiposa showing intracytoplasmic membrane system.

x 35000. $KMnO_4$ fixed.

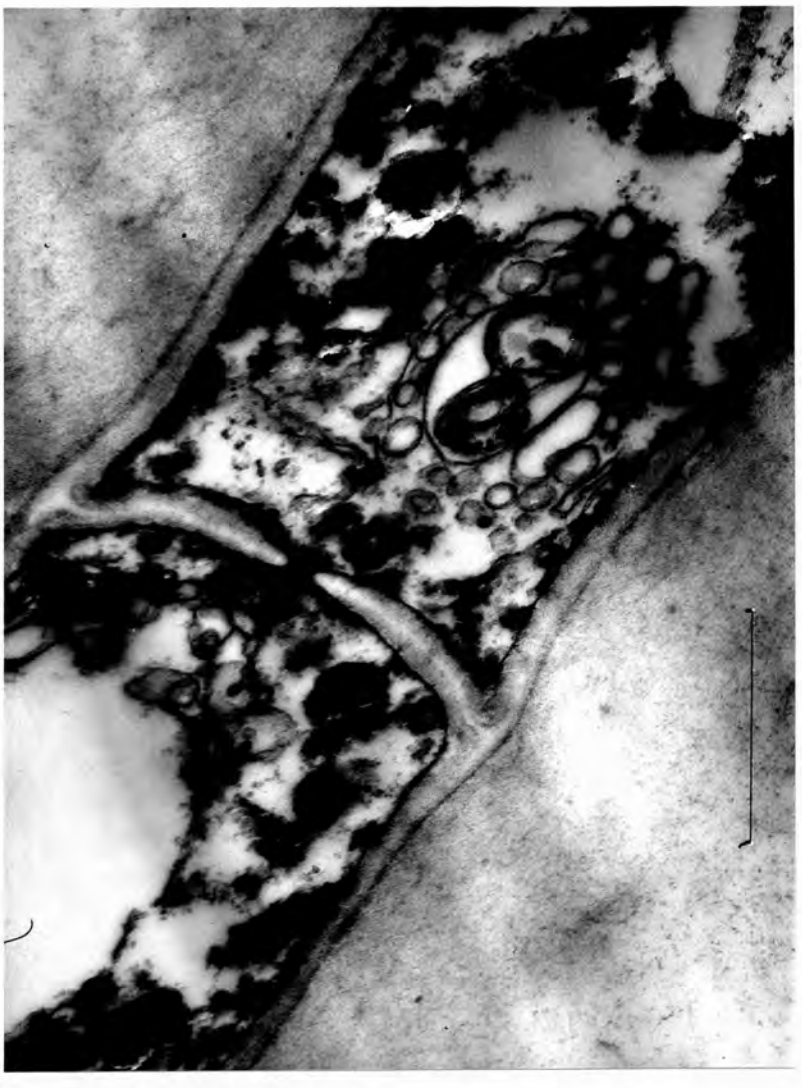


Fig. 43

Longitudinal section through a plugged septum of Ceratocystis adiposa.

x 44000. glutaraldehyde/osmium fixed.

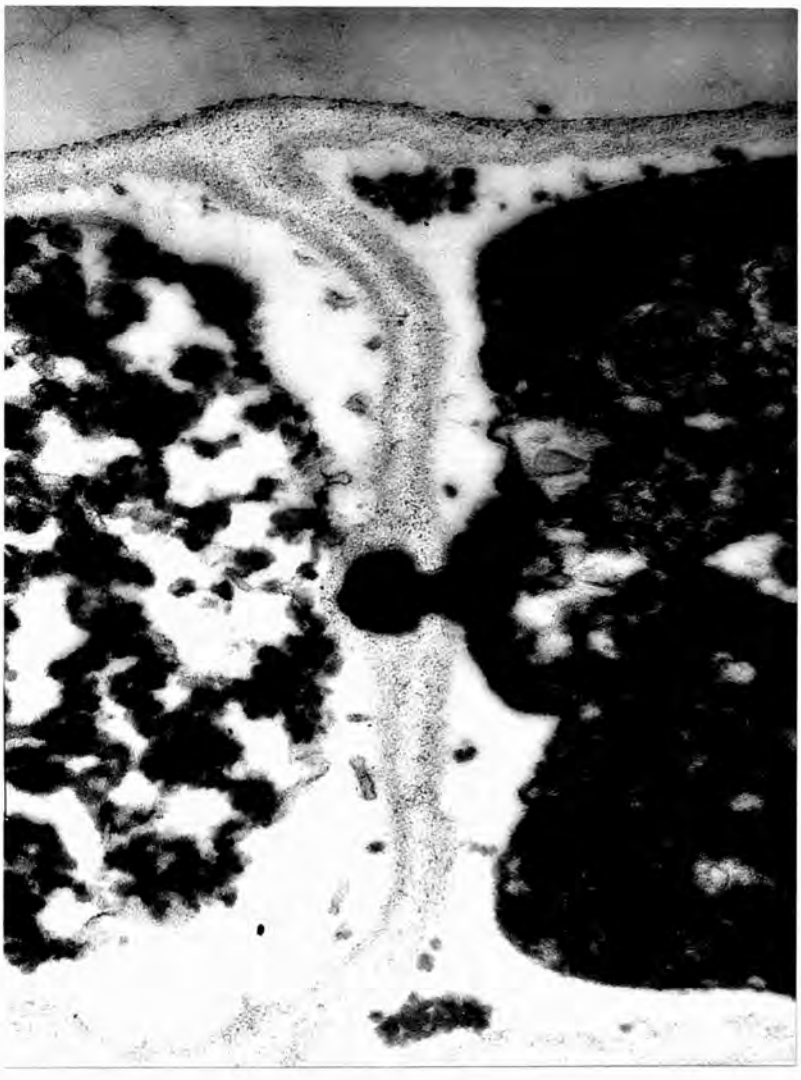


Fig. 44

Longitudinal section of a hypha of Ceratocystis adiposa
x 12 000 glutaraldehyde/osmium fixed.

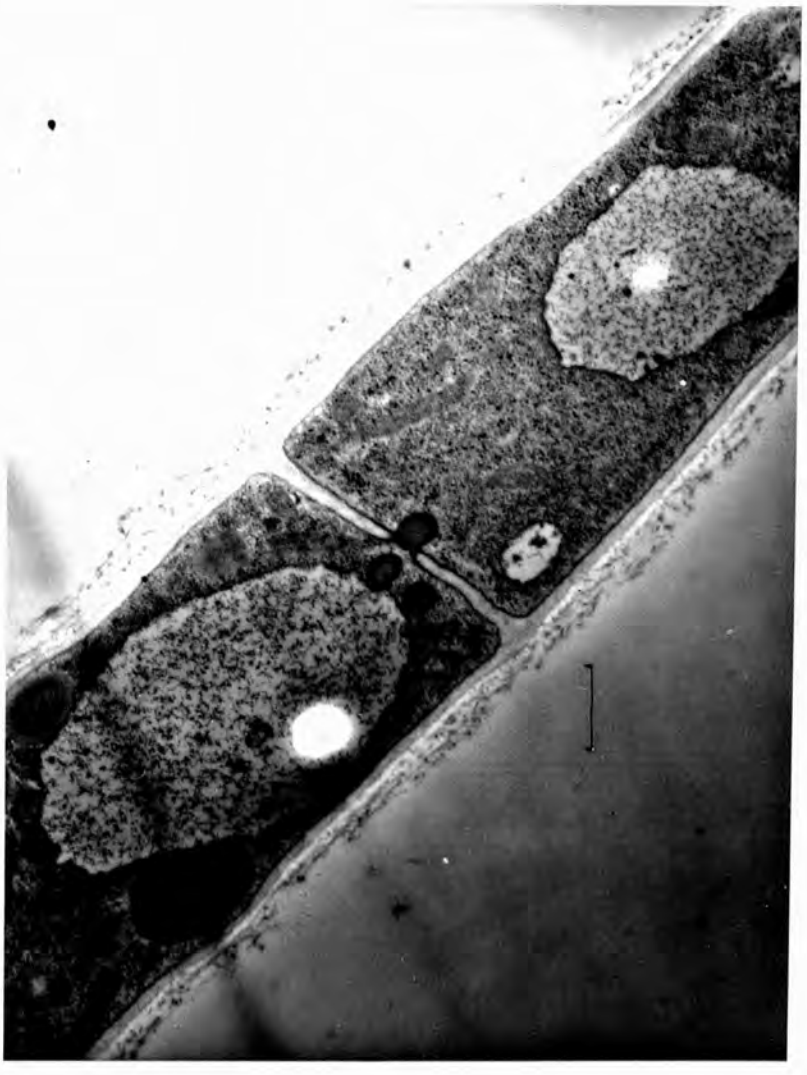
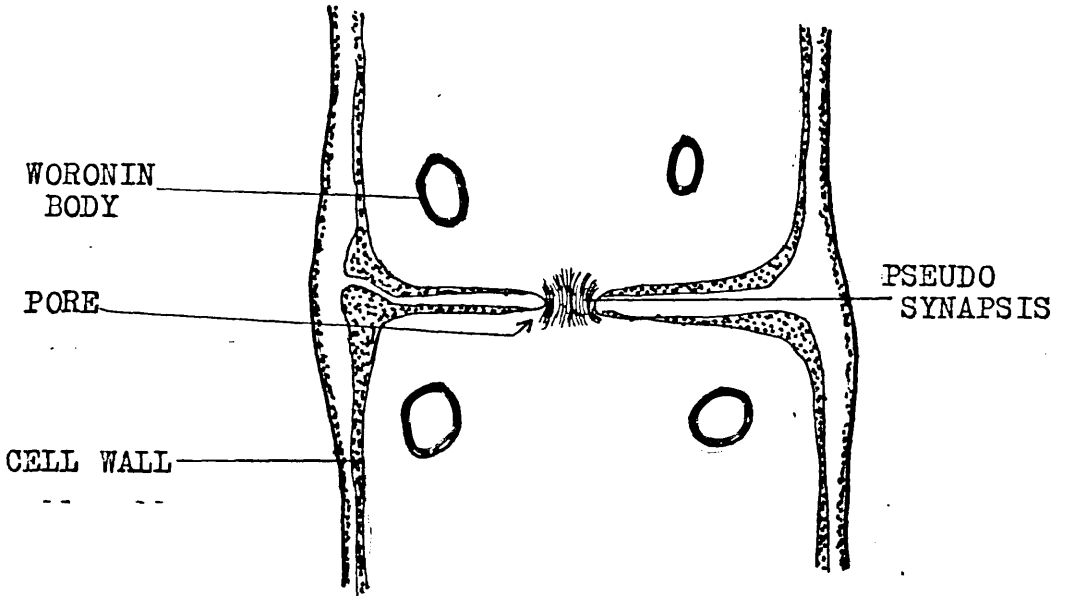


FIGURE 45.

A possible structure of the septum of Ceratocystis adiposa which appears very similar to that of Sporothrix schenckii (Thibaut 1970). (Nicot and Mariat 73).



There appear to be two Woronin bodies in each side of the septum. On occasions, these may block the septal pore (Fig 43 & 44) and so isolate one cell from another. When the pore is open, fibrils appear to pass through the pore. These may arise from the small dark bodies on each side of the pore. Together these form the pseudo synapsis or appareil synaptique (Thibaut 1970b)

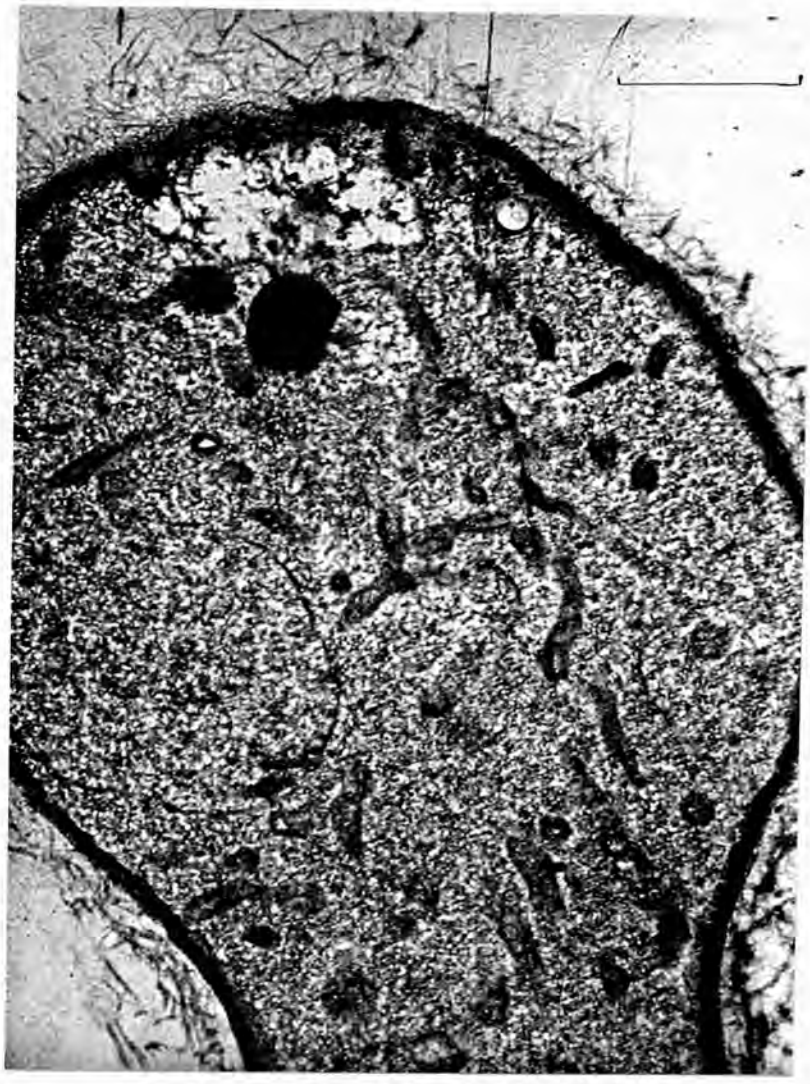
THE HYPHAL TIP.

Hyphal tips are shown in Fig. 47 with a large dark body near the tip. These appear similar to Woronin bodies found near septa in this fungus and may therefore have some regulatory function concerned with cell wall synthesis. The rest of the cytoplasm is dense and contains many nitochondria. A nucleus is shown in Fig. 47 about 2μm from the tip and 1.5μm in diameter.

(Page 211 missing due to change of pagination)

Fig. 47

Section through a hyphal tip of Ceratocystis adiposa
x 26000 glutaraldehyde/osmium fixed.



METHODS.

Giemsa Stain for nuclei.

1. A spore suspension was centrifiged and the supernatant medium decanted off.
2. 5 mls of freshly prepared Ethanol = Acetic Acid (3 = 1) were added to the spores for 30 minutes, centrifuged and decanted off.
3. The spores were covered with N. HCl at 60°C and kept at 60°C in a water bath for 10 minutes, cooled and washed twice with distilled water.
4. The spores were washed in M/200 phosphate buffer, pH 7.0.
5. Stain was prepared by adding 0.5 ml Giemsa R66 to 10 ml of phosphate buffer.
6. 1 - 2 ml of prepared stain were added to the spores and left to stain for 30 minutes. A further drop of Giemsa R66 was added and left for another 30 minutes. A drop of the spore suspension was placed on a clean glass slide and observed.

Acridine orange for nuclei.

Acridine orange (50 mgm/litre water) was used to stain nuclei. Preparations were placed in the dark for 10 minutes and then were observed using the G & S Fluorescence microscope.

Sudan IV for fat.

Sudan IV (5g) was dissolved in 95 ml of 70% alcohol.

Conidia were stained for 30 minutes.

Orcein-proprionic acid stain (Speller 1969).

2% orcein in 60% proprionic acid was refluxed for 2 minutes over a low bunsen flame, allowed to cool and filtered.

Conidia were fixed in 60% proprionic acid for 10 minutes, washed in distilled water and hydrolysed for 2 minutes in N HCl. They were then washed twice and transferred to stain.

Conidia were germinated in 1% glucose and treatments of 0.25M sorbose, PEG 200 and 2.5×10^{-4} M sodium deoxycholate, and fixed after 5 & 7 hours. Nuclear distribution was observed. Table. 24 . Fig. 53 .

To determine the nutrients necessary for germination.

Distilled water, 1% glucose and SSM were inoculated with a suspension of washed conidia to give 5×10^{4} spores per ml and incubated on an orbital shaker at 25°C and 200 rpm. The flasks were observed after 18 hours.

To determine the effect of sodium deoxycholate on % germination on semi-solid medium.

Three replicate sets of agar plates of SSM containing a known concentration of sodium deoxycholate were spread with 0.1 ml of a spore suspension containing up to 300 conidia per ml on plates up to 7×10^{-4} M and 3000 per ml on 8×10^{-4} M and greater concentrations of sodium deoxycholate.

The plates were incubated invertedly at 25°C and the colonies counted over a period of up to 5 days to allow for a delay in germination at higher concentrations.

To determine spore size.

Spore size and shape was observed from drawings made using a projection headed microscope. Fig. 48 .

To determine % germination with time in SSM.

100 ml flasks containing 25 ml SSM were inoculated with conidia to give $\sim 10^{4}$ per ml. The flasks were incubated at 25°C and 200 rpm on an orbital shaker and samples removed at intervals and 100 conidia scored for germination. When

Fig. 48

Conidia of Ceratocystis adiposa

(a) To show range of shape and size.

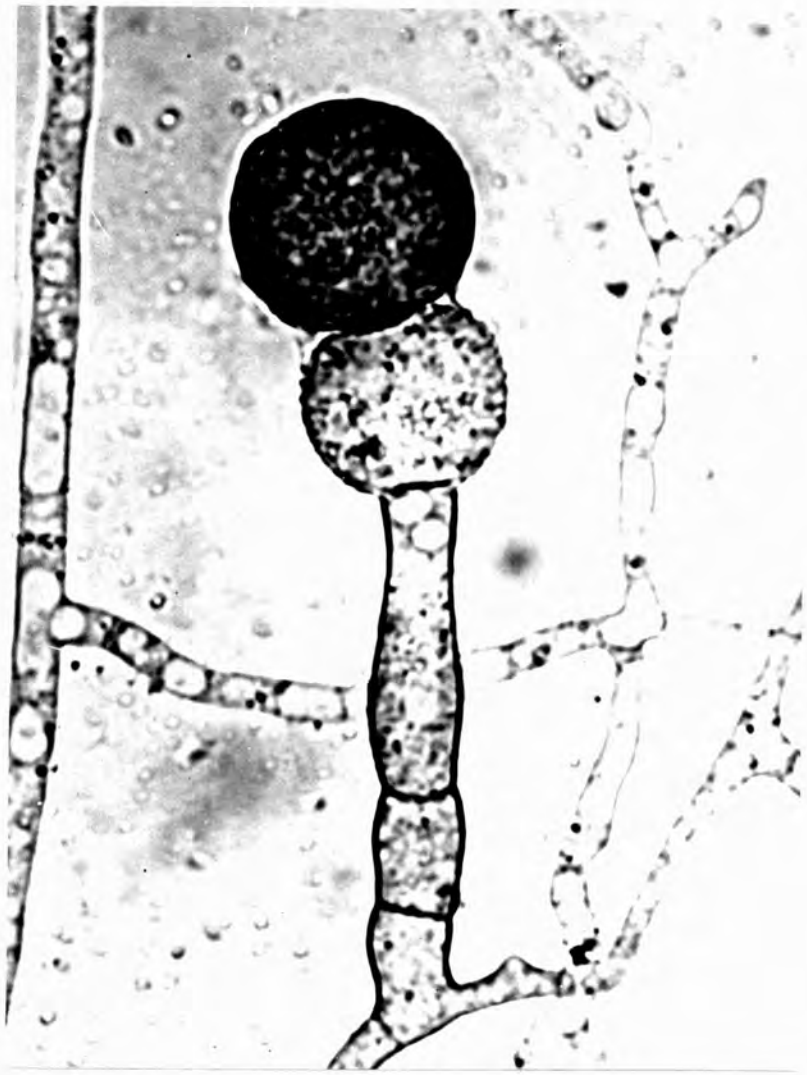
(b) To show detail of thick walled conidia.

(c) To show conidia on a conidiophore.

a)

b)

c)



germination reached 70%, the germ tubes of conidia had tangled together making it impossible to count the number of conidia in a clump accurately.

Coulter Counter Analysis of spore size distribution.

As germination may be considered in two phases, swelling and germ tube formation, it was decided to investigate the effects of paramorphogens on the two phases.

To test for swelling, the Coulter Counter, Model A, was used with a 100 μm aperture tube. The Coulter Counter was calibrated using paper mulberry pollen (13.31 μm diameter) and settings of the threshold and gain switch calculated to give the following lower limits to the size classes.

Settings			Volume ($\mu m^3 \times 10^{-3}$)	Equivalent diameter (μm).
tL 40	I	1	2.0	15.6
31		1	1.6	14.5
55		2	1.4	13.9
47		2	1.2	13.2
39		2	1.0	12.4
31		2	0.8	11.5
23		2	0.6	10.5
39		3	0.5	9.8
31		3	0.4	9.1
23		3	0.3	8.3
15		3	0.2	7.2
8		3	0.1	5.7

The spore suspension was prepared by shaking the spores from a slope with 10 ml of Tween (1 in 10^4). The suspension was filtered through a double layer of muslin to remove fragments of mycelium and large clumps of spores. Chains of conidia were separated by blending the suspension in a ground glass tube

and plunger. The final suspension contained mainly single spores. This suspension was used directly in the Coulter Counter or inoculated into flasks and incubated before being analyzed.

The sample, usually 1 ml of a dense spore suspension, was added to 300 ml of membrane filtered 0.5% Na Cl. The manometer was filled with the sample which was stirred continuously to prevent the spores settling out and 0.5 ml of the sample was counted immediately as some shrinkage occurred in the 0.5% Na Cl, see Fig. 55 . A raw data sheet is shown in Table 40

To test whether inhibition by Na De & PEG is reversible.

A dense spore suspension was inoculated into SSM containing 10^{-3} M Na De or 1 M PEG to give final concentrations of 3×10^{5} and 3×10^{4} spores/ml. After 1 and 2 days, 0.1 ml of each was plated on to both and SSM plates and growth on the plates observed after up to 5 days. After three days, the flasks with PEG had a few germinated conidia but no pellets formed until 6 days. No growth was observed in Na De flasks.

To see if the irreversible inhibition of conidia by 10^{-3} M Na De was due to membrane damage, a spore suspension was inoculated into a 1% glucose with 2×10^{-3} M sodium deoxycholate or 1 M PEG. After 24 hours, the spores were filtered from the media which was rotary evaporated. This now concentrated media was tested with ninhydrin for amino acids. To establish which amino acids are present in conidia, they were treated with boiling 80% ethanol for 10 minutes. The extract was concentrated.

Both samples were centrifuged to remove any precipitate and analyzed on an amino acid analyzer by the Biochemistry Department.

Whether the reduction in the spore volume observed was due to shrinkage of the protoplast or of the whole cell is not clear as it was not ascertained whether the electrolyte penetrates the cell wall. However, from visual measurements, it would seem that total cell volume was being recorded.

Nuclei.

Nuclei were stained blue by Giemsa and conidia contained between 2 and 4 nuclei. Fluorescence emitted by the nucleic acid/acridine orange complex formed by stained nuclei was observed through an orange filter and again 2 - 4 nuclei per conidium were observed. The nucleus is about 1 μm diameter. Plate 62 - E.M. Control).

Fat droplets.

The conidia contained one or two large fat droplets which stained with Sudan IV. The fat droplets are > 3 μm diameter.

Nutrients required for germination.

The spores did not germinate in distilled water but required a carbon source, glucose, before they germinated. Polyethylene glycol inhibited germination for 18 hours in both glucose and SSM. 10^{-3} M Na De appeared not to inhibit germination when used with glucose alone.

Solution	Germination
H_2O	.
H_2O + 1 M Peg.	.
H_2O + 2 x 10^{-3} M Na De	.
Glucose	✓
Glucose + 1 M Peg.	.
Glucose 10^{-3} M Na De	✓
SSM -	✓
+ 1 M Peg.	.
10^{-3} M Na De	.
SSM + 2.5 x 10^{-5} M Na De	✓

% germination on sodium deoxycholate media.

As shown in Table 39 and Figs. 49 and 50 increasing concentrations of Na De decrease the % germination of spores. The apparent increase in % germination at 10^{-4} M is probably due to restriction of Kr so fewer conidia are overgrown by germ tubes from early germinated spores. At 2×10^{-3} M , no growth was observed on any of the plates.

At low concentrations of Na De $< 10^{-4}$ M spore germination was apparently uninhibited whilst Kr was slightly reduced. The The "kill curve", Fig. 49 shows a usually inverted S shaped curve which was converted to a straight line by plotting probits. Fig 50.

Germination with time.

% germinated conidia with time in SSM is shown in Fig. 51 . The lag phase for Ceratocystis adiposa conidia was about 3 hours and then % germination increased rapidly to 70% at 5 hours. Above this % germination, the germ tubes of conidia which had germinated early were tangled together and clumps of germinated conidia and trapped ungerminated conidia were formed. These tended to remain at the bottom of the centrifuge tube and were not always taken up in a pipette. Even when these clumps were observed, it was difficult to count the numbers of germinated and ungerminated conidia.

The growth of germ tubes was measured from spread 2% malt agar plates and the ln length (μm) plotted against time. The increase in length of the germ tubes was logarithmic for some time and so α_g , germ tube specific growth rate could be calculated (Trinci, 1971(b)). This was found to have a maximum value of 0.8 hr^{-1} which corresponds to a doubling time of 52 minutes. Fig 53 Table 41.

Fig 49

% Germination of Ceratocystis adiposa conidia on sodium deoxycholate in SSM at 25°C

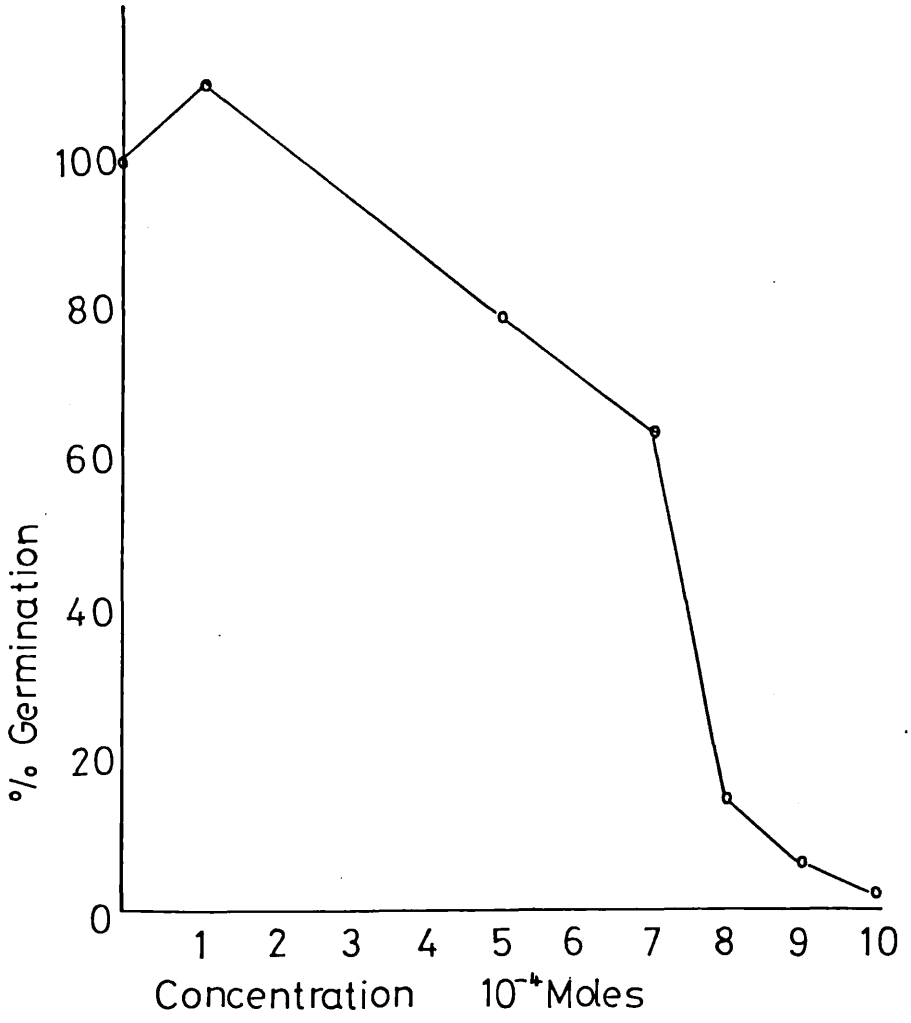


Fig 50

Germination of C. adiposa (SSM; 25°C)

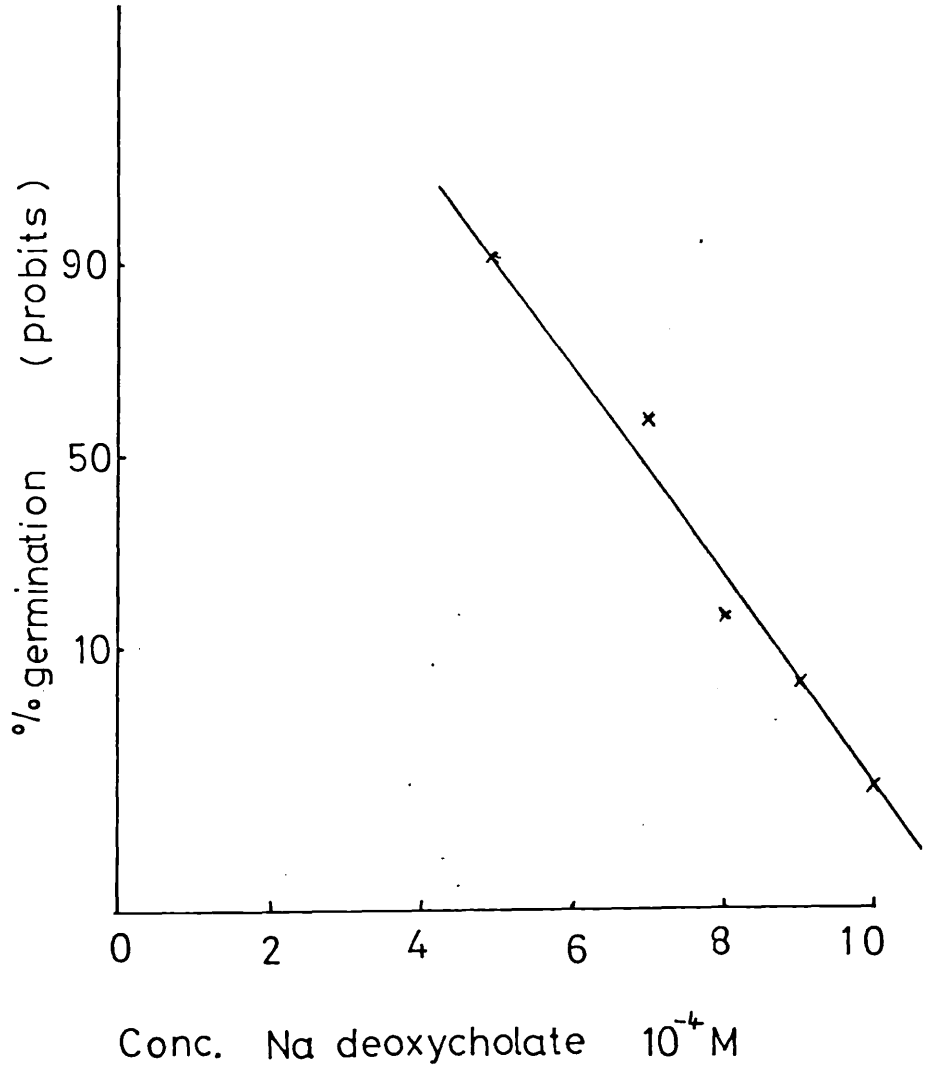


Fig 51 % Germination of Ceratocystis adiposa conidia (SSM, 25°C)

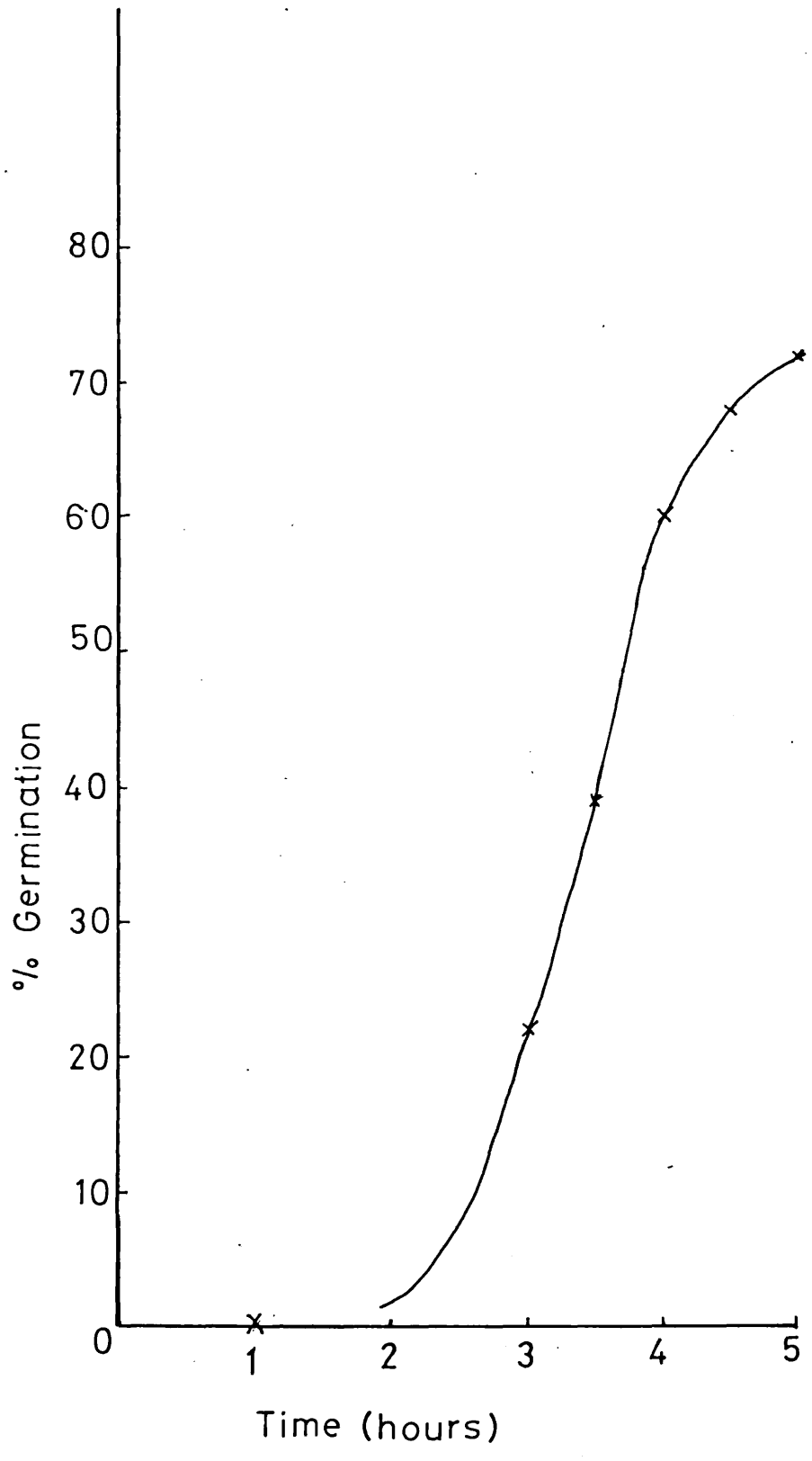
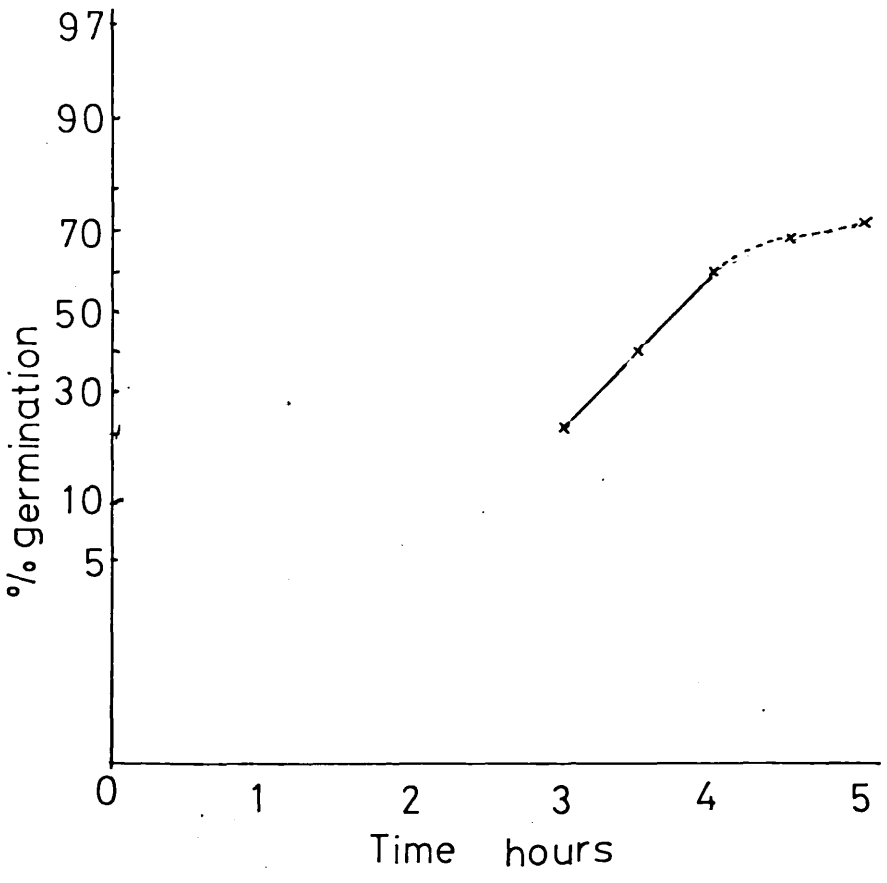


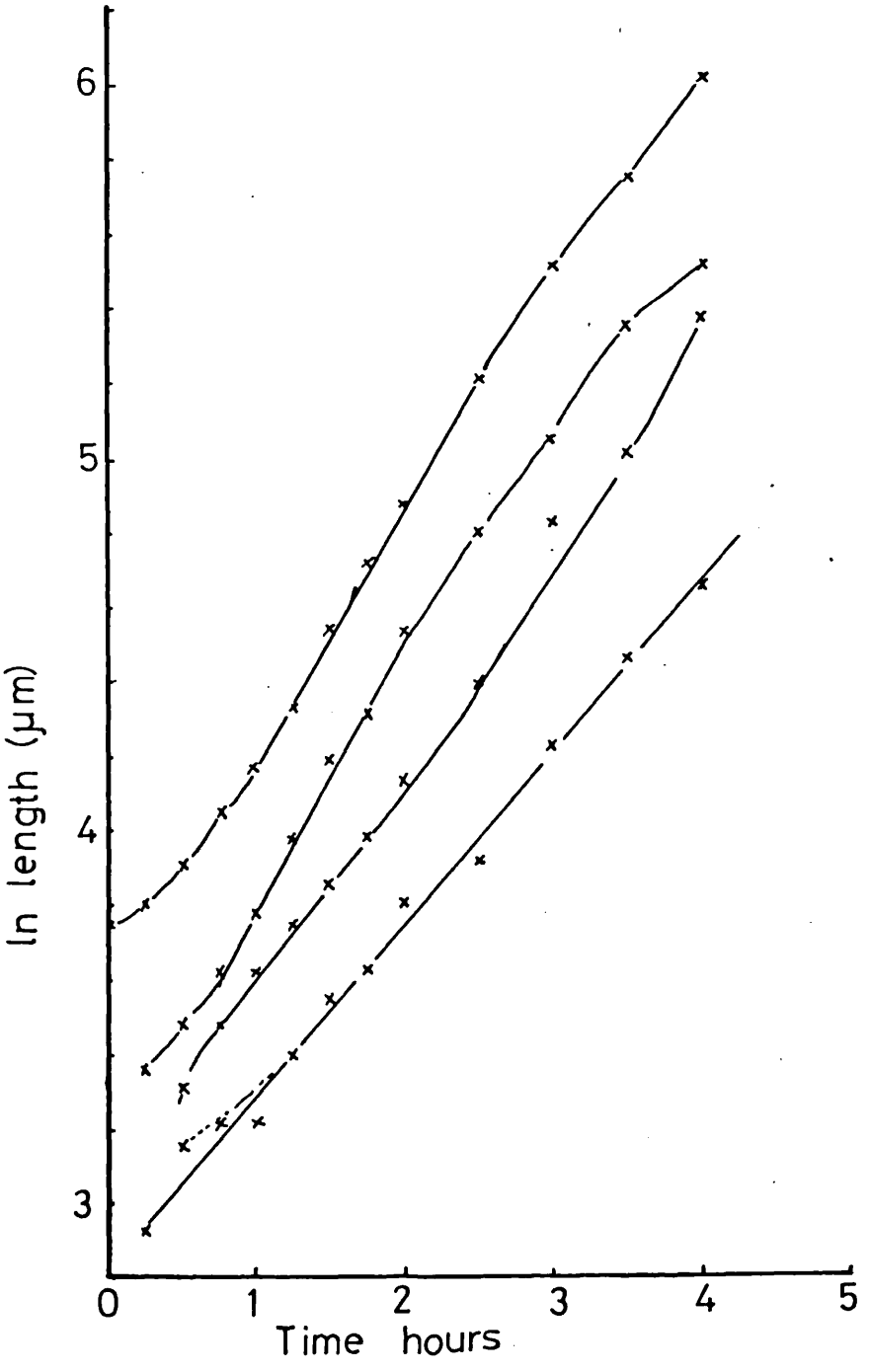
Fig 52

Conidium germination of C. adiposa (SSM, 25°C)



Plot using probits (Waggoner & Parlange 1974)

Fig 53 Germ tube growth rates of Ceratocystis adiposa on 2% malt agar 25°C



Prior to germination, the mean number of nuclei per spore was 3.9 ± 2.8, the modal value being 4 (Table 24). After 5 hours, germination nuclei from conidia in glucose (SSM) and PEG media did not appear to have divided while those from sodium deoxycholate and sorbose had started to divide. Nuclei in conidia from all treatments had divided after 7 hours and some in glucose, sorbose and sodium deoxycholate appeared to have divided twice.

A conidium and germ tube from a five-hour culture was observed to have 56 nuclei. Assuming a lag phase of 3 hours from the previous experiment and an initial nuclear number of 4, then each generation must have been ~ ½ hour.

(i.e., 8 16, 32 64 nuclei.)

From the Table 24 , most conidia from SSM have still only 4 nuclei at 5 hours but appeared to have divided twice by 7 hours to give 9 - 14 nuclei, a doubling time of about one hour.

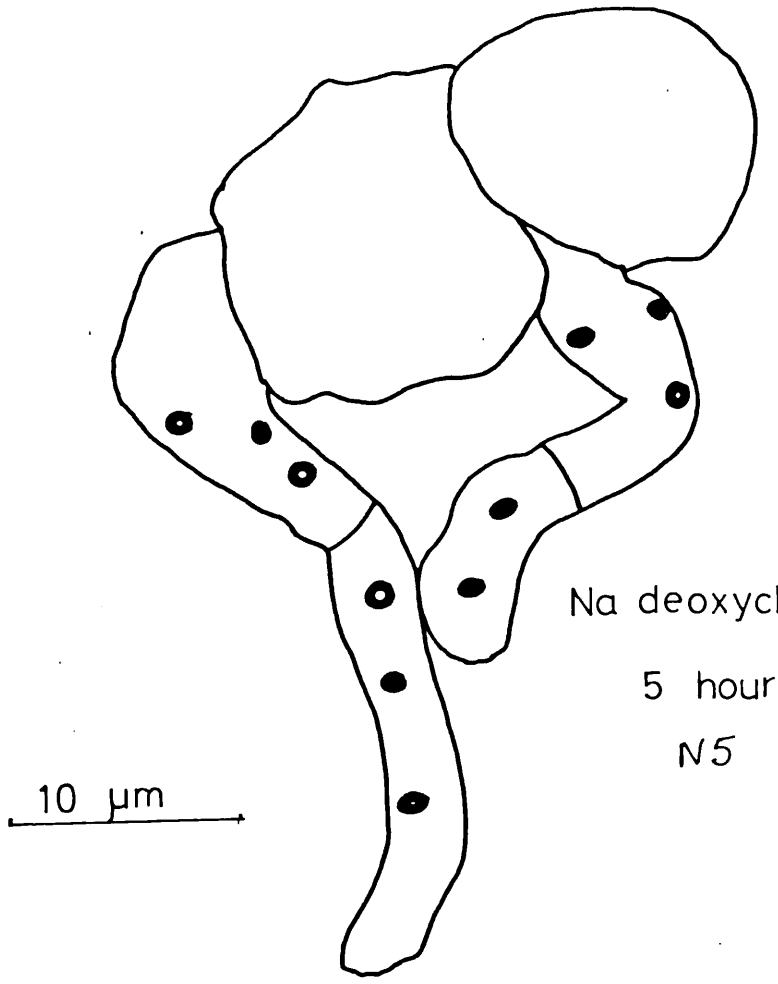
The number of nuclei associated with unit volume of cytoplasm varied but was not significantly different between the treatments. From Figs. 54 , the cross sectional area of cytoplasm per nucleus was found to be in arbitrary units.

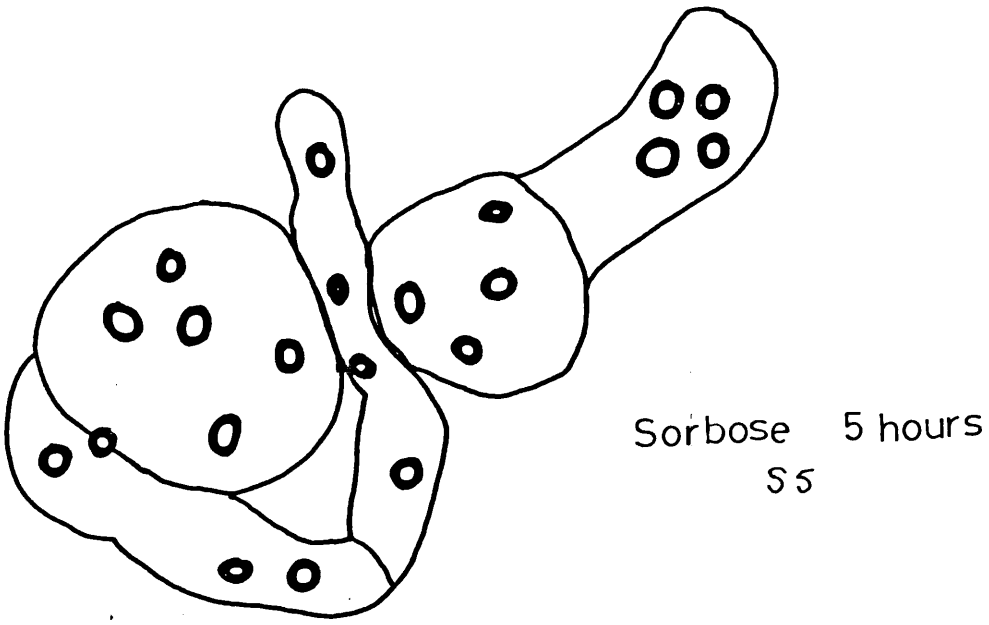
C 7 = 99, 163, 119. - SSM

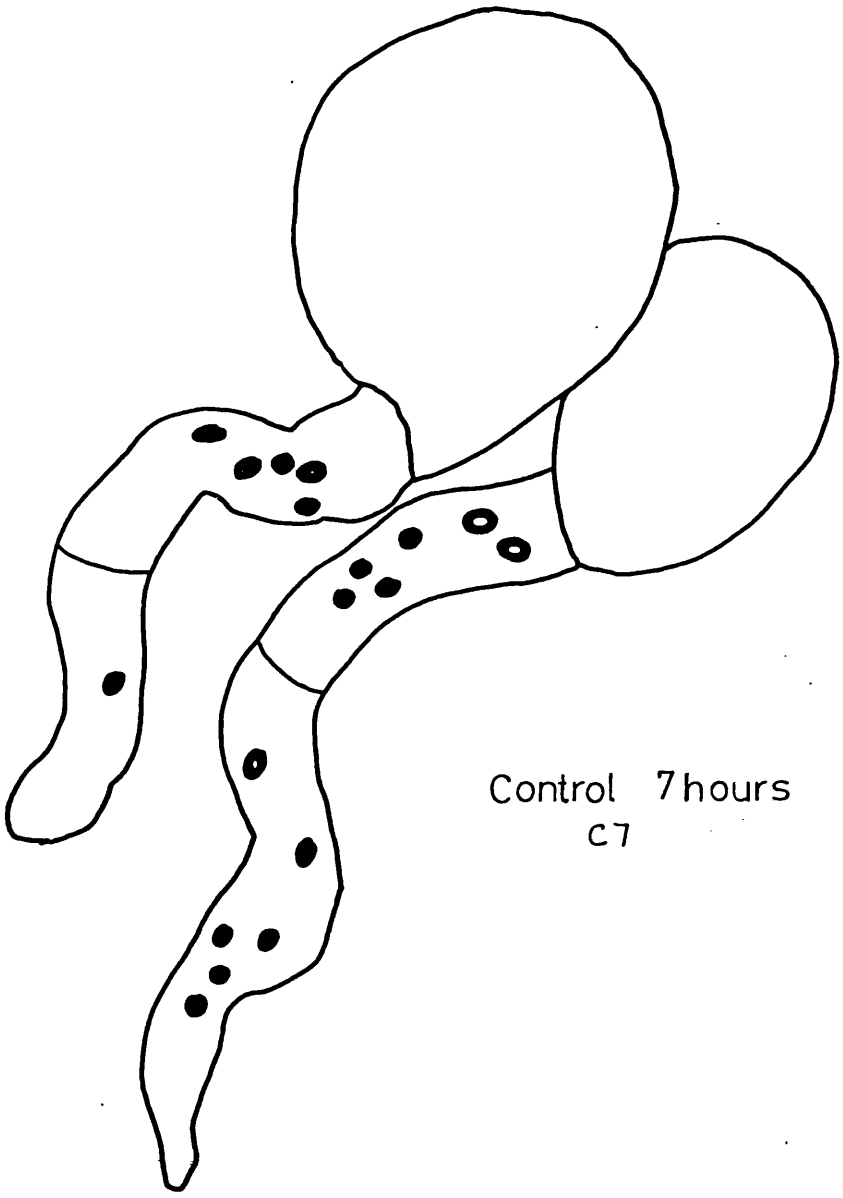
S 5 127, 131 - SSM + 0.25M Sorbose

N 5 141, 193 - SSM + 0.25×10^{-4} Na deoxycholate

Fig 54 Nuclear distribution in germ tubes of Ceratocystis adiposa.







Control 7 hours
C7

TABLE 24.

Number of Nuclei in conidia and germ tubes of Ceratocystis adiposa.

Number nuclei	Time 0	5 hrs. C	P	N	S	7 hrs C	P	N	S
2	2	3	-	-	-		-		-
3	7	6	2	-	-		1		-
4	9	10	3	1	1		-		1
5	-	3	2	3	4		1		-
6	2			4	2		1	1	1
7	-			3	3	2	3	1	3
8	1			1	2	-	3	1	1
9					-	1		1	1
10					1	1		-	1
11						1		1	-
12				1		2		-	1
13						1		-	
14						2		1	
15								-	
16								1	
Mean	3.9	3.6	4.0	6.5	6.4	10.9	6.6	10.1	7.8

C = SSM
P = SSM + 0·25M PEG
N = SSM + 0·25 × 10^{-4}M Na De
S = SSM + 0·25M Sorbose

The range of size, shape and wall thickness of conidia of Ceratocystis adiposa is shown in Plate 48 . The spore suspensions obtained from cultures grown at 30°C contained predominantly thin-walled elliptical conidia found to be between 5.0 - 7.5 μm x 10 - 15 μm (vol. as cylinder 200 - 650 μm^3). The spherical thick-walled conidia ranged from 10 - 20 μm in diameter (500 - 4000 μm^3).

The effect of 0.5% Na Cl solution used as the electrolyte was to cause a reduction the spore volume (Fig. 56). Therefore, unless the counts obtained at each setting were very different (10%), only two counts were made to reduce the time taken to do an analysis and the error due to shrinkage of the conidia.

A preliminary analysis of a spore suspension from a culture grown at 25°C using a 200 μm tube is shown in Fig. 55 The peaks came at 9 μm diameter and 21 μm diameter which correspond to the visual measurements.

1. Age of Culture.

Spores were taken from slopes which had been incubated at 30°C for varying lengths of time and analyzed. There was very little variation in the size distribution with age up to 15 days. (Fig. 57).

2. Temperature of incubation.

Cultures which had been incubated at 25°C and 30°C were analyzed at 8 days old and a 2 week culture grown at 25°C was analyzed. (Fig. 57). There was a number of differences between the distributions. At 30°C, the peak came between 2 - 3 μm^3 x 10^{-3} ≡ 7 - 8 μm diameter. At 25°C, it came between 4 - 5 or 5 - 6 μm^3 x 10^{-3} while in the cultures from 9 - 10.5 μm diameter Fig 57.

Fig 55 Preliminary analysis of a 25°C spore suspension of C. adiposa with a 200 µ aperture

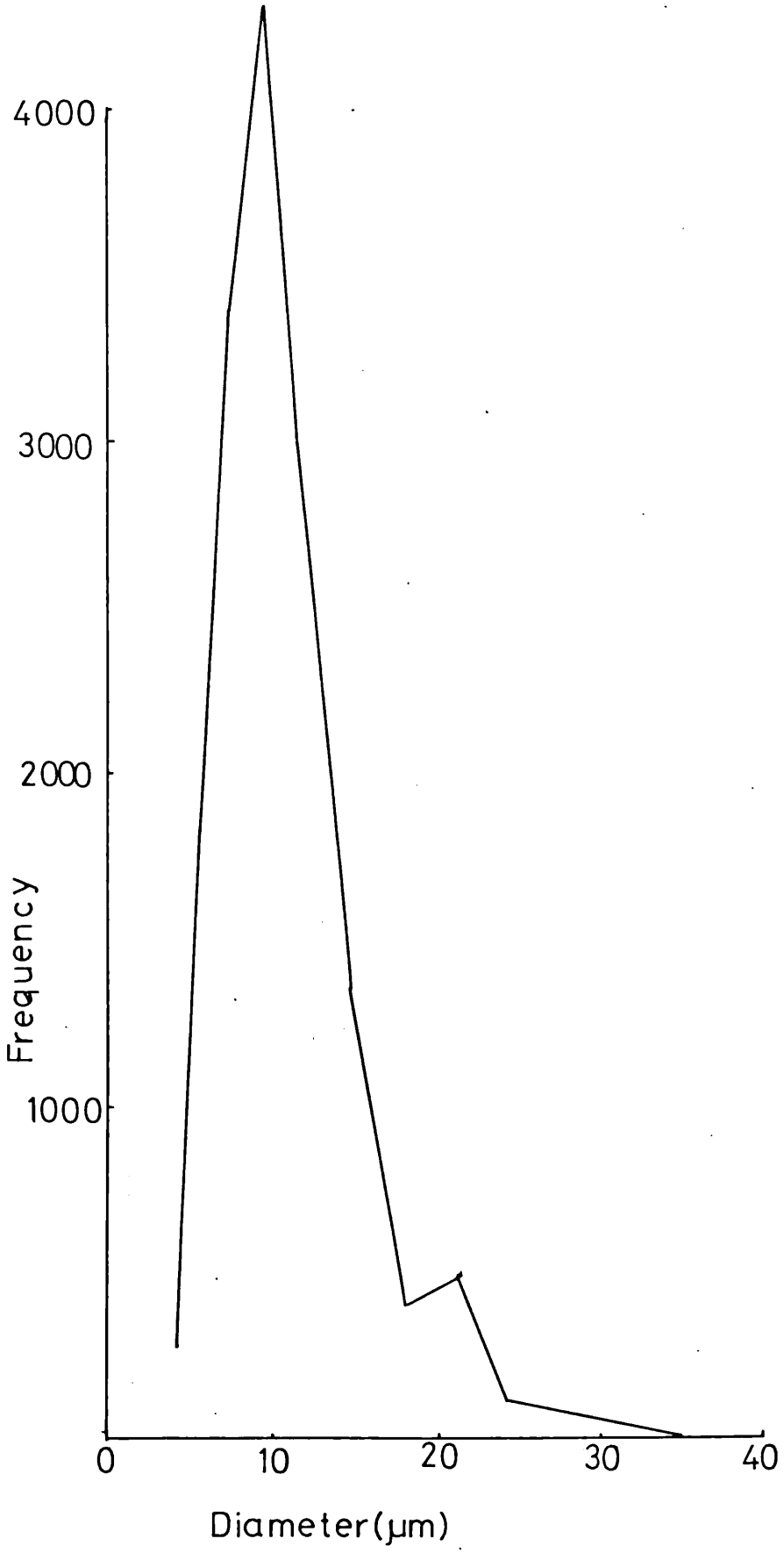


Fig 56 To show the effect of electrolyte on spore size distribution after 10 mins

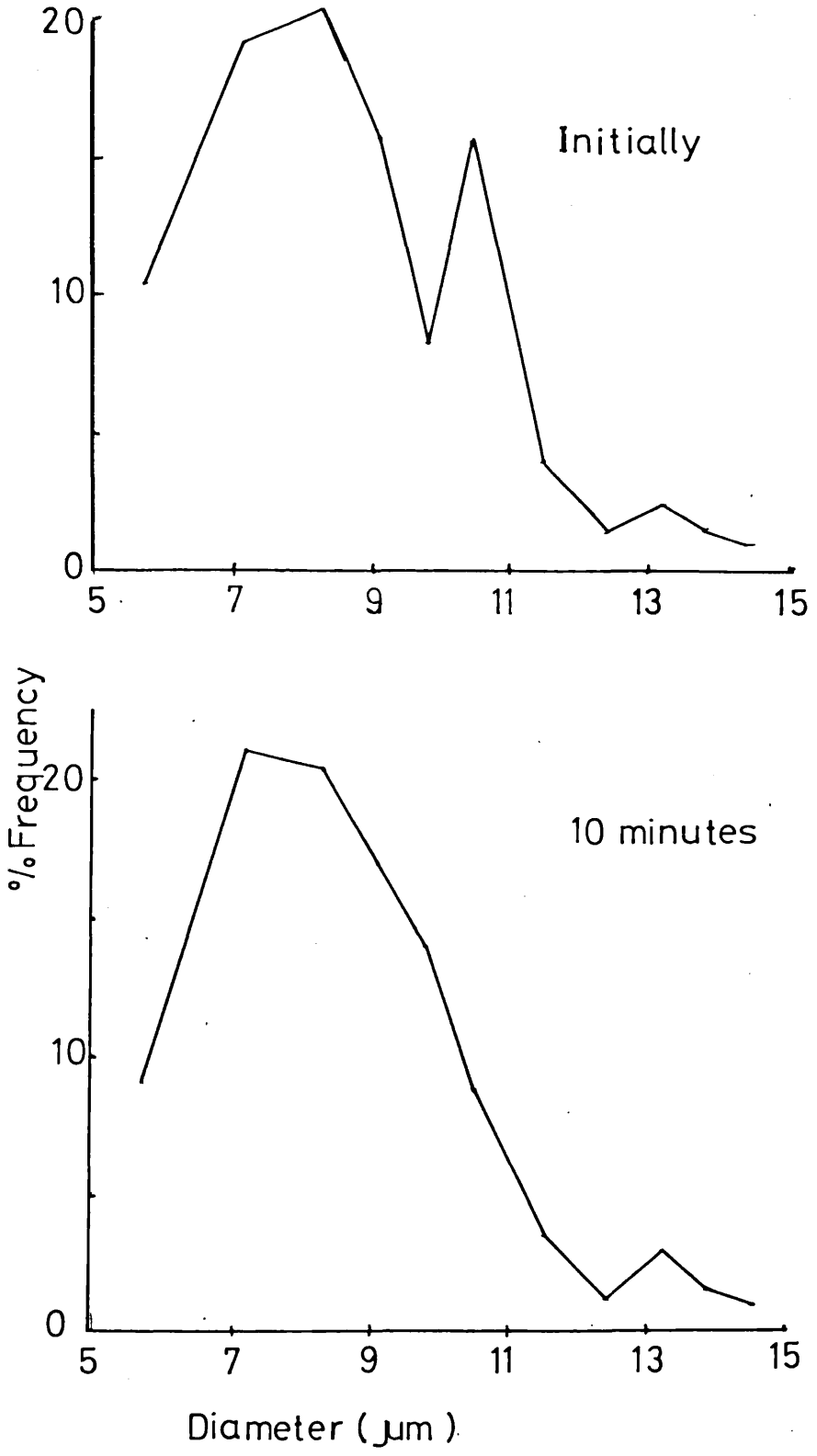
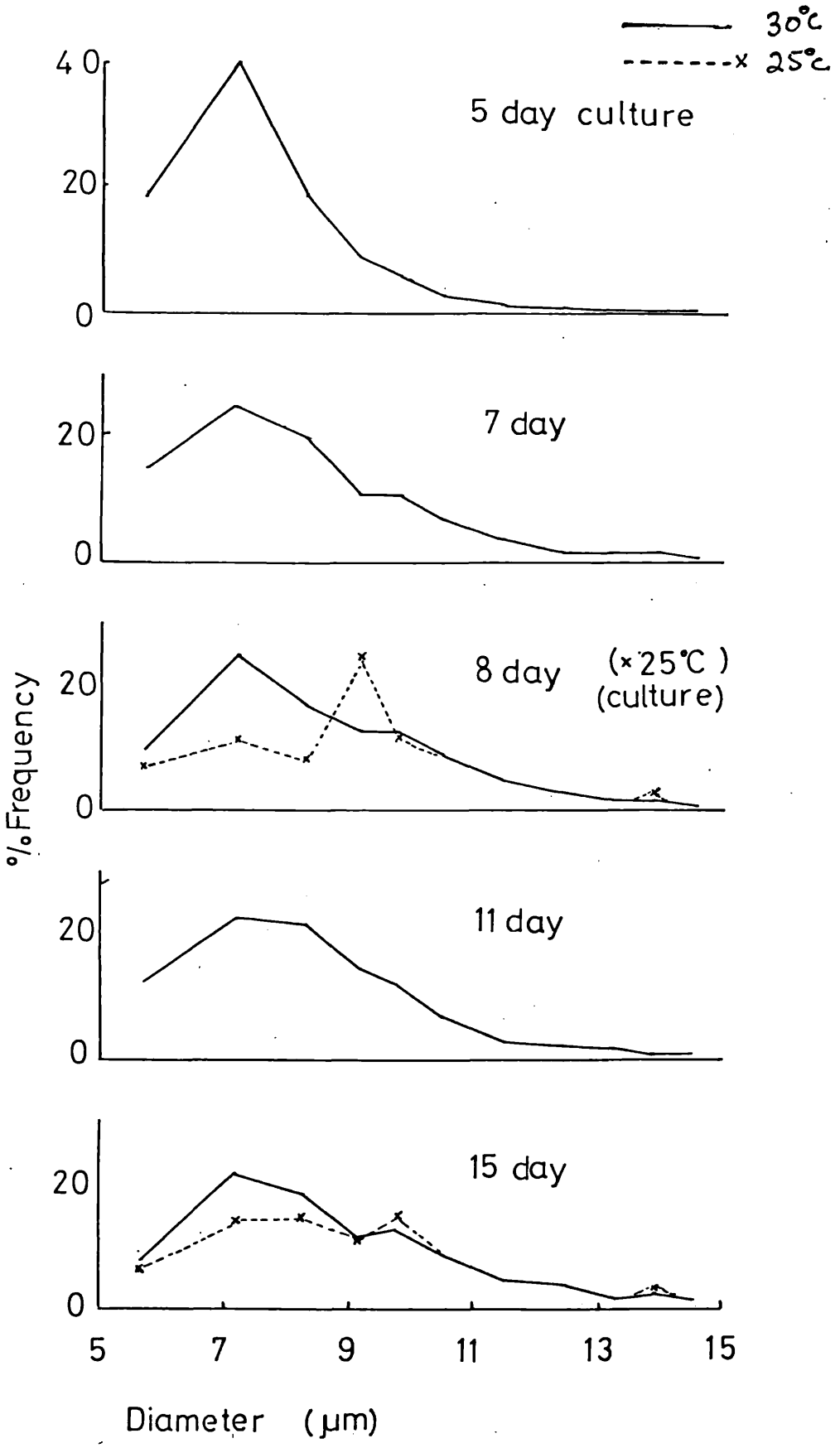


Fig 57 Size distribution of C. adiposa conidia from different aged cultures.



30°C there was only a shoulder in this position. There was a second small peak between 14 - 16 $\mu m^3 \times 10^{-3}$ (14 - 14.5 μm diameter.

3. Pattern of germination.

To follow the pattern of germination in SSM, 20 ml of the medium was inoculated with a dense spore suspension and incubated at 25°C at 200 rpm or an orbital shaker. A 1 ml sample was removed immediately and analyzed. (Fig. 58). Another sample was removed after 3 hours and analyzed. There appeared to have been an increase in size of the spores. The next sample was removed after 7 hours by which time some spores had germinated and the germ tubes had tangled together. These large clumps would not pass through the 100 μm aperture. The analysis was therefore done on the free spores which remained ungerminated. These appeared to be the smaller spores which had not swollen. After 11 hours, these spores had begun to swell.

4. Effect of inhibitors.

Spores were incubated for 5 and 10 hours in 1 M Peg and 1 M Glucose and 10^{-3} Na De in 1% Glucose at 25°C and 200 rpm (as SSM precipitates sodium deoxycholate). Fig. 56 shows the initial size distribution. After 5 hours in the control medium, the spores appeared to have shrunk but some spores had germinated and if compared with Fig. 58 , it shows a stage between 3 and 7 hours. Fig 59

Spores in 1 M Peg and Glucose had not begun to germinate (swell) and they showed shrinkage but in Na De the spores appeared to swell.

After 10 hours, Fig. 60 , only the Na De treated spores showed any signs of germination and had a distribution similar to that of the control at 11 hours. (Fig. 58). While

Fig 58 Size distribution of C. adiposa conidia during germination in SSM at 25°C

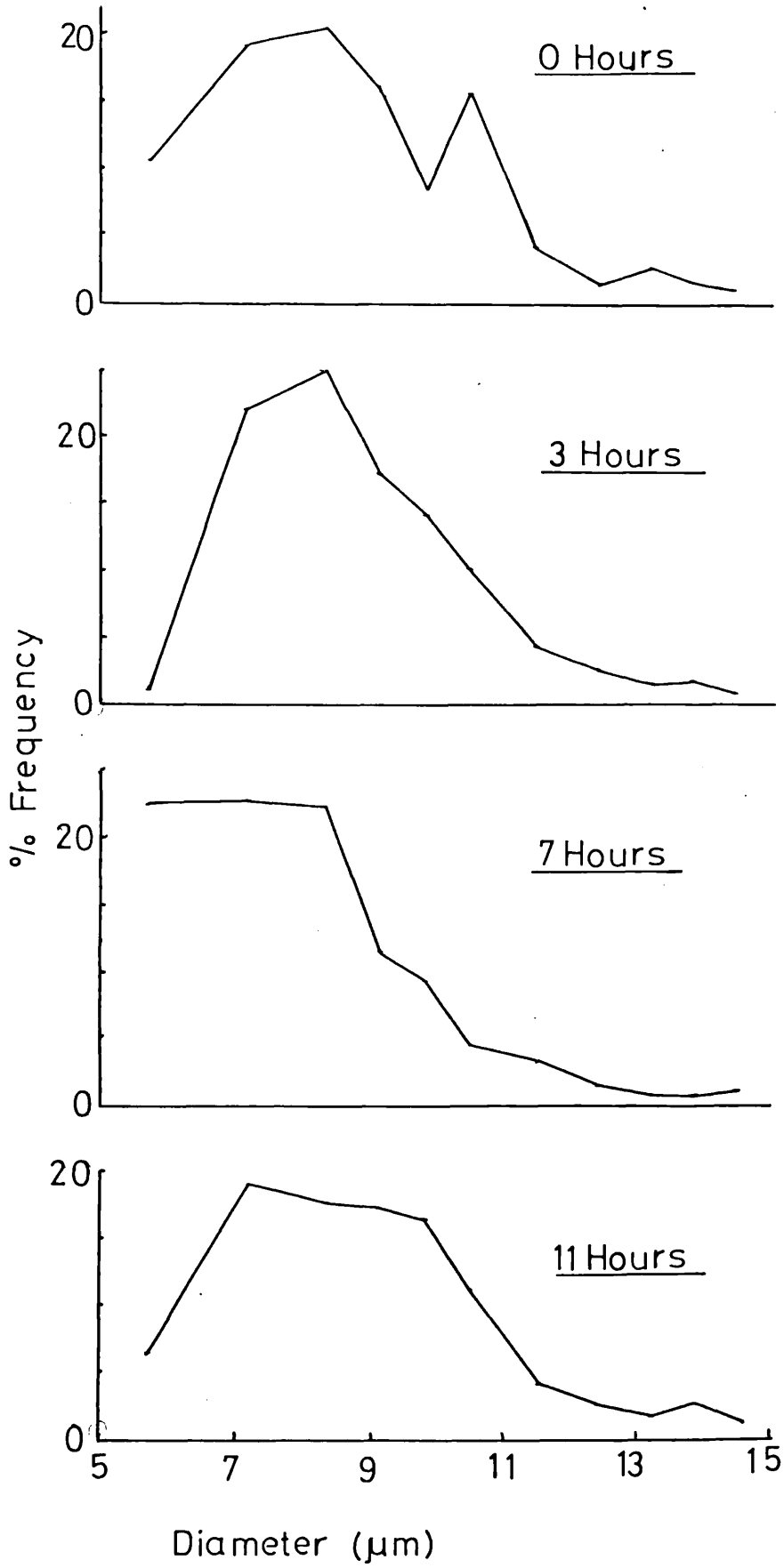


Fig 59 Size distribution of C. adiposa conidia after 5 hours incubation

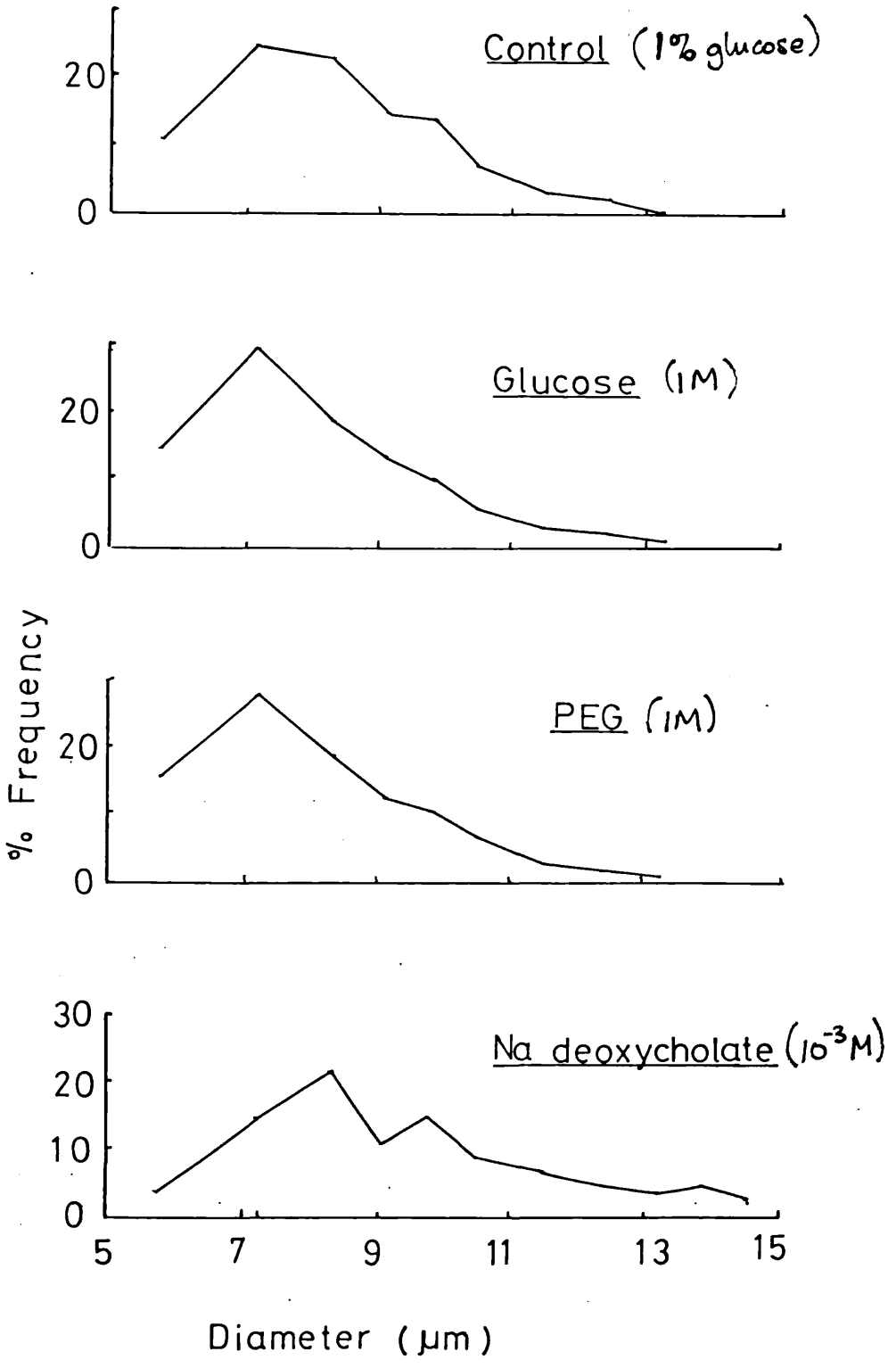
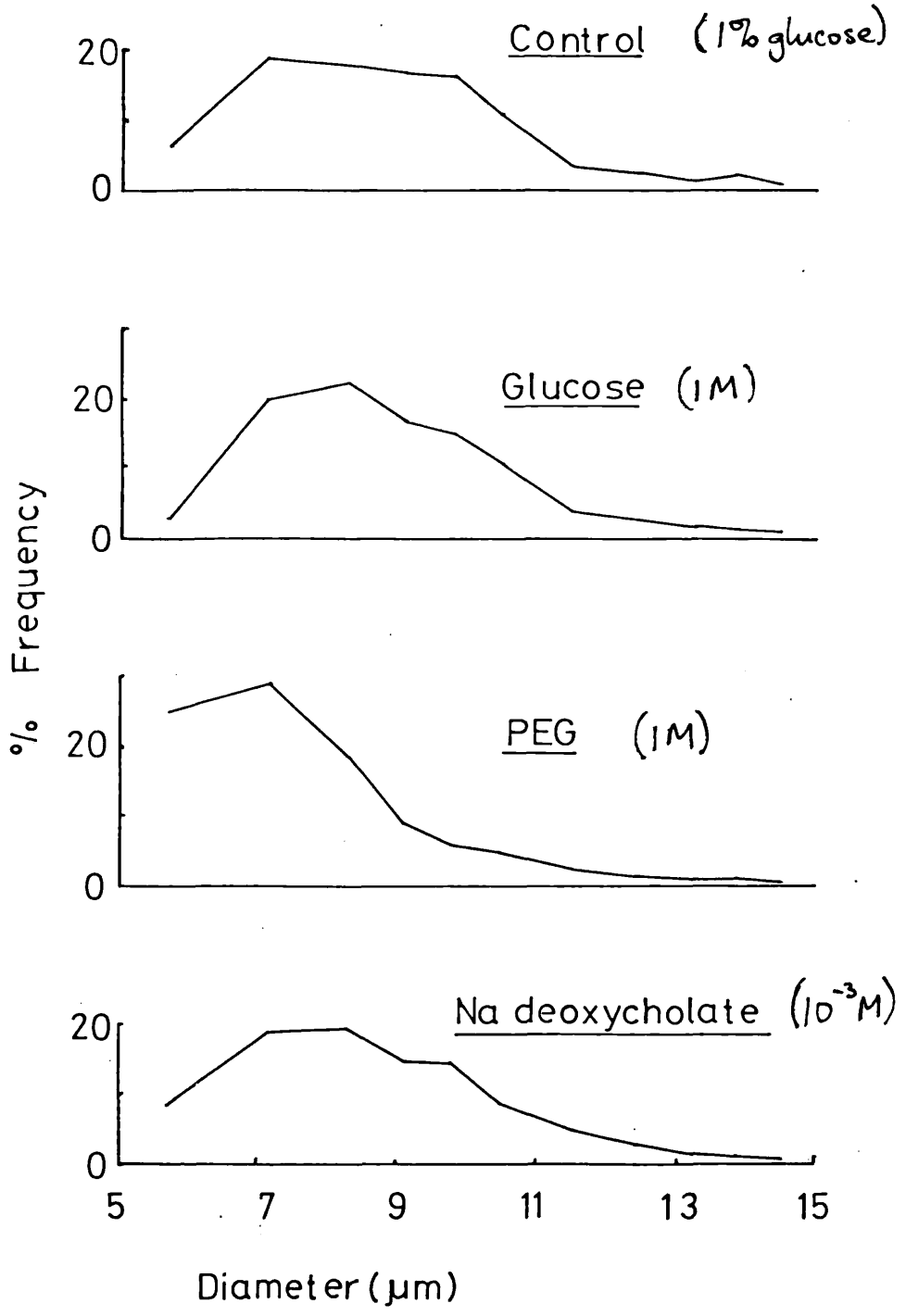


Fig 60 Size distribution of C. adiposa conidia after 10 hours incubation



neither the spores in 1M Glucose or Peg had germinated, the ones in Glucose had begun to swell while those in Peg continued to shrink.

TABLE 25 .

To show reversibility of inhibition of conidial germination by 10^{-3} M sodium deoxycholate and 1.0M PEG 200.

Time in treatment.	Concentration of spores on plate	2% Malt.			SSM		
		2days	3days	5days	2days	3days	5days.
1 day Na De	$3x10^3$	g	m	m	g	m	m
Peg	$3x10^3$	g g	m m	m m	g g	m m	m m
2 days Na De	$3x10^4$	-	1	2	-	2	2
	$3x10^3$	-	-	-	-	-	-
PEG	$3x10^4$	g g	m m	m m	g g	m m	m m
	$3x10^3$	g g	m m	m m	g g	m m	m m

g = few spores germinated. g g = many spores germinated.

m = thin mycelium. m m = dense mycelium.

As shown in Table 25 , the spores germinated after only 1 day's treatment when transferred to 2% Malt agar or SSM. Therefore, after one day the effect of Peg or Na De was reversible. After 2 days, the spores in Na De failed to germinate. The action of Peg was still reversible after 2 days.

The concentrated medium (1% Glucose + Na De or Peg) when tested with ninhydrin gave a red colour from Na De and coagulated. There was no reaction from the Peg medium. This indicated the

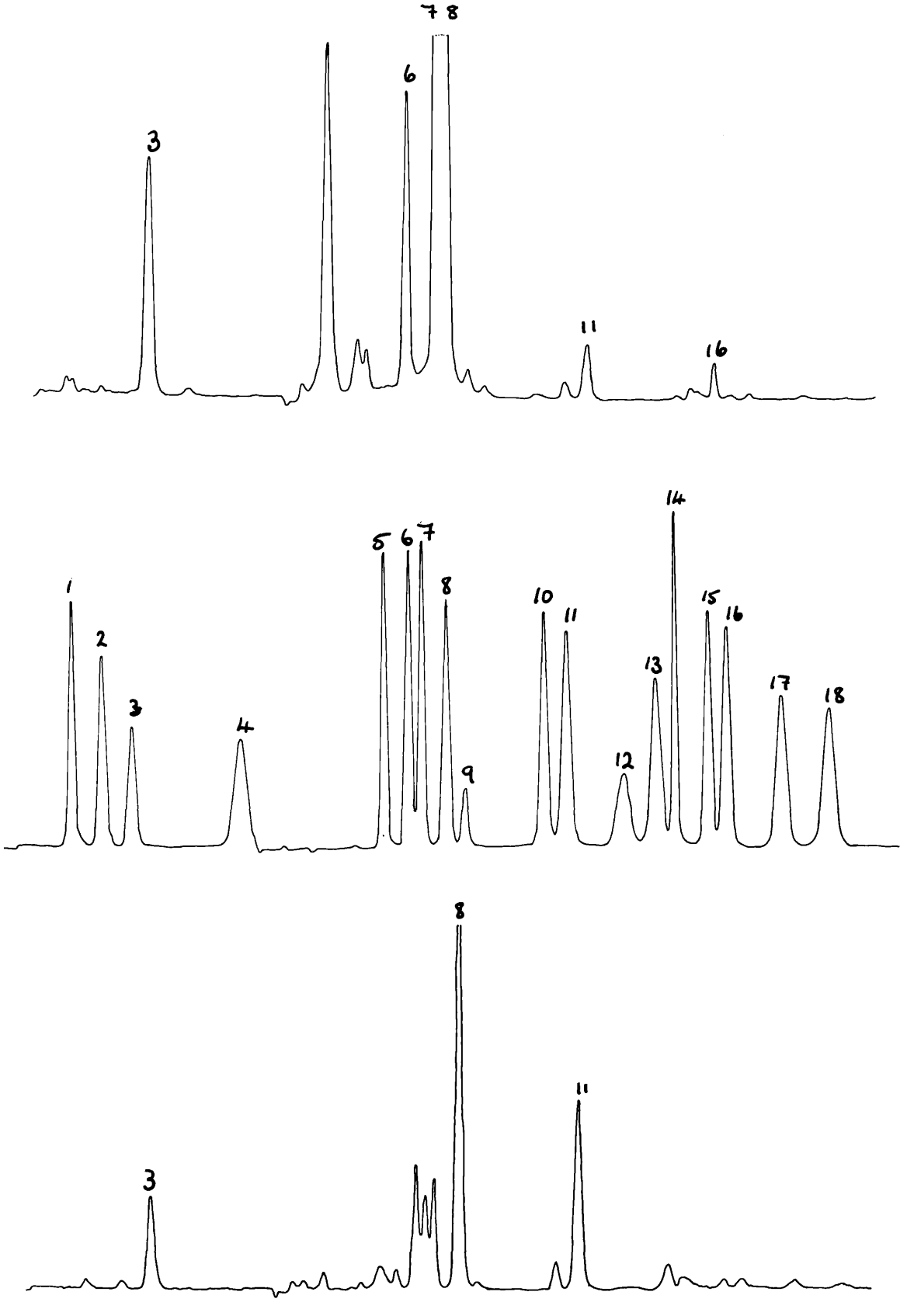
TABLE 26.

Amino acids from Conidia of Ceratocystis adiposa obtained in sodium deoxycholate medium and alcohol.

Standards	Na De	Alcohol
1. Lysine	✓	✓
2. Histidine	✓	✓
3. NH_3	✓	✓
4. Argenine	–	–
5. Aspartic Acid	✓	✓
6. Threonine	✓	✓
7. Serine	✓	✓
8. Glutamic acid	✓	✓
9. Proline	✓	✓
10. Glycine	✓	✓
11. Alanine	✓	✓
12. Cysteine	–	–
13. Valine	–	✓
14. Methionine	–	✓
15. Isoluecine	✓	✓
16. Leucine	✓	✓
17. Tyrosine	–	✓
18. Phenylalanine	–	✓

Fig. 61

Amino acid analysis of spore extracts by
(a) Sodium deoxycholate (b) Standards (c) alcohol.



loss of amino acids from sodium deoxycholate treated spores so the medium was analyzed for individual amino acids and compared with the alcoholic extraction.

E.M. study of spore inhibition.

Two spores from control medium which had not yet formed germ tubes are shown in Plate 62 . A nucleus (possibly 2) and many mitochondria may be seen in the sections. These spores would have germinated if they had been left in the medium.

The spores from glucose, peg and Na De media would have taken much longer to germinate, if at all. These spores (Plates 63 - 65) showed many large, densely-stained bodies containing phospho lipid and/or unsaturated fats (stained with glutaraldehyde and also with Sudan IV). In the polyethylene glycol treated sample (see Plate 64), the spore which had formed a germ tube contained only 2 such bodies and another germinating spore did not contain them.

Fig. 62

Sections of two conidia of Ceratocystis adipose from standard synthetic medium.

x 28000 glutaraldehyde/osmium fixed.

Fig. 63

An ungerminated conidium of Ceratocystis adiposa from glucose medium.

x 14000 glutaraldehyde/osmium fixed.

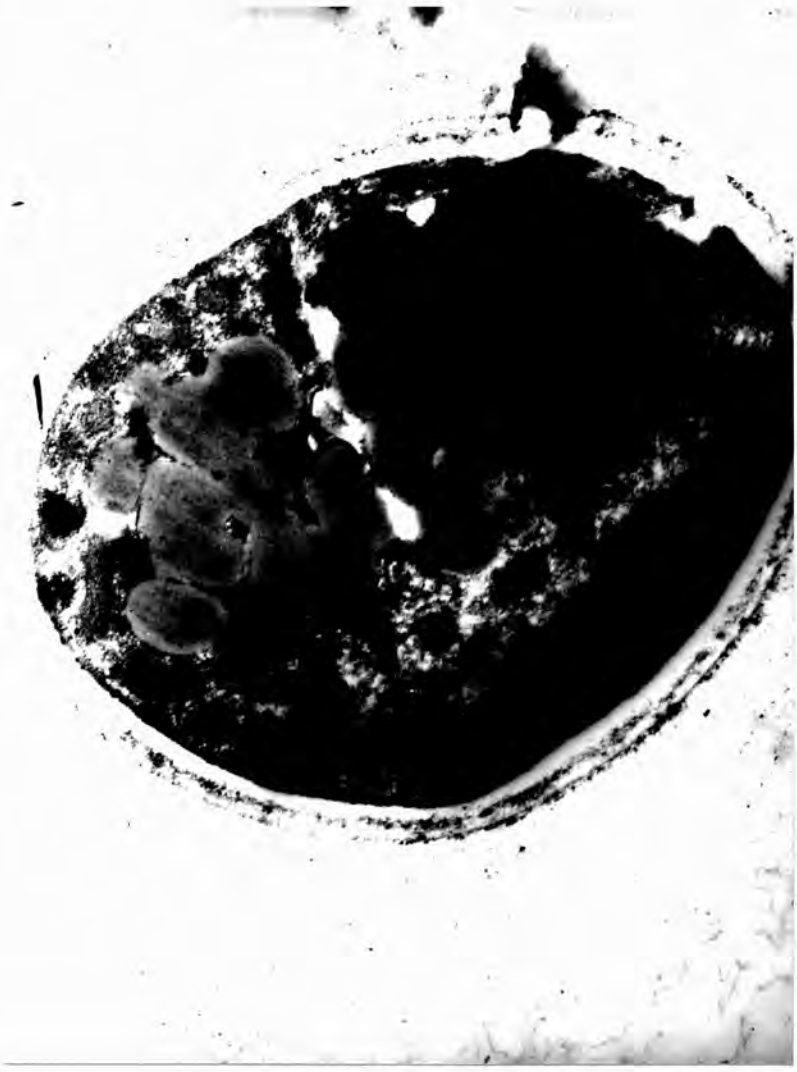


Fig. 64

Conidia of Ceratocystis adiposa from polyethylene glycol 200 medium.

x 4500 glutaraldehyde/osmium fixed

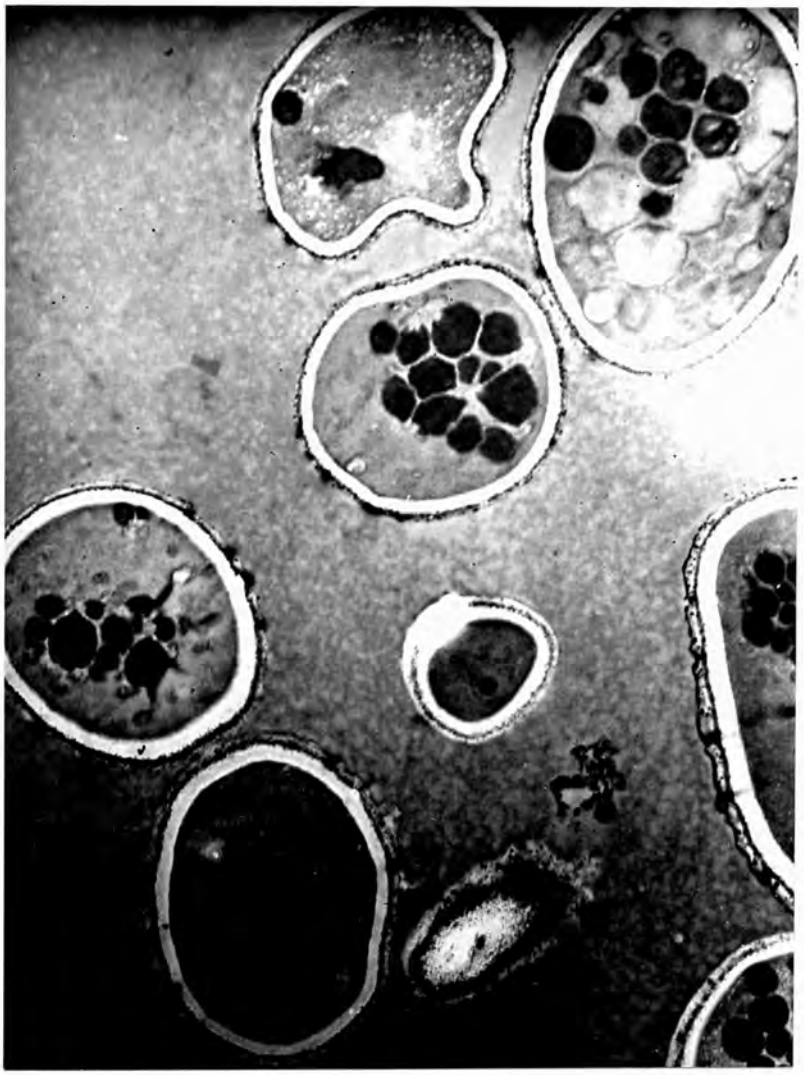
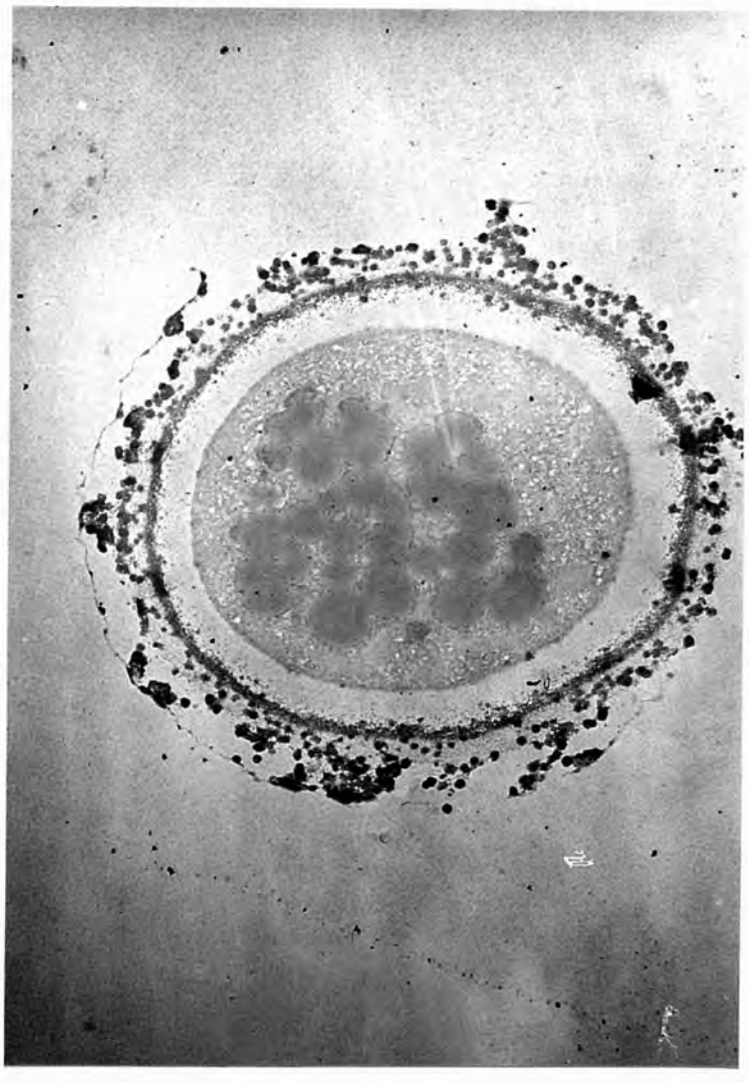


Fig. 65

An ungerminated conidium of Ceratocystis adiposa from sodium deoxycholate medium.

x 10600 glutaraldehyde/osmium fixed.



The spore wall is much thicker than the hyphal, being about 700 nm thick (Fig 67). In the mature wall, there is a dark layer in the middle and dark patches in the outer layer. The inner layer remains less darkly stained. At the junction between two spores is a membrane which holds the spores together (Fig 66).

The cross wall between developing spores (Fig 68) appears to be contructed like a septum. The outer wall consists of four layers, the inner two of which continue into the septum. At the centre of the septum is a large dark body blocking the pore.

When the spore germinates, the outer wall breaks and does not form part of the germ tube wall (Fig 69).

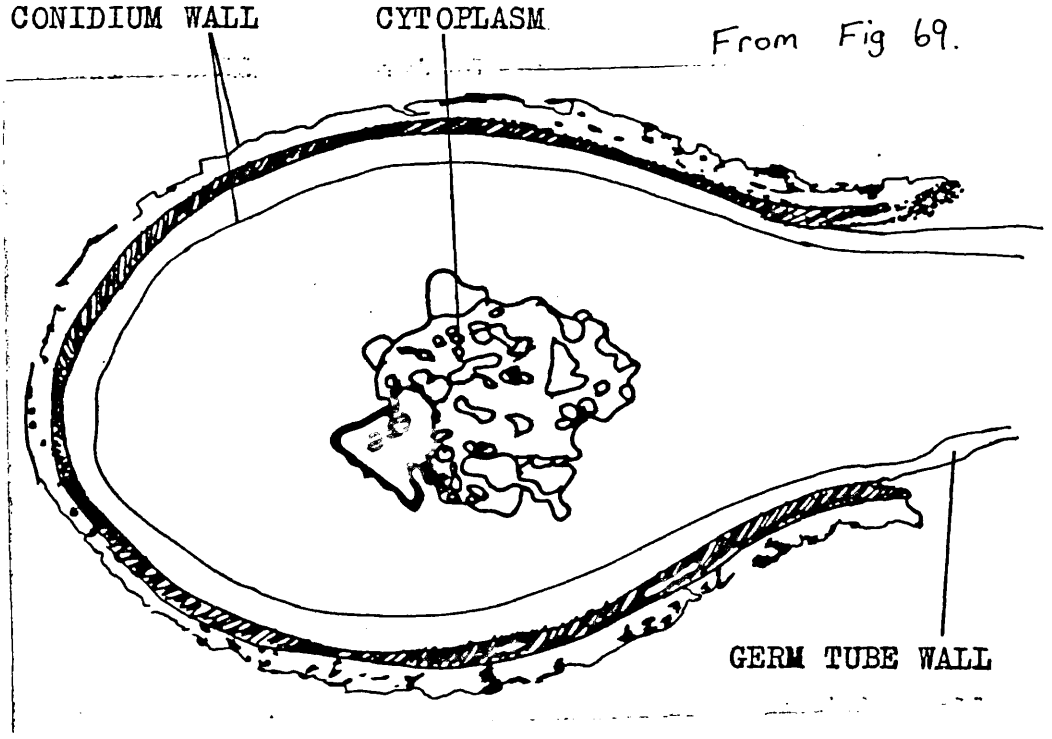


Fig. 66

Chain of three conidia of Ceratocystis adiposa
x 4250 glutaraldehyde/osmium fixed.

Fig. 67

Longitudinal section through a conidium of Ceratocystis adiposa

x 14000 glutaraldehyde/osmium fixed.

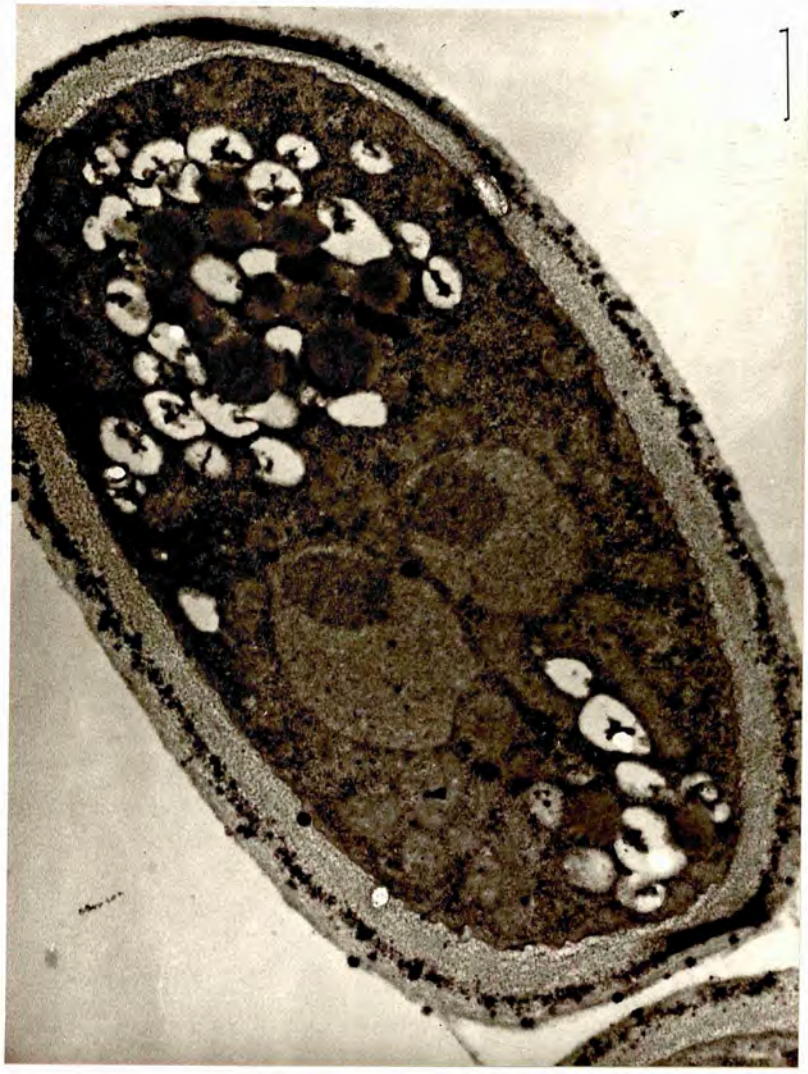


Fig. 68

Longitudinal section through the septum between two conidia of Ceratocystis adiposa.

x 32500 glutaraldehyde/osmium fixed.

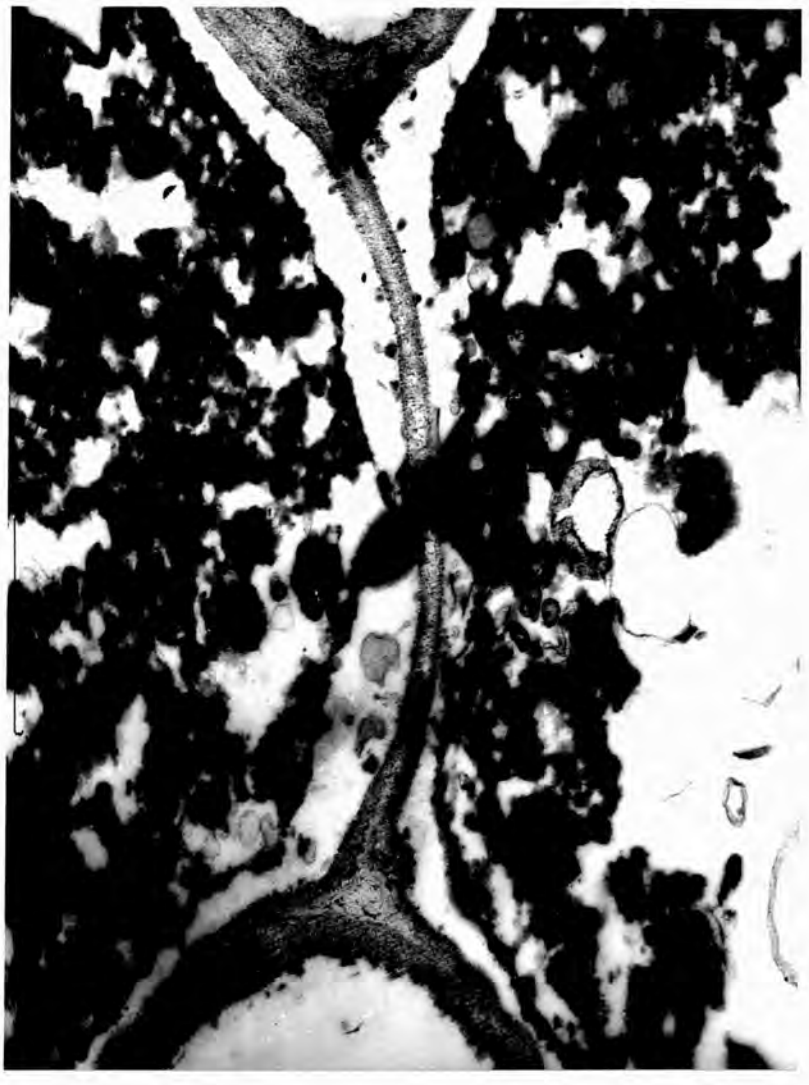
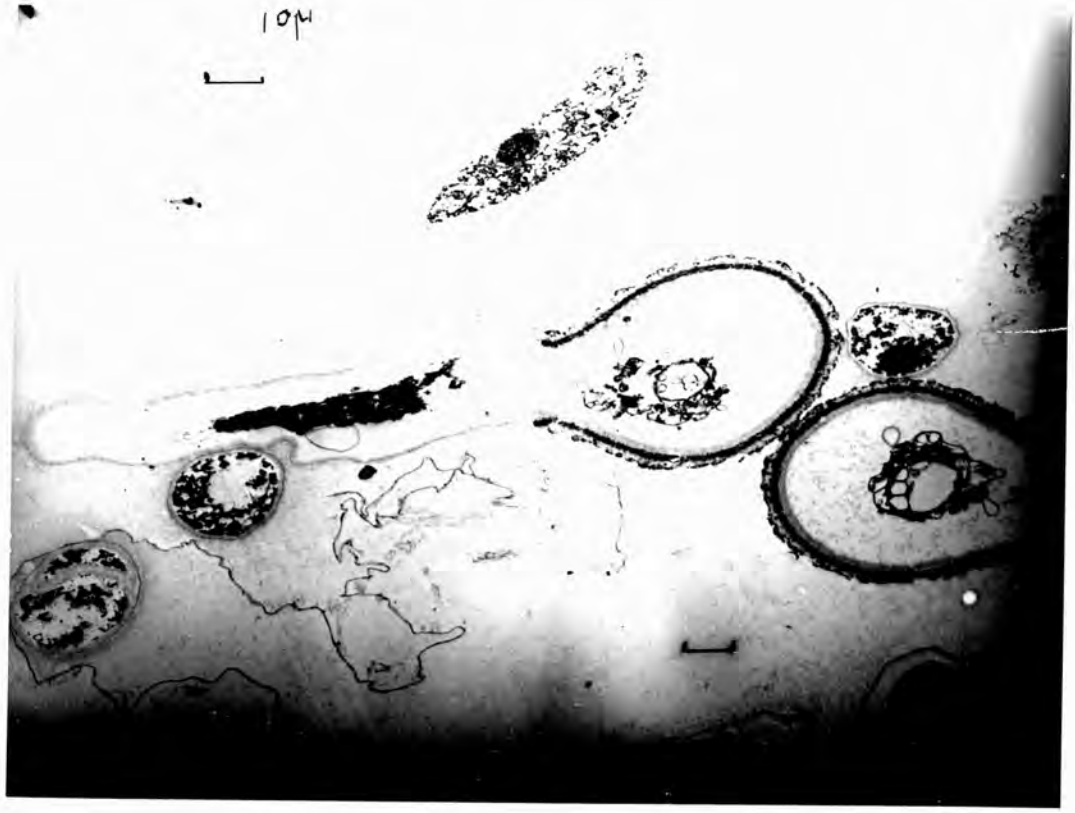


Fig. 69

Section through a germinating conidium of Ceratocystis adiposa.

x 800 glutaraldehyde/osmium fixed.



The most obvious effect of paramorphogenetic substances is the alteration they induce in growth rates of surface colonies grown on semi-solid media leading to large and obvious differences in colony size between control and experimental treatments. The measure of colony diameters and the estimation of radial growth rates, Kr, was chosen as the initial method of assessing paramorphogenetic effects.

It was found that replication of growth rate values within an experiment was good but between experiments replication was not good and values for Kr or SSM at different times were significantly different (Appendix Table 31). (Ryan et al 1943).

The variation in growth rate may have been due to small variations in temperature caused by opening an incubator during an experiment or to a lack of sensitivity in the thermostatic control of the incubator. Brancato & Golding (1953) pointed out that temperature and medium concentration require careful control when radial growth rates are being investigated.

Ceratocystis spp. have been reported to be sensitive to fluctuations in temperature, the radial growth rate being reduced in proportion to the amplitude of fluctuation. (Reynolds et al 1972). Ceratocystis adiposa grew best at 25°C (Kr = 17.5 mm/day on PDA). Growth was reduced from 17.5 mm/day at constant 25°C to 16.25 mm/day with a 3°C fluctuation over 24 hours, 13.75 mm/day with 6°C and 11.5 mm/day with 9°C . At a mean of 30°C, Kr was 12.5 mm/day at constant temperature and reduced to 5.0 mm/day with 9°C fluctuation.

The composition of different batches of media may have varied slightly which would also cause differences in Kr between

experiments. This is shown by the increased values of Kr by C. adiposa at low concentrations of glucose.

At 25°C, perithecia did not always produce mature spore drops and again this was probably attributable to variations in the environment.

Difficulty was encountered in regulating the temperature of orbital shakers to 25°C which caused differences in the rates of increase of dry matter from 6.4 mgm/hr to 2.6 mgm/hr for growth in SSM. Where experiments had to be done in two batches, a control was included in each.

Radial growth rate could be estimated by measuring the rate of growth of many individual hyphae but their growth rates varied depending on whether they were primary, secondary or tertiary hyphae. (Butler, 1961; Steele & Trinci, 1975)b. The extension rate of an individual hypha depended on many factors :-

Diameter (Butler 1961)

Hyphal growth unit (Morrison & Righelato 1974,) (Steele & Trinci 1975 (b).)

Peripheral growth zone (Trinci 1971 (a)).

Extension zone (Steele & Trinci 1975(a)).

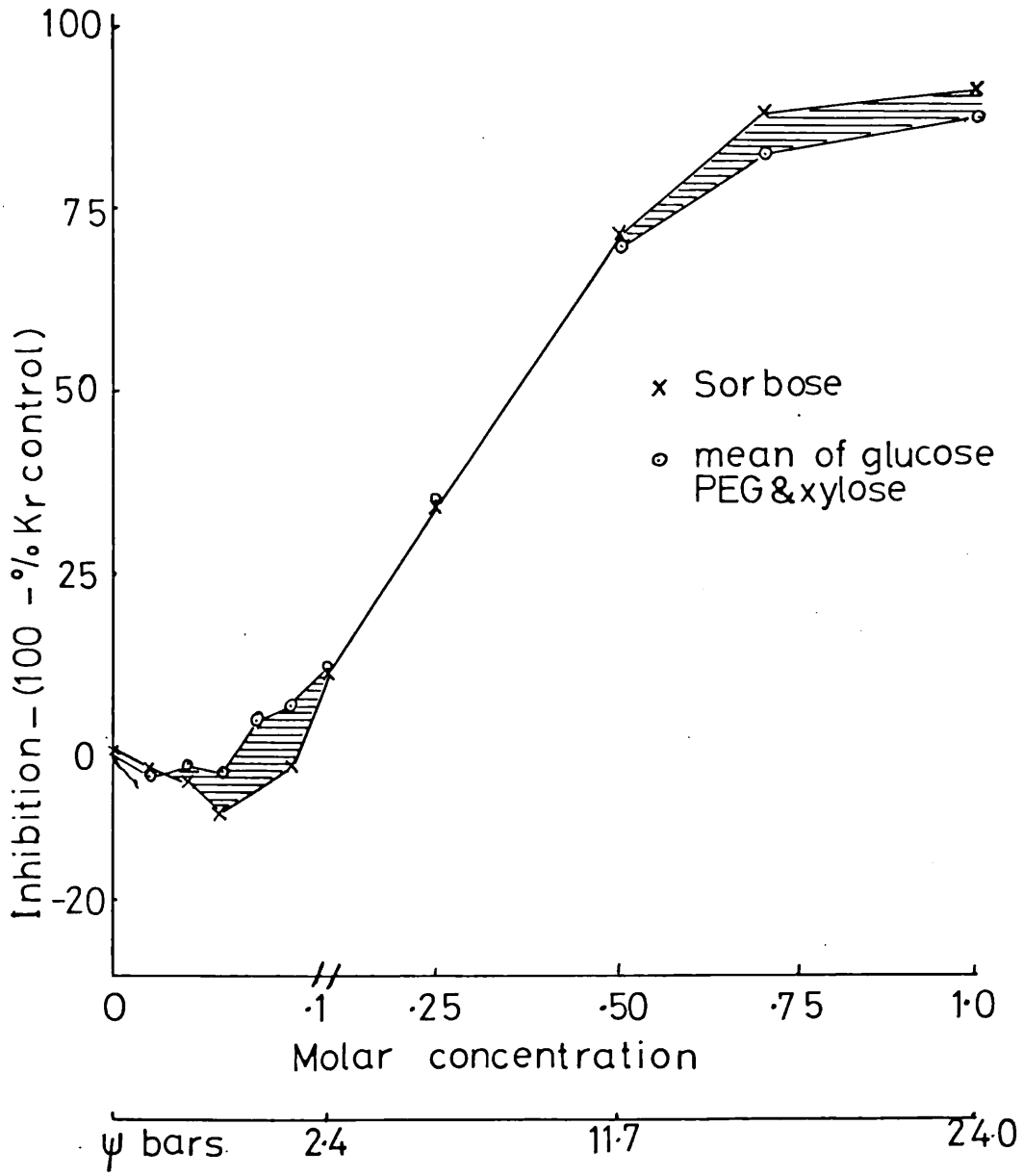
Rate of division of apical cells (Brancato & Golding 1953).

It was easier and required less replication to obtain consistent results to use colony diameter rather than individual hyphal growth rates to measure Kr.

Sorbose caused a reduction in Kr at 0.04 M and the values remained less than those for Kr on glucose, xylose and polyethylene glycol up to 0.1 M (Figs. 9 and 70). This indicated that sorbose caused inhibition of Ceratocystis adiposa above that due to increased water potential and is shown by the shaded area in Fig. 70 . Between 0.1 M and 0.5 M, there was no difference between the inhibition caused by sorbose and the

Fig 70

To show the % inhibition of Kr caused by sorbose and low water potential ψ



other paramorphogens indicating that the effect of water potential had masked that of sorbose. Above 0.5 M, sorbose caused less reduction than that caused by water potential. Fig. 70 .

Botrytis fabae was very sensitive to low concentrations of sorbose (.01M) but was less sensitive than C. adiposa to glucose and polyethylene glycol. Fig. 12 .

Chaetomium aureum was sensitive to Sorbose and Na Deoxycholate, Sorbose was effective at < 1% S and Na De < 0.1%, the most effective concentrations being 0.7% and 0.006 % with pH adjusted to pH 6.5 (Ghora 1973).

Sorbose and 3.0 Me Glucose increased branching to give short distorted compartments and decreased Kr at 0.44 m mol and 5.24 m mol respectively. Dry wt. production was little effected and sorbose was metabolized to CO_2.

The sorbose reaction said to be mediated through CAMP in Dendryphiella salina. 3.0MG directs carbohydrate metabolism towards polysaccharide and away from sugar alcohols. Glycogen breakdown was inhibited. (Galpin et al 1977).

Glucose was readily metabolized and caused a more sustained increase in Kr with concentration. At very high concentrations (>0.75M) there was a "tailing off" of effect with C. adiposa but not B. fabae.

At concentrations from 0.05M to 1.0M, the logarithmic plot for Kr against concentration of xylose is almost straight for C. adiposa. Fig. 9 . As this sugar was not metabolized, its effect may be considered to be solely osmotic. This indicated that the relation of Kr to water potential for C. adiposa may be logarithmic as water potential is directly proportional to concentration of sugars. Fig. 71 .

C. adiposa did not grow at concentrations of polyethylene

3-O-MG . 3-O-methyl glucose

glycol 200 above 0.5M, the extrapolated line cut the axis at 0.7M. However, on xylose, growth occurred at 1.0M maybe because it was slowly metabolized in the presence of glucose. Polyethylene glycol 200 may have been slightly toxic as it reduced Kr of Aspergillus chevalieri at all concentrations used (0.5M - 2.0M) and reduced α_s of C. adiposa. If polyethylene glycol is toxic, then xylose would be the better non-metabolized substrate to use for controlling water potential of the medium.

The optimum glucose concentration for Aspergillus chevalieri, if maximum Kr is taken as the criterion for determining the optimum substrate concentration, was 2.5M glucose. Kr was stimulated by concentrations up to 2.5M (45%) and inhibited at higher concentrations.

A similar phenomenon was observed in Fusarium roseum on media supplemented with salts or sucrose (Cook et al 1972). However, polyethylene glycol reduced the growth rate of A. chevalieri at concentrations between 0.5M to 2.0M.

Stimulation of growth in C. adiposa by low concentrations of all paramorphogens indicated that SSM may have a higher water potential than the optimum for growth and with glucose, there may have been an effect due to increased nutrient content of the medium.

Trinci (1969) found that Kr of Aspergillus nidulans on solid media was a reliable indicator of changes in α_s induced by temperature but not when the glucose concentration was varied. Sterne & McCarver (1978) reported that Kr was not a reliable indicator of α_s for three soil fungi on different osmotically adjusted media. The values of Kr/α_s for Rhizoctonia solani were 11 - 25, Pythuim ultimum 39 - 47, and Verticillium dahliae 10 - 16. The ratios were not directly related to osmotic potential. Kr was useful as an indicator of the range of

osmotic potential values which would permit growth of an organism.

The influence of water potential on the growth of zerophilic fungi was investigated by Pitt & Hocking (1977) who found that solute type influenced growth but pH less so. Sodium chloride inhibited growth but glycerol and glucose/fructose did not.

Mycelial growth of Whetzelinia (Sclerotinia) sclerotiorum was stimulated as water potential was decreased from -1 to -14 bars but was reduced at lower water potentials. Kr was reduced to 50% at -37 to -47 bars. The different osmotica used, KCl sucrose and a salt mixture produced similar effects at comparable water potentials. (Grogan & Abawi 1975). These results differed from those of Pitt & Hocking (1977) who reported that the solutes used produced different effects at comparable water potentials.

The relationship between concentration and water potential is shown in Fig. 71 . For glucose, the linear relationship may be expressed

ψ (-bars) = 43.75 mM concentration glucose,

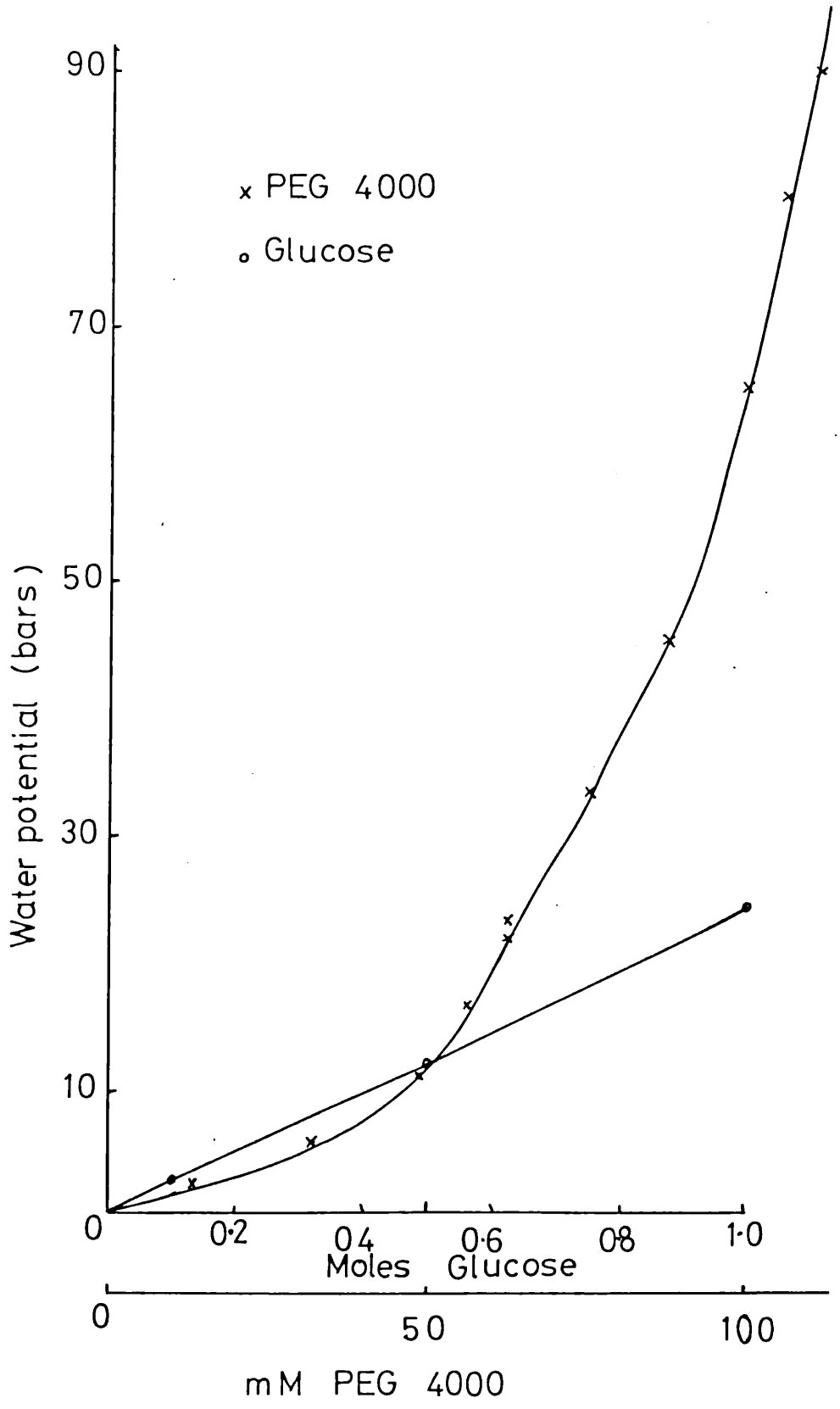
while for PEG 4000 the relationship is logarithmic.

log ψ (-bars) = 24.62x - 5.73 x = % concentration.

log ψ (-bars) = 61.55x - 14.32 x = mM "

The water potential of solutions of glucose and the other small molecules is exerted by the osmotic potential. For PEG 4000 there is a matric potential acting as well as the osmotic potential so the relationship between water potential and concentration is not linear. The relationship between water potential and relative humidity is shown in Fig. 4 . It should be noted that a water potential of -100 bars represents a

Fig 71 Relationship between concentration and water potential



relative humidity above 90%, -70 bars ≡ RH 95% and -14 bars ≡ 99%. (Leyton 1975).

Water potential exerted a considerable influence on Kr. Fig. 9 . Growth of C. adiposa ceased between 0.5 and 1.0M PEG 200 (-15 to -24 bars) but at 0.025M PEG 4000 (-4 bars). However, on sugar growth took place at 1.0M (-24 bars). Ceratocystis montia ceased growth at -20 bars induced by PEG 4000 which is equivalent to ⩾ 98% relative humidity. (Kidd et al 1977). It would appear that C. adiposa was less able to tolerate low water potentials when they had a matric component than when they were solely osmotic potentials.

Adebayo & Harris (1971) found that growth was inhibited at matric water potentials which were only $\frac{1}{2}$ - $\frac{1}{3}$ that of limiting osmotic potentials. If an organism was subjected to matric water potentials or an osmotic potential induced by a solute which could not be accumulated by cells of the organism, the cells were not able to come to osmotic equilibrium.

The morphology of Aspergillus wentii altered little above -61.2 bars (Adebayo et al 1971). A. niger showed optimum growth at 93% RH and grew over a range of 76 - 100% RH. Hunter et al (1975) reported a 50% reduction in growth by -30 bars induced by salts in Endothia while Verticillium was reduced 50% by -49 bars and completely by -119 bars. (Congley & Hall 1976).

The growth limiting humidity of A. ochraceus depended on the solute used and pH of the media, e.g. with Na Cl it is 0.84 - 0.81 with glucose/fructose 0.79, glycerol 0.80 and for Wallemia sebi, values are 0.81 - 0.75, 0.69 - 0.71 and 0.80 - 0.70. All these values are equivalent to water potentials of less than -100 bars. (Pitt & Hocking 1977).

Hyphae of C. adiposa grown on semi-solid media with low

water potentials appeared meandering and distorted (Fig. 18+19). Fomes fomentarius grown at -20 bars showed meandering hyphae which became shorter, bulged and produced a thicker cell wall. Cells vacuolated and resembled old forms of mycelium sooner (Vincent 1969). These observations were similar to those reported for Neurospora crassa grown on sorbose. (Trinci & Collinge 1973 (a)).

Hyphal tips have a lower water potential than their surroundings and hyphal extension is dependent on this difference. (Park & Robinson 1966). Tips equilibrate to applied hypotonic or hypertonic solutions to re-establish the pressure difference to one consistent with the rate of wall synthesis at the apex. When a hypertonic solution is applied, the apical cells lose turgor and cease to extend although wall synthesis continues thus occluding the apex. Growth may proceed by sub-apical branching when turgor is re-established.

This effect of low water potential may explain the meandering appearance of C. adiposa hyphae when grown on concentrated media. The hyphal growth rate, Kr, was reduced because the fungus was unable to generate a great enough pressure difference between its apical cells and the medium to push the apex forward at the normal rate. However, if cell wall synthesis continued at more or less the same rate as in normal hyphae, the apex became occluded. Eventually, a great enough pressure, due to the increased volume of cytoplasm, developed and growth continued either from a true sub-apical branch or from an area to one side of the apical dome. If this is true, the hyphal extension would be pulsed and each pulse of new growth would take place to one side of the apical dome, i.e., asymmetrical wall synthesis at the tip. This mode of growth would probably lead to swollen, meandering hyphae.

A. chevalieri showed an increase in the lag before growth commenced on solid media with decreased water potential. The inoculum was a mixture of hyphal fragments and spores. This lag may have been to allow the hyphal cells to regain turgor lost when transferred to the concentrated medium by accumulating or synthesizing solutes. It is also likely that the lag phase of spore germination was extended by low water potentials.

At 5.0M glucose, the lag was 100 hours to allow for hyphal repair and spore germination. Snow (1949) reported latent periods of up to 2 years for A. glaucus spores to germinate at 65 - 75% RH so absorption of water at these low humidities was very slow. The induction of permease systems for the uptake of solutes from the medium would not take more than a few days, e.g., sorbose permease (Klingmuller 1967)4 Glucose may have been metabolized to polyols to keep the osmolarity of the cytoplasm at a sufficient level to maintain turgor and allow growth.

As well as concentration, the molecular weight of PEG also had a significant effect on C. adiposa Kr. (Fig. 28). Kr was reduced more by equimolar concentrations of high molecular weight polymers than by low molecular weight polymers. (Fig. 28) This may have been a reflection of the ease with which the molecules enter the cell and so caused the cell to maintain turgor. PEG 200 had been reported to enter root systems of plants (Lawlor 1970) and roots had a low permeability to PEG $>$ 1000. Some mycorrhizal fungi absorbed PEG 4000 but it was not metabolized. (Mexal & Reid 1973). Rapid necrosis and dessication were caused by very high molecular weight PEG (20000) in plants and this may have been due to the transpiration pathway and water movement being blocked.

Low molecular weight PEG may have been absorbed by C. adiposa to provide an osmotic equilibrium with the medium although if

PEG 200 was slightly toxic, it may have further reduced Kr and αs. High molecular weight PEG were probably absorbed slower than PEG 200 if at all, so for the mycelium to maintain turgor, it must have absorbed more nutrients from the medium which were not then available for growth.

Anand & Brown (1968) reported that increasing weights of PEG caused a reduction in growth rate and this may have been due to physico-chemical properties of molecular weight other than osmotic potential, e.g. matric potential. The solubility of oxygen in solutions of PEG decreases with increasing concentration, e.g. only 25% of the oxygen is present in a PEG 4000 solution of -20 bars compared to normal liquid media. (Mexal & Reid 1973).

High sugar concentrations maintained a mycelium in a fermentative condition which showed in both Krebs and glyoxalate cycles (Turian 1973) and in repression of mitochondrial structures (Brown & Johnson 1970). An increase in glucose concentration from 2 - 10 g/l induced a decrease in oxidative degradation of glucose by yeast and a possible decrease in mitochondrial lipid. (Brown & Johnson 1971). Neurospora crassa grew as single cells in 3.22M glycerol media and were disrupted by an abrupt decrease in osmotic pressure of the medium.

Mitochondrial fractions of these cells have a low cytochrome content. (Bates & Wilson 1974). This may be related to the findings of Wilson & Griffin (1975) who studied soil fungi at low water potentials. Kr and respiration were decreased and more oxygen was needed to produce unit growth. Two possible reasons are that either more energy was required for osmoregulation or at low water potentials, respiration became partly uncoupled from energy production.

The effect of low water potential on growth is far from

simple. It is dependent on whether the potential is osmotic or matric, whether growth is prevented and occlusion of the apex occurs. Lower growth rates may be induced by interference with the respiration pathways of the organism or by oxygen starvation due to decreased solubility in low water potential solutions.

The differentiation of vegetative mycelium to form fruit bodies was also influenced by water potential. A. glaucus produced cleistothecia at -450 bars and conidial production was enhanced down to - 150 bars (Curran 1971). Abnormal fruit bodies were produced by Penicillium baarnense on media in which the water potential was reduced with diethylene glycol (Bouvier 1973). Normal fruiting was restored when the water potential was increased by diluting the medium.

Perithecia were not formed by Neurospora tetrasperma on medium containing sucrose but if sucrose was replaced by xylitol or ribitol, then perithecial production was stimulated up to 0.5M. However, PEG 400, sodium chloride or increased sucrose concentration had no effect on fruiting. (Vizwanath-Reddy & Turian 1975). It is probable that the sugar alcohols influence fruiting by being a slowly metabolizable carbon source rather than by increasing the osmotic pressure or carbon source of the medium.

C. adiposa ceased perithecial production at 30°C and it was decided to investigate whether any of the paramorphogens used in previous studies influenced the production of perithecia with temperature. Although sorbose was used more slowly than glucose as a carbon source, both sugars decreased perithecial production when their concentration was increased to 0.25M (4.5%) at 20°C and 25°C. The optimum temperature for perithecial production was 20°C.

From the experiments in which cultures were moved from 20°C to 30°C and vice versa, it appeared that a culture required

2 days at 20°C to initiate fruiting as areas of mycelium grown for less than 2 days at 20°C before being moved to 30°C produced no perithecia. (Fig.32). When plates were incubated first at 30°C, they produced more perithecia over the while plate when removed to 20°C than cultures incubated for the whole time at 20°C. This may be due to nutrient depletion as mycelial growth was more vigorous at 30°C than at 20°C. The peak of perithecial production on the 3rd day corresponded to the removal of the culture to a temperature which permitted fruiting.

After 3 days at 30°C, the culture on PEG medium showed no perithecia on the first two days growth of mycelium. This confirmed the observation that a culture required 2 days to initiate perithecial production or inhibit it.

Although the paramorphogens influenced the pattern of fruiting of C. adiposa, they did not induce fruiting at 30°C. PEG appeared to enhance the effect of temperature in totally inhibiting fruiting at the centre of cultures incubated at 30°C for 3 days whereas the other substances did not cause this marked response to temperature. Glucose and sorbose tended to reduce fruiting because of an increase in the carbon source of the medium. Sodium deoxycholate reduced the number of perithecia produced on cultures started at 30°C and removed to 20°C but did not totally inhibit perithecial production at the centre of these colonies.

In order to estimate Ks, substrate affinity, at various substrate concentrations using Kr, the following conditions must be fulfilled; that the width of the peripheral growth zone does not change when α_s changes with substrate concentration. (Pirt 1973). The hyphal growth unit increased as α_s decreased when substrate concentration was reduced. This would cause the peripheral growth zone to increase and hence account for the

increase in Kr at low substrate concentrations. Therefore Kr is not a good estimate of Ks.

When substrate affinity was determined using Kr, it was found that growth on sorbose did not follow Michaelis - Menten kinetics (Fig. 26). This was probably due to the inhibitory effect of sorbose on the growth of C. adiposa. The value of Ks for C. adiposa grown on glucose was 0.05 mM. This value is similar to that obtained by Schneider & Wiley (1971 (a)) for Neurospora transport system 11 which was between 0.01 mM and 0.04 mM. As substrate uptake by an organism must depend upon its transport processes, substrate affinity must depend to some extent on the affinity of the carriers of the transport process for that substrate.

Schneider & Wiley (1971) characterized two transport systems for the uptake of glucose by Neurospora crassa and that regulation of sugar uptake was by repression and de-repression of System 11.

In 1967, Crocken & Tatum reported that sorbose did not enter cells against a concentration gradient but that it reached an equilibrium with the external medium in 60 minutes in yeast and 9 hours in Neurospora, both at 30°C. The Michaelis - Menten kinetics for sorbose uptake were :-

Neurospora	Km	116 mM	Vmax	15 n mol	ml^{-1} min^{-1}
Yeast	Km	244 mM	Vmax	900 n mol	ml^{-1} min^{-1}

Sorbose was not metabolized by yeast cells but Neurospora converted it to glucose via sorbitol. When glucose was added to sorbose-equilibrated-cells, it caused an efflux of sorbose against a sorbose concentration gradient and a carrier was suggested as the possible mechanism.

Conidia of C. adiposa germinated after 3 hours in SSM. However, using sintered glass crucibles to obtain dry weight

measurements, it was not possible to detect any increase in dry weight for 12 hours. (Figs.72+73). When the dry weight was obtained at 18, 24, and 42 hours, the points fell in approximately a straight line rather than a curve. Therefore, the true logarithmic phase of growth occurred between 12 and 24 hours after inoculation.

As the conidia clumped together when they began to germinate, they formed pellets at a very early stage. Gull & Trinci 1971 reported that spores of Botrytis cinerea, soon after germination, showed mucilage covering the germ tube. Mucilage was also observed around hyphae and in the medium around C. adiposa. If the mucilage caused a decreased rate of diffusion of oxygen and nutrients, then staling conditions would occur sooner in the centre of pellets.

The theoretical value for $\alpha_s \sim 2.0\ hr^{-1}$ is greater than that for most fungi (Table 6). These two conditions, i.e. a very fast growth rate and many conidia clumped together, would lead to pellets which would soon exceed the diameter at which the whole of the mycelium was able to contribute to growth.

As the fungus grows quickly, a plot using the equation $y = \text{Log}_e \left(\frac{m}{1-m} \right)$ (after Van der Blank 1975) was drawn Fig.73 This would have allowed for the depleting nutrients as the mycelium grew but this plot still did not give a straight line between 12 - 24 hours.

The specific growth rate α_s of C. adiposa grown in SSM was always higher than when any paramorphogen was added. Glucose and sorbose did not cause a significant reduction but PEG and sodium deoxycholate reduced α_s significantly.

True sorbose paramorphs are characterized by a reduction in Kr while α_s remains constant, (Trinci 1973(a)) due to a variation in branch frequency. Although C. adiposa was not

Fig 72 Dry wt, pH & titratable acid from cultures of Ceratocystis adiposa

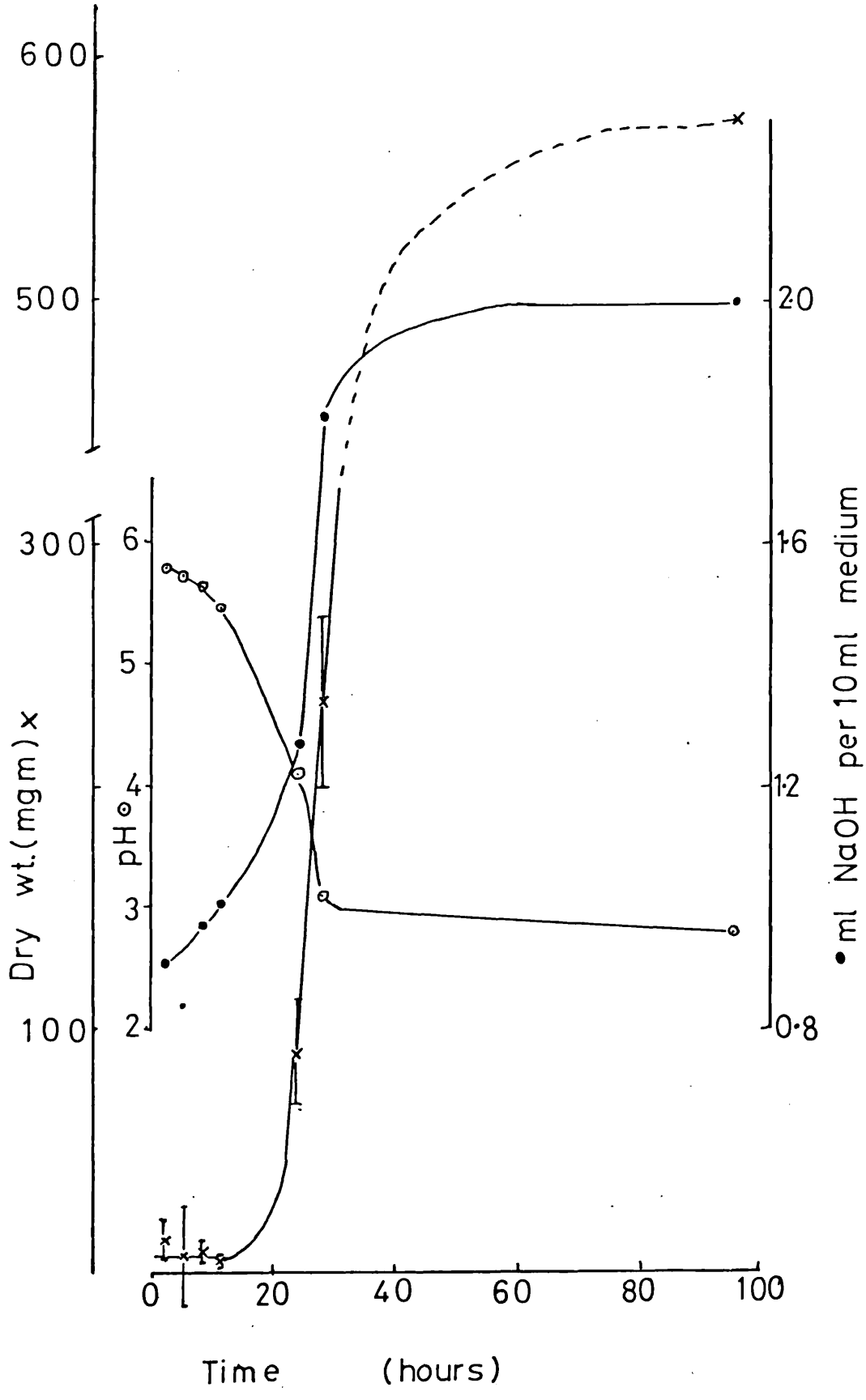
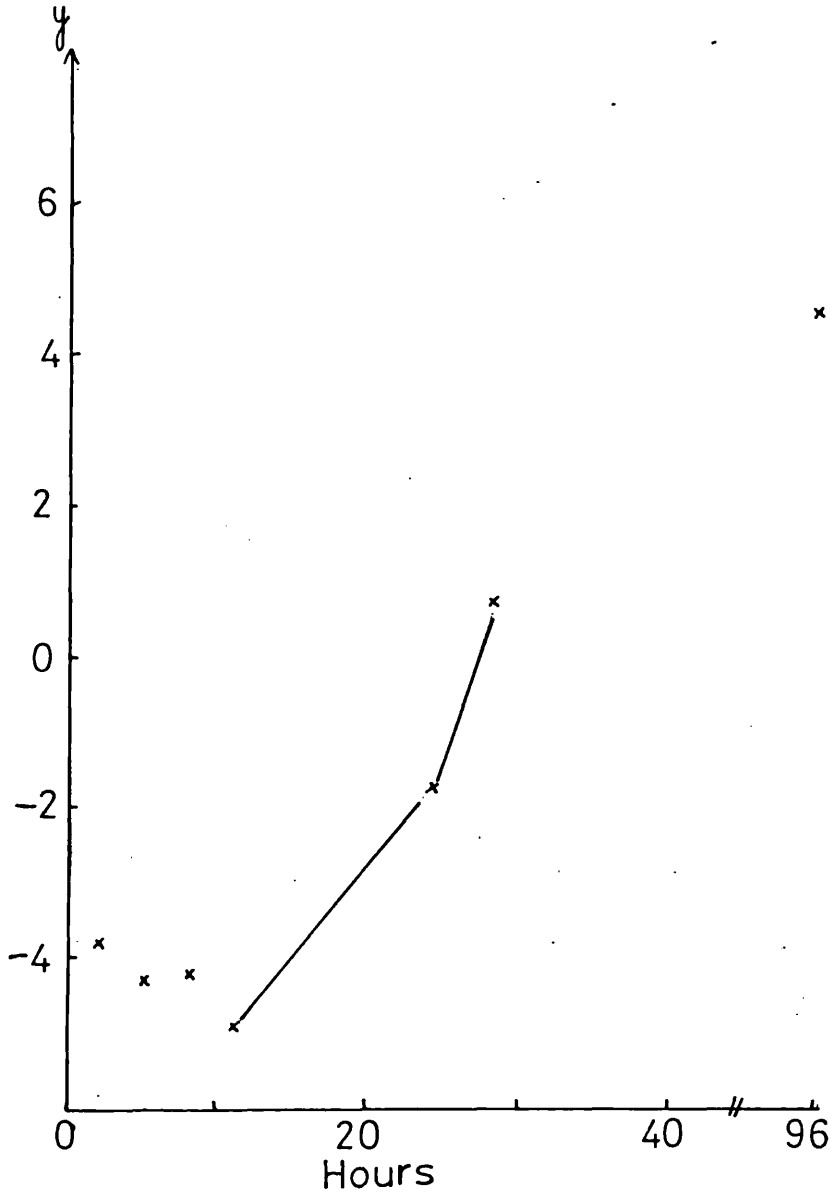


Fig 73
Increase in mycelial dry weight with time.



$$y = \log_e \left(\frac{m}{1-m}\right)$$

m = proportion of max. dry wt.

(van der Plank, 1975)

very sensitive to sorbose, the concentration used was probably having an osmotic effect (0.25M) as well as competitively inhibiting glucose uptake and metabolism. PEG and sodium deoxycholate may be toxic as they caused a significant reduction in α_s .

When sorbose was the carbon source in SSM, the value for was much lower than for glucose. However, if the experiment had been allowed to continue, the growth rate may have increased as Fig. 27d may not be a straight line but represent a long lag phase while the necessary enzymes for sorbose metabolism were induced before the true value of α_s was reached.

Sorbose agar appeared to support as much growth as other readily metabolised sugars but the colony had a longer lag phase. This may have been due to the induction of a sorbose permease system to allow the uptake and use of sorbose as a carbon source. (Klingmuller 1967(a)).

Opinions vary as to whether mycelial growth can be treated as logarithmic, cube root or arithmetic. Unless very many accurate results are taken, it is probably difficult to distinguish which is the best fit. On the results obtained in this study, the difference between the values of r for the three types of plot are shown in Table 15 and an arithmetic plot is the best fit in most cases.

If growth is pelleted, autocatalytic growth does not occur at the centre of the pellet as hyphae are starved of oxygen and nutrients (Pirt 1966). The size and number of pellets will effect the rate of growth and so, if germination was inhibited as in sodium deoxycholate, fewer colonies developed and αs would be reduced when compared with a control. This was because few large pellets will have less surface area for growth to take place than will many smaller pellets. However, α_s is independent of inoculum size and is a measure of td, doubling time, unlike an arithmetic plot of weight against time which is dependent on inoculum size and the percentage germination of the spore inoculum.

On the whole, the arithmetic plot gave the best straight line which indicated that the log phase of growth was not sampled. Growth occurred on the flask sides and was included in the dry weights although the conditions which prevail on the flask side were very different from those within the liquid medium.

To allow comparison of the results obtained in this study with those of other workers, values for αs and td were obtained from a logarithmic plot of the data. These values were also compared with theoretical values obtained from Kr, ω, (peripheral growth zone) and apical cell length measurements. (Table 27).

The values for td ranged from 6.35 hours to 7.06 hours when calculated from the dry weight determinations but the theoretical values for C. adiposa ranged from 14 - 26 minutes when calculated on Kr/ω or 19-56 minutes from apical cell length range/Kr. (Bainbridge 1976 and Trinci 1971(a)).

Although no increase in dry weight was detectable for 12 hours by weighing dried samples in sintered glass crucibles, the

TABLE 27

Comparison of experimental and theoretical values for Kr, αs, ω and td of *Ceratocystis adiposa* on various media at 25°C.

	α_s hr^{-1}	Kr $\mu m hr^{-1}$	ω Pgz μm	Kr/Pgz td	Doubling time td	HGU μm
Control						
theor	(a) 2.83			(b) 18 min	(c) 26 min	
expt.	.1029	396	140	6.74 hours		160.5
	.1059			6.55 hours		
Glucose						
theor	2.88			14 min	30 min	
expt	.1091	245	85	6.35 hours		88.2
Sorbose						
theor	3.01			14 min	19 min	
expt	.1089	244	81	6.36 hours		121.8
Poly-ethylene glycol						
theor	1.92			22 min	29 min	
expt	.1037	177	92	6.68 hours		172.2
Sodium deoxy-cholate						
theor	1.60			26 min	56 min	
expt	.0982	146	91	7.06 hours		114.6

(a) αs calculated from Kr/Pgz (Trinci 1971(a)).

(b) td calculated from ln 2/αs. (Trinci 1973(a)).

(c) Bainbridge (1976) (4 x Standard deviation of apical cell length).

pH of the medium fell indicating that components of the medium were being used by the germinating spores. (Fig 72).

As the peripheral growth zone is $\ll$ 140 μm, a pellet of diameter $>$ 280μm will have a region in the centre which is not contributing to growth. As the value of Kr is 396 μm/hr, this state of logarithmic growth may last for only a very short time ($\sim$ 30 mins).

Trinci (1973(a)) found that Kr measured in growth tubes was higher for some strains of N. crassa than that obtained for circular colonies and may be correlated with the fact that circular colonies have to generate new leading hyphae to maintain a constant hyphal density at the colony margin. It could be argued therefore that spherical pellets may show a lower radial growth rate than circular colonies. However, the mutant spco 4 formed dense hemispherical colonies embedded in the medium and when prevented from penetrating the medium by growing the colony on cellophane, the value of Kr was reduced. (Trinci 1973(a)). Kr was also found to increase with medium depth, therefore the radial growth rate of spco 4 depended on material synthesized by mycelium embedded in the medium. This situation would be analogous to pellets in liquid medium and so values for pellet Kr would have to be measured for the particular fungus and conditions being used. The value of Kr of surface colonies on semi-solid medium may not be a true measure of pellet Kr in liquid medium.

Branching was estimated by using the value of hyphal growth unit (HGU). (Trinci 1973(b)). This may be considered as the length of hypha associated with a hyphal tip. As the hypha extends, the length contributing to extension increases until the rate of synthesis is sufficient to support two hyphal tips growth and a branch is formed. (See Katz et al 1972). It was

shown that for the control and glucose treated colonies that HGU was slightly greater than ω while for the other treatments it is considerably greater. (Table 27). The values for ω/HGU are 87.2%, 96.4%, 66.5%, 53.5% and 79.4% respectively for control, glucose, sorbose, PEG and sodium deoxycholate treatments.

The value of HGU for C. adiposa was 160.5 μm and of ω was 140 μm . Therefore, once a hypha had exceeded 140 μm a branch was formed and logarithmic growth could continue by this process until the hyphal density at the centre of the pellet was too great to allow oxygen to diffuse to cells in the centre.

The values for the growth parameters of C. adiposa in SSM were Kr = 396 μm/hour, α_s = 0.8 experimental and 2.8 theoretical, ω = 140 μm, HGU 160 μm . When these values were compared with those obtained for other fungi, C. adiposa has a much greater theoretical α_s than any reported in Table 7 .

The value for Kr was similar to that for Absidia (379 μm hr^{-1}) and Mucor hiemalis (377 - 498 μm hr^{-1}) but the peripheral growth zone was very small, very few ω reported were less than 500 μm . HGU of 160 μm was greater than most, some mutants of N. crassa had larger values. (Table 7). C. adiposa is therefore characterized by a small volume of cytoplasm which contributes to growth (ω) but its rate of synthesis is very high and the hyphae branch comparatively infrequently.

Kr was decreased by sorbose and if the rate of synthesis remained the same (α_s not significantly reduced) then the products of synthesis must be directed into branching. In C. adiposa HGU and Kr were correlated (Fig. 21). This was also observed by Morrison & Righelato (1974). At high concentrations of paramorphogen, Kr was decreased and branch

frequency increased (HGU decreased).

However, the osmotolerant fungus A. chevalieri behaved differently. Branch frequency decreased at high concentrations of glucose and PEG. Kr decreased at concentrations above and below the optimum of 2.5M glucose. PEG was more inhibitory than glucose and may be toxic. Fig 25

Under adverse osmotic conditions, i.e., high solute concentration, C. adiposa and A. chevalieri showed different responses. This may reflect an adaptation of A. chevalieri metabolism to survive at low water potentials or more probably at high sugar concentrations.

It was suggested that branch frequency is an important determinant of the width of the peripheral growth zone of fungal colonies and hence Kr (Morrison & Righelato 1974). However, it is equally likely that branch frequency is determined by ω, α_s and Kr.

Where sorbose causes a reduction in Kr solely by its competitive inhibition of glucose metabolism of the cell wall, the values of α_s may not be much reduced. However, in the case of C. adiposa, higher concentrations (20x) were needed to obtain a reduction in Kr and therefore osmotic effects must have been involved as well. The explanation becomes more complicated because high osmotic pressure causes a reduction in yield, while sorbose can be utilized as a carbon source to increase the yield.

A soluble extra cellular polygalacturonic acid polymer appeared to be excreted by the cells of C. adiposa into the liquid medium. This may well have accumulated in the centres of the pellets and caused the very slow filtering in dry weight estimations and cell wall preparations.

When the cell wall was cleaned, the washing procedure removed this polysaccharide with the cytoplasm.

Reissig & Glasgow (1971) reported that a colonial mutant of *N. crassa* produced a mucopoly-saccharide which inhibited growth and caused hyphal agglutination.

The amount of cell wall in the mycelium was found to be reduced by glucose, sorbose and PEG, i.e., media with low water potentials. This could be because the cell wall was thinner or less dense. The thickness of the cell walls for control, glucose, sorbose, PEG and sodium deoxycholate treated cultures were 200 nm, 100 nm, 200 nm, 150 nm and 100 nm respectively. These measurements were made on photographs of LS which passed through a septal pore or TS at the thinnest part of the wall to allow for any obliqueness in the section.

These values only give an approximate measure of cell wall thickness as this varies with age because of secondary thickening. (Trinci & Collinge 1975).

Loss of material during analysis varied but has been reported by Edson (1972) to be 10% of material lost in the first step and 30% in the second step of *N. crassa* wall analysis. Korn & Northcote (1960) reported 98% recovery and Mahadevan & Tatum (1965) reported loss of serine and threonine during fractionation.

Some values for 24 hour wall were more than 100% recovery due to the over-estimation of Fraction 11. In 72 hour cell wall, the recovery was always less than 75%. It is only possible to compare each treatment within an experiment but not between them. Comparing the control cell wall from 24 and 72 hour cultures, the recovery of cell wall was 85% and 88% so within the same experiment it seems results are comparable. The main change with age is that Fraction 11 decreased and 1V increased. When the experiments were done separately, then values were different, e.g., 24 hour wall in two experiments.

Fraction 1	8.9%	& 5.9%	
11	33.6%	51.7%	* overestimate.
			method used.
111	29.8%	5.2%	
1V	27.7%	37.2%	

There is considerable variation in the chemical composition of ascomycete cell walls. Table 28 . Carbohydrate is the major component, accounting for between 56 - 83% of the cell. Protein may contribute up to 14% and lipid up to 17%.

Within the genus Ceratocystis there are two groups of species; those which contain cellulose and rhamnose and those which do not. (Rosinski & Campana 1964, Smith et al 1967, Jewell 1974). Ceratocystis adiposa was reported to contain glucose and mannose but not cellulose, rhamnose or galactose. The results of this investigation confirmed these findings in that glucose and mannose were the only monosaccharides detected.

A comparison of N. crassa with C. adiposa cell wall shows lower levels of Fractions 1 and 111, while a higher level in Fractions 11 and 1V. The much higher value of Fraction 1V from C. adiposa may be accounted for by the presence of melanin associated with chitin which prevented chemical and enzymic degradation of the cell wall..

Fraction	% yields	
	N. crassa Mahadevan & Tatum 1965.	C.adiposa (72 hour)
1	11.8 - 16.0	0.6 - 2.1
11	9.0 - 13.2	11.6 - 26.6
111	15.0 - 20.0	1.5 - 4.9
1V	6.3 - 11.5	22.6 - 55.2
Yield	Max 60.7	36.3 - 74.1

TABLE 28.

Major components of some ascomycete cell walls.

FUNGUS	Ref	Component (%)						
		Carbo-hydrate	Pro-tein	Lipid	Glu-cose	Mann-ose	Galac-tose	Amino sugar
Aspergillus niger	1	73-83	0.5-2.5	2.7	✓	✓	✓	9-13
A. nidulans	2	57.6	10.5 10	4.6 9	28.9	2.8	3.8	19-1 14-3
Ceratocystis ulmi	4	56.0	7.9	4.5	43.5	✓	1.8	3.95
Fusarium sulphureum	5	66	7.3	5.5	14	✓	✓	39
Penicillium rubrum	6	67.4	7.2	17.0	44.6	0.6	5.4	15.4
Neurospora crassa	7		14		56.4-58.2			6.4-7.1
	8				48.6			✓
	9				60.0	4.0	3.6	16

✓ reported present in cell wall.

1. Johnson (1965)
2. Zonneveld (1971)
3. Bull (1970(a))
4. Harris & Taber (1973)
5. Barran et al (1975)
6. Unger & Hayes (1975)
7. de Terra & Tatum (1963)
8. Potgieter & Alexander (1965)
9. Edson (1972).

Glucose appeared to increase the Fractions 1 - 111 after 72 hours, i.e., the non-chitin containing fractions although in wall from 24 hour cultures, Fractions 1 & 1V are increased. Sorbose caused low values in 72 hour wall in Fractions 1 - 111 and an increase in 1V as reported for N. crassa by de Terra & Tatum (1961). In N. crassa sorbose caused levels of glucosamine 184% and glucose 68% and the ratio of glucosamine to glucose was 0.12 in sucrose medium and 0.32 in sorbose medium. Crocken & Tatum (1968) reported that sorbose caused a decrease in the level of β) 1-3 glucan in the cell wall.

C^{14} sorbose was traced as it was used by N. crassa and no phosphorylated sorbose compounds were found but an increase in sorbitol occurred. The sorbose label was found as ^{14}C glucose in all cell wall fractions (Mahadevan & Tatum 1965). Sorbitol supported growth without inducing paramorphs.

It may be that C. adiposa has a more efficient method of converting sorbose to sorbitol which thereby reduced the paramorphic effect of sorbose.

Crocken & Tatum (1968) reported that sorbose caused higher incorporation of ^{14}C glucose into amino acids and therefore into Fraction 1 of the cell wall; less efficient utilization of glucose, more carbon being lost as CO_2 ; alteration in the cell wall and the inability to conidiate in N. crassa. Sorbose did not prevent conidiation of C. adiposa although it did alter the cell wall.

Sorbose acted on the surface of cells, aerial hyphae possessed normal morphology and if sorbose was injected into hyphae, it had no effect. (de Terra & Tatum 1961 & Wilson 1970). It seemed that sorbose needed contact with the cell wall to effect a morphological alteration, therefore it was not influencing internal metabolism possibly because it was converted to

sorbitol. It must act by inhibiting the uptake of glucose by competing for the sugar carrier or by competitively inhibiting the glucan synthetase enzymes responsible for building glucose units into cell wall polymers.

The total recovery from 72 hour PEG cultures was very low. As PEG is not a carbon source, the organism may have exhausted the available glucose and be mobilizing cell wall material from old cells for growth by autolysis (Trinci & Righelato 1970). If this was the case, then the sodium deoxycholate would be expected to have a low yield but this was not the case.

The specific activity of all the enzymes used was not determined so absolute amounts of substances released cannot be related. Small amounts of glucose, N-acetyl glucosamine and protein were released from C. adiposa cell wall when incubated in distilled water, possibly as a result of the action of lytic enzymes remaining within the cell wall after cleaning. The results (Fig.35) indicated that the glucan in the wall may be both β-1,3 and β-1,4 linked as it was susceptible to glucanase and cellulase. As glucose and protein were released by digesting the cell wall with chitinase, it would seem that glucan and protein were associated, possibly intermeshed with the chitin fibrils. Chitin formed framework and was not digested by enzymes other than chitinase. Melanin in the cell wall prevented a total dissolution of cell wall by enzymes.

The uptake of ^{14}C glucose was more rapid than the uptake of ^{14}C sorbose from media in which they were the sole carbon sources. This reflected the difference in the specific growth rates of C. adiposa in SSM in which the carbon sources are glucose and sorbose; 2.56 mgm dry weight per hour in glucose and 0.58 mgm in sorbose. The rate of increase in cpm between 6 and 24 hours was 4 cpm/hour in glucose and 1·4 cpm/hour in sorbose. The

relative efficiency of substrate utilization by C. adiposa was 1.56 cpm/mgm dry weight in glucose and 2.41 cpm/mgm dry weight in sorbose. C. adiposa had a slower growth rate and less efficient utilization of carbon source when this was supplied by sorbose compared to glucose.

In Neurospora, sorbose caused a less efficient utilization of glucose in that less glucose was incorporated into the cell and more is lost as CO_2. (Crocken & Tatum 1968). The results for $^{14}CO_2$ evolution by C. adiposa were not reliable so no conclusions could be made about respiration rates.

The lower concentration of sorbose used (0.67%) did not reduce the growth rate significantly but caused a reduction in the cpm indicating inhibition of glucose uptake. However, at the higher concentration (1.33%) the cpm from ^{14}C glucose was higher. As sorbose altered the cell wall composition and reduced the amount of cell wall produced by the fungus it may have increased the permeability of the wall and allowed glucose to enter the cell more rapidly.

The wall of N. crassa allowed the smaller weight isozyme of invertase to penetrate but kept the larger weight isozyme in the cell. (Trevithick & Metzenberg 1966). An increase in secretion and decrease in fractionation was observed in mutants which was explained by an increased porosity of the wall. (Trevithick et al 1966).

It was proposed that the mutant had abnormally large pores which allowed molecules up to 18500 molecular weight to pass through compared to the wild type size limit of 4750.

Pores have been reported in the cell wall of N. crassa 25 - 30 Å and Pythium 40 - 80 Å by Manocha & Colvin (1967 & 1968).

Sodium deoxycholate, a bile salt with detergent properties

was much more effective in reducing Kr than were sugars; the values for Kr_{50} were about 0.25 x 10^{-4} M with sodium deoxycholate and 0.25M with sugars for C. adiposa. It was noticed that a reduction in inhibition of Kr occurred at 6 - 8 x 10^{-5} M for C. adiposa and at 7.5 - 10 x 10^{-5} M for B. fabae. This was not due to a change in pH of the medium. Sodium deoxycholate was precipitated as needle-like crystals by various components of the medium ($H_2PO_4^-$, Mg^{2+} , Zn^{2+} Fe^{3+}, Mn^{2+}) so the actual concentration of deoxycholate in solution was not known.

When conidia were shaken in glucose + deoxycholate solution, they germinated rapidly. If the same concentration of deoxycholate (10^{-3} M) was added to SSM, crystals appeared to be deposited on the conidia and germination prevented.

Inhibitory concentrations of deoxycholate caused conidia to lose almost all their amino acids (Fig. 61.). Considering the uses of deoxycholate in releasing RNA from membranes and that it has detergent properties; it is not surprising that the permeability of the cell membrane is altered.

Sodium deoxycholate is an anionic, surface-wetting agent, therefore it may increase the permeability of the cell wall allowing molecules to pass through to the cell membrane. Bile salts such as this form micelles with lipids and phospho lipids, (Mahler & Cordes 1966) through interaction of the hydrophobic face with the non-polar lipid, leaving the polar face and side chain exposed to interact with an aqueous environment. It is the formation of these micelles that is the basis for the action of bile salts as emulsifying agents.

The cell membrane contains a lipid layer which may be attacked by sodium deoxycholate especially at the apex where vesicles are fusing with the membrane (Grove & Bracker 1970) and the lipid layer may be exposed. The interference with the apex

of the hypha may induce increased lateral branching.

The conidial development of Penicillium, Ceratocystis and Fusarium was placed in Section IV of the classification by Hughes (1953), (Cole & Kendrick 1969). Conidium development in the genus Penicillium differed from that of Aspergillus in that the spore wall was formed round the already delimited conidial protoplast between the protoplast and the original phialide wall (Fletcher 1971). The septum arose from the spore wall to form the roof of the next spore (Zachariah & Fitz James, 1967). Microconidia, macro conidia and the phialide tip of Fusarium oxysporum stained with antisera to micro conidia while the hyphal did not.

Neurospora formed chains of conidia (Turian 1966). A fissure in the interconidial wall represented the first stage in separation of the conidia. There was a deepening of the constriction furrows into the plasticized wall leaving a central pore to maintain cytoplasmic continuity between adjacent spores. Eventually, the pore was occluded by a plug of electron dense material preceeding complete occlusion of the pore by cell wall material in the released mature conidium.

The development of conidia of Ceratocystis adiposa has recently been studied by Hawes & Beckett (1977). They reported that conidia were produced in chains which may easily fragment or persist. Conidia in persistent chains were surrounded by a membranous sheath. This was confirmed in this study, see Fig. 66 . If the sheath was disrupted, then the chain remained intact indicating that the sheath was not solely responsible for holding the conidia together. Campbell (1972 & 1975) suggested that the main factor in keeping chains of conidia intact was the stage at which the septum between adjacent conidia split. Hawes & Beckett's observations also suggested

that the septum was important in holding chains together. In this study, Fig. 68 shows the septum between two conidia which is plugged but shows no sign of splitting.

There was no difference in conidium ontogeny between the hyaline and deeply-pigmented conidia which could be found in the same permanent chain. The large globose pigmented conidia took longer to form possibly because of the increase in deposition and elaboration of wall material. The shape of the conidium depended on the depth at which it formed in the conidiogenous cell. Those which formed totally within the cell are cylindrical while those which expand above the tope of the cell are round of pear-shaped. (Hawes & Beckett 1977(a)), Fig. 48 , (a), (b), (c).

The first conidium produced by the conidiogenous cell was formed by blowing out the tip of the cell to form the conidium which was surrounded by a new wall. The wall of the conidiogenous cell broke down to release the conidium.

The conidiogenous cell contained a single nucleus, endoplasmic reticulum, mitochondria, ribosomes and young conidia often contained lipid droplets. (Hawes & Beckett 1977(b)). The septum which delimited the spore was single layered at first but became multi-layered as the conidium wall matured. The mature conidium wall was found to be three-layered but the outer wall layer did not form part of the septum.

It is possible that Fig. 47 shows a hyphal tip being blown out to form a conidium. Unfortunately, most of the conidiogenous cell was obscured by a bar of the copper grid but the outer wall may be seen at the edge of the plate.

C. adiposa appeared to contain 1 or 2 large lipid droplets when stained with Sudan IV. However, in the TEM, these appeared to be made up of many smaller droplets. (Fig. 64, 65) This may have been due to fixation and staining methods which

had caused the large droplets to fragment.

Conidia of N. crassa contained lipid bodies 0.12 - 1.0 μm diameter as well as sparse endoplasmic reticulum, amoeboid mitochondria and 4 - 6 nuclei (Weiss 1963). In Fusarium culmorum spores, lipid bodies decreased in volume with age as they were used for respiration. (Marchant 1966(a)). Botrytis cinerea conidia contained whorled structures, spherules, granules and membrane loops resembling phospho lipid inclusions. These may have provided the material for assembling membranous organelles and therefore myelin figures may not be evidence of degenerate membrane systems (Freeman 1964) but of potential organelles (Buckley et al 1966) in spores. Concentric membranes were also reported in spores of Aspergillus nidulans (Florance et al 1972) and Trichoderma viride (Rosen et al 1974).

Most of the conidia shown in Fig. 64 from PEG medium show lipid droplets except one which had apparently begun to germinate. Conidia from SSM show nuclei, numerous mitochondria and no lipid droplets. (Fig. 62). It appears therefore that before a spore could germinate, the darkly-stained lipid bodies must have disappeared. In the ungerminated spores, very few other cell organelles were seen and it may be that mitochondria and other membrane organelles need to be formed or at least multiply before germination can take place. The lipid material in the darkly-stained bodies could be used for membrane synthesis as well as respiration.

Spore swelling and water uptake are associated with the early stages of germination. Water may be required for activating enzymes necessary for organelle synthesis and cell wall formation. In media of low water potential, germination was prevented by the inability of the spores to obtain water against a large osmotic gradient. When the spores were placed on a

suitable medium and could obtain water, they germinated. After three or four days in molar glucose or sorbose, conidia were observed to germinate probably because the two sugars were accumulated by the cell either actively with sugar carriers or passively. PEG may have been accumulated passively but be toxic at osmotic equilibrium concentrations within the cells (Lawlor 1970).

Swelling was associated with the re-appearance of endoplasmic reticulum in Trichoderma viride. Metabolic activity became evident from the re-appearance of electron dense bodies and the change in shape and numbers of mitochondria while lipid granules disappeared (Rosen et al 1974). Remsen et al (1967) ~~also~~ also reported a decrease in lipid content of Penicillium megasporum spores during germination. However, a three-fold increase in the lipid content of oidia of Coprinus lagopus on germination was suggested by Heintz & Niederpruem (1971) to be caused by the mobilization of lipid at the time of germ tube formation.

Membranes of the endoplasmic reticulum were sparse or not visible in dormant spores. On germination, the membranes increased in amount. (Aitken & Niederpruen, 1970; Buckley et al, 1966; Fletcher,1971; Florance et al,1972; Hawker & Abbott,1963; Hawker & Hendy,1963; Lowry & Sussmen,1968; Marchant, 1966(a); Martin et al, 1973; Richmond & Pring, 1971(b)); Rosen et al, 1974; Williams & Ledingham, 1964.

Mitochondria of Penicillium notatum and Sphaerotheca macularis appeared dumbell shaped as if they multiplied by fission. (Martin et al,1973; Mitchell & McKeen,1970).

Mitochondria in Geotrichum candidum spores altered in shape early in germination from short oval to filamentous preceding an increase in oxygen uptake (Park & Robinson,1970).

An increase in the number of mitochondria on germination of Aspergillus oryzae (Tanaka,1966) and an increase in size and number in Penicillium griseofulvum (Fletcher 1971) spores correlated well with the increase in respiration. This increase in mitochondria also occurred in A. nidulans (Florance et al, 1972), Neurospora tetrasperma ascospores (Lowry & Sussman, 1968) and in Geotrichum candidum where short oval mitochondria became filamentous preceding an increase in oxygen uptake. (Park & Robinson,1970).

In a study of the origin of yeast mitochondria by Linnane et al,(1962) aerobic and anaerobic grown cells were used. The basic cytoplasmic features of both types of cells were similar but anaerobic cells had no mitochondria. Instead they showed multi membrane systems reminiscent of endoplasmic reticulum and of myelin type concentric membranes. When the cells were fractionated, aerobic cells gave mitochondria in the particulate fraction while anaerobic cells gave fragments of the reticular system. Anaerobic cells, when aerated showed the membranes lined up in a parallel fashion, fused and then infolded to form primitive mitochondria with a few cristae and cytochromes synthesized thus showing a relationship between reticular membranes and mitochondria.

As C. adiposa germinated, the emergence of the germ tube was observed Fig. 69 . It appeared that only the inner layer of the conidium wall was continuous with the germ tube wall. The outer layers of the conidium wall ruptured.

In other spores, the germ tube appeared to be continuous with the inner layer of the spore wall, e.g., Botrytis fabae (Richmond & Pring,1971(a)), Penicillium notatum (Martin et al, 1973(b), Trichoderma viride (Rosen et al,1974), Botrytis cinerea (Hawker & Hendy,1963), P. megasporum (Remsen et al,1967) and

Neurospora tetrasperma ascospores (Lowry & Sussman,1968).

The cell wall of dry dormant conidia of Aspergillus nidulans appeared to be three-layered but on hydration showed five layers. This probably reflected changes in the existing cell wall rather than the formation of new layers (Border & Trinci,1970; Florance et al,1972). As the conidium expanded, the outer two wall layers ruptured, the middle layer decreased in thickness as it was stretched and the innermost layers were continuous with the germ tube.

Conidia of Botrytis cinerea had a two-layered wall, the outer layer of which ruptured on germination. The germ tube was surrounded by the elastic inner layer and a mucilage sheath (Hawker & Hendy 1963) which condensed to give an electron dense outer zone to the wall at the base of the germ tube. There was no mucilage around germ tubes of Fusarium culmorum although there was on the spore (Marchant 1966(b)). Cell organelles divided early in germination and as the germ tube elongated, the contents of the spore flowed into it causing a large vacuole to form in the spore cytoplasm. Buckley et al (1966) considered vacuolization to be due to the utilization of storage material and probably both events contributed to the loss of material from the spore cytoplasm.

Fusarium culmorum conidia during germination produced a new wall layer in the region of the germ tube which led to stretching of the conidial wall. However, the diffuse appearance of the wall around the base of the germ tube suggested enzyme dissolution rather than mechanical rupture. (Marchant 1966 (a) & (b)), see p.251

Microsporum gypseum macroconidia contained more of the following than did the mycelium : carbohydrate-protein ester, disulphide bonds, acid soluble and insoluble phosphates.

These decreased on germination resulting in changes in the spore coat properties. Germination was initiated by alkaline protease and β, 1-3 glucanase followed by ethyl esterase, phosphodiesterase and chitinase activity.

These hydrolases were compartmentalized in lysosomes and vesicles inserted into the maturing wall. On hydration, the vesicles presumably burst and released their contents into the wall where the lytic activity of the enzymes weakened the wall so providing a passage for the germ tube to emerge. (Page & Stock, 1974). During germination of Neurospora crassa, there was a specifically greater removal of β, 1-3 glucan and a little chitin than when germination was prevented when there was a different type of enzyme degradation. In a mutant which showed early germination, the first type of degradation is more pronounced probably due to the higher amount of enzymes present. (Mahadevan & Rao 1970).

There appeared to be two forms of conidia produced by C. adiposa. There were small thin walled conidia, larger thick walled conidia and a range of size and wall thickness between the two (Fig.48). Most conidia produced at 30°C were of the smaller type and these were the ones studied. At 25°C, more larger conidia were formed as shown by the secondary peak on Fig. 55.

Glucose was found to be necessary for germination. An interesting observation was that while germination occurred in a solution of glucose and sodium deoxycholate, it was inhibited by SSM containing the same concentration (10^{-3} M). Sodium deoxycholate remained in solution in glucose but was precipitated by SSM as needle-like crystals which adhered to the spores. It may be that the spores provided a nucleus on which the crystals formed or that the crystals stuck to the mucilage around the

spore. In either case, the crystals may have provided a localized high concentration of deoxycholate around the spore so inhibiting germination.

When 10^{-3} M sodium deoxycholate was incorporated into semi-solid media (SSM) germination was reduced to 5% even though the concentration was evenly distributed. Possibly the component which complexed with deoxycholate formed a more toxic compound than sodium deoxycholate itself. The curve for inhibition of C. adiposa by Na deoxycholate was a sigmoid dosage mortality curve and was converted to a straight line using probits. (Bliss in Fisher & Yates 1957).

When investigated with the Coulter Counter, the spores were found to swell in sodium deoxycholate + glucose more markedly than the control while 1.OM glucose and PEG did not swell. It was not possible to run a sample from SSM + deoxycholate because the crystals blocked the aperture.

After one day in sodium deoxycholate solution, the spores germinated when plated onto SSM or 2% malt agar but after two days in deoxycholate very few spores were able to germinate. (Table 25). Conidia from 1.OM PEG were still viable after two days and germinated on SSM. This was probably because spores in deoxycholate solution have lost amino acids. (Table 26).

Charlang & Horowitz (1971) reported that fungal spores lost a germination factor in media of a low water potential. This was not the case with C. adiposa, as in PEG there was no loss of a germination factor or viability.

Sodium deoxycholate is a detergent and probably caused damage to the cell membrane which became more permeable thus allowing amino acids and probably other endogenous nutrients to diffuse out of the cell.

During the first hour of germination, nearly all the amino

acid pools decreased and very little was secreted into the medium.

Charlang & Horowitz (1971 & 1974) proposed that reduced pyridine nucleotides are produced by the degradation of the large glutamic acid pool therefore if amino acids are lost, many biochemical events early in germination cannot take place. The germination factor in Neurospora crassa may perform an analogous function to the germination enzyme described by Gould et al (1966) in Bacillus cereus.

Coulter Counter analysis showed that the electrolyte (0.5 % Na Cl) used caused slight shrinkage of the conidia. (Fig.56) Therefore, each analysis was done as quickly as possible to minimise the shrinkage.

The effect of the age of the culture from which the spores were obtained did not appear to influence the size distribution of conidia at 30°C (Fig. 57). However, temperature influenced size distribution. Cultures grown at 25°C produced larger, thick walled spores which caused a pronounced secondary peak at 14-16 μm (Fig. 57). The range of spore size corresponds to that obtained by Hawes & Beckett (1977) of 4.5 μm to 17.0 μm diameter.

When conidia were allowed to germinate in SSM, an increase in size was observed at first (Fig.58) but after 7 hours incubation, the distribution showed a greater proportion of small spores. When the conidia had germinated, their germ tubes tangled and the clumps would not pass through the aperture of the Coulter Counter tube. Therefore, after 7 hours, those spores which had germinated and clumped together have not been counted and only those which had not germinated were counted in the 7 hour sample (Fig. 58). It would seem that smaller spores are slower to germinate. This was also observed by Hobot & Gull (1977) for Syncephalastrum racemosum and in this organism, swelling was not a pre-requisite to germ tube formation.

Ceratocystis adiposa conidia did not appear to swell markedly before germination. (Fig.58).

In submerged culture, conidia of Aspergillus niger showed agglutination which commenced at the same time as swelling and was associated with changes in the spore wall surface at that time. Clumping continued through the intertwining of germ tubes (Galbraith & Smith 1969). Clumping of hyphae was also reported by Reissig & Glasgow (1971) and was caused by a mucopolysaccharide produced by a colonial mutant of Neurospora crassa.

The behaviour of the nucleus in conidium formation was described by Hawes (1978). Vegetative cells and cells of the conidiophore were uninucleate and nuclei were 1 - 2 μm diameter. Occasionally, two nuclei were reported present in a conidiogenous cell, one at the apex inside the conidium initial and the other nearer the base. Once a septum had formed, the conidium was uninucleate but underwent nuclear division to produce 1 - 6 nuclei. (Hawes & Beckett,1977(a),(b)).

Most of the conidia in this study had 4 nuclei (range 2 - 8). It appeared that after 5 hours, nuclei of conidia in sorbose and Na deoxycholate had divided (6.4 & 6.5 nuclei per conidium). After 7 hours, it appeared that sorbose and PEG 200 inhibited nuclear division (mean number of nuclei being 7.8 and 6.6) as control and Na deoxycholate treated conidia were in the second phase of division (mean nuclear numbers 10.9 and 10.1). Control conidia which had divided twice in two hours therefore had a generation time of one hour.

It should be remembered that Na deoxycholate in glucose was much less inhibitory than Na deoxycholate in SSM and possibly stimulated nuclear division after 5 hours in glucose. This correlated with the swelling observed in the Coulter analysis where deoxycholate caused greater swelling than the control at

5 hours (Fig.59).

It may have been that Na deoxycholate disrupted the cell membrane to release inhibitors from the conidium or to allow the more rapid entry of water or glucose for germination. When added to SSM however, Na deoxycholate caused a loss of amino acids which would have been expected to reduce germination.

The growth of the germ tube was found to be logarithmic between one and two hours after emergence. (Fig.53). The maximum rate for C. adiposa was 0.8 $hour^{-1}$ which had a doubling time of 52 minutes. Trinci (1971) reported logarithmic growth for various fungal germ tubes. Generally non-septate germ tubes had a higher germ tube specific growth rate (αg) than septate species.

Mucor racemosus = 0.809 hr^{-1} Geotrichum lactis = 0.417 hr^{-1} Aspergillus wentii = 0.303 hr^{-1} A.niger = 0.282 hr^{-1} and Penicillium chrysogenum = 0.292 hr^{-1} . Therefore, C. adiposa had a higher α g than most septate fungi. However, this increase in volume did not necessarily mean an increase in dry weight. Mitchison (1963) reported that during the cell cycle of Schizosaccharomyces pombe, dry weight increased at a constant rate while cell volume grew at an increasing rate and cell concentration fluctuated.

αg was always greater than αs and may represent the maximum growth rate supported by complex organic substances from spore reserves while vegetative hyphae have only the relatively simple compounds in the medium.

TABLE 29.

The extent of swelling of some fungal spores.

Fungus	Swelling	Reference
Cunninghamella elegans	10.5 μm - 12.0 μm diameter	Hawker(1966)
Fusarium culmorum	1.25 x volume	Marchant & White(1966)
Penicillium chrysogenum	2.4 μm to 5.7 μm diameter	McCoy et al (1971)
Rhizopus arrhizus	4.5 x 5.5 to 8.5 x 8.5 μm 3x volume	Hawker (1966) Ekundayo & Carlile (1964)
Trichoderma lignorum & P.notatum.	2 x volume	Burguillo et al (1972)
Aspergillus nidulans.	3 μm to 5 μm diameter.	Border & Trinci (1970)
Botrytis cinerea	10.3 - 12.5 μm diameter.	Gull & Trinci (1971)

As the spores of P. chrysogenum and Aspergillus oryzae swelled, invaginations of the plasma membrane disappeared and the wall decreased in thickness from 0.22μm to 0.15μm and 0.22 - 0.26μm to 0.15 - 0.20μm respectively (McCoy et al (1971) Tanaka (1966)).

Hydration of spores prior to germ tube formation is not accompanied by vacuolation so that water is probably bound rather than free (Burnett 1976). The structure of enzyme molecules is dependent on bound water molecules and as activation of spores involves the association of trehalase and trehalose, hydration of the enzyme molecule may be the removal of a barrier required for activation. (Hill & Sussman 1964).

Spores of Penicillium atrovenetum were reported to swell

before producing a germ tube (Hobot & Gull (1977)) and this preliminary phase of germination required intense metabolic activity and a supply of glucose and phosphate as exogenous reserves. Di- and poly saccharides were unable to provide a carbon source because the spores had no hydrolytic enzymes. Nitrogen was found to be essential for germ tube formation but not for swelling.

Swelling is mainly due to water uptake. In Fusarium culmorum there was a 21.1% increase in water content and only 6.2% increase in dry weight of spores (Marchant & White,1966) and in Syncephalastrum racemosum, dry weight only increased at germ tube emergence. (Hobot & Gull,1977).

Swelling occurs to varying extents (Table 29). The normal extent of swelling in conidia of Trichoderma lignorum and Penicillium notatum in Czapek-Dox medium was to double in size. When $(NH_4)_2\ SO_4$ was used as the nitrogen source conidia increased in size 20 - 30 fold. This was found to be caused by a large drop in pH to pH 1 from pH 5.5 after 36 hours. The same happened when asparagine was used as the nitrogen source and the pH adjusted to pH 1. The low pH induced wall softening by breaking some of the S - S cross links in the cell wall. Cells then extended under turgor pressure and eventually burst if a low pH was maintained. (Burguillo et al,1972).

Some spores contain sufficient endogenous nutrients to enable them to germinate in distilled water, e.g., uredospores of Puccinia graminis tritici (Allen 1955 & 1957), Neurospora tetrasperma ascospores (Lowry & Sussman,1968). Most spores require some nutrients to germinate.

TABLE 30.

Nutrients required for germination by some fungi.

Fungus	Nutrients	Reference
Botrytis cinerea	Simple sugars amino acids	Blakeman 1975.
Fusarium culmorum	carbon nitrogen	Marchant & White 1966
Glomerella cingulata	glucose Mg SO_4 KNO_3 KH_2PO_4	Lin 1945.
Penicillium atrovenetum	glucose, nitrate phosphate	Gottlieb & Tripathi 1968.
Penicillium griseofulvum	glucose nitrate phosphate	Fletcher & Morton 1970.
Penicillium notatum	carbon, nitrogen.	Martin & Nicolas 1970.
Rhizopus arrhizus	glucose	Ekundayo 1966
Syncephalastrum racemosum	glucose	Hobot & Gull 1977.
Trichoderma lignorum	carbon nitrogen	Martin & Nicolas 1970.
Hypocrea pulvinata	carbon phosphorus nitrogen	Speller 1969

Ceratocystis adiposa conidia would not germinate in water but needed glucose as an exogenous carbon source. Some spores contain sufficient endogenous reserves to allow them to germinate in water with no exogenous nutrients.

Ascospores of Neurospora tetrasperma contain 25% lipid and 33% carbohydrate as endogenous nutrients. Lipid serves as the energy source during dormancy and carbohydrate is not used until the spores are activated by heat shock when respiration increases 20 to 30 fold. (Lingappa & Sussman,1959). The total lipid content of Rhizopus spores decreases during the first two hours of germination and the proportion of neutral lipid decreases while polar lipid increases. Gunasekaran et al (1972) suggest that free fatty acids are converted into glycerides during early stages of germination.

During the first hour of germination, the amino acid pools in Neurospora conidia decrease, e.g. glutamic acid decreases by 70%. Cell reserves of trehalose, mannitol, arabitol, were depleted during the lag phase of Schizophyllum commune basidio spore germination but exogenous carbon and nitrogen were required to metabolize polyol reserves. This was accompanied by an increase in RNA and protein levels and inhibition of protein synthesis arrested the depletion of polyol reserves.

When ^{14}C glucose was supplied to P. atrovenetum, it was metabolised to the following:- (Gottlieb & Tripathi,1968).

15% CO_2	3.2% polysaccharides & nucleotides.
27.7% RNA	5.4% lipid.
13.2% DNA	9.5% protein.
12% amino acids and sugars.	11.8% cell wall.

The increase in RNA was necessary to form enzymes needed for protein synthesis. Both RNA synthesis and swelling were inhibited by Na N_3 and protein synthesis and swelling were

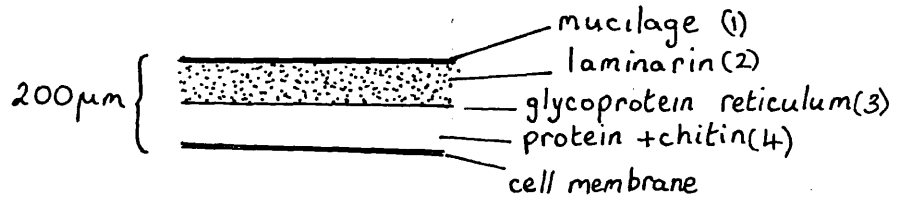
prevented by Actidione, Blasticidin S and Puromycin indicating that protein synthesis was necessary for swelling.

Germination of Aspergillus niger conidia was considered in three stages by Yanagita (1957); endogenous swelling, exogenous swelling and sprouting. Nucleic acid synthesis occurred in the exogenous swelling stage at the end of which glutamic acid and alanine were accumulated. Oxygen uptake increased and protein synthesis began when the germ tube sprouted.

The swelling of P. roqueforti spores took place after 4 hours incubation during which time the spore had assembled the required synthetic enzymes, RNA and protein. Unsaturated fatty acid synthesis, nuclear division and DNA replication occurred giving a dry weight increase of 2 mgm hr^{-1} per 10^9 spores. Fatty acid synthesis reached its maximum at germ tube emergence reflecting the proliferation of cellular and membrane material. (Fan & Kinsella,1976).

Assuming that the sections were from mycelium of C. adiposa with a mature wall, it should be comparable with the mature wall of N. crassa (Hunsley & Kay,1976). The wall of N. crassa showed:- An outer amorphous layer of laminarin (Fraction 111) with a reticulum embedded in it (Fraction 1). Below this lay a layer of easily removable protein followed by a discrete layer of protein overlying the inner chitin-protein layer.

Fig 74 CELL WALL ULTRASTRUCTURE.



In the wall of C. adiposa (Fig.74) there appeared to be 4 layers. The outermost dark layer may have been continuous with the mucilage (1). The narrow dark band (3) was probably glycoprotein and the outer layer (2) a mixture of glucans and protein while the inner layer (4) was chitin protein and melanin.

The width of the wall varied from 100 - 200 nm which was within the range obtained for N. crassa 50 - 270 nm and Geotrichum 70 nm (Trinci & Collinge,1975).

Because of the variation in cell wall appearance observed for the same treatment, it was not possible to deduce any difference between the treatments in cell wall thickness or appearance. However, a reduction in Fractions 1 - 111 as % weight of cell wall may not be observed as a reduction in thickness of the outer layer if a protein component provided the frame work, possibly by lining the walls of micro pores within the cell wall. (Manocha & Colvin,1967). The wall may also be more diffuse but not necessarily thinner.

Glucan may be embedded among the chitin micro fibrils as chitinase released glucose from C. adiposa cell wall.

At the septum there appeared to be an extra innermost dark layer which formed part of the septum.

The mycelial form of Sporothrix schenckii showed hyphae 1.0 - 1.8 μm diameter with walls 80 - 140 nm thick. The cell wall of C. stenoceras was 120 nm wide in mycelial cells. (Garrison et al 1976).

The conidium wall shown in Figs. 67 + 69 showed three layers. The outer layer contained areas of electron dense material within an electron transparent material. This outer layer governs the surface ornamentation of conidia as it is thick on the highly ornamented walls and thin on the smooth, less heavily pigmented conidia. (Hawes 1979).

The electron transparent material was found to be alkali soluble α - 1, 3 - glucan (Hawes,1979). The chitin microfibrils were only disrupted by chitinase after pronase treatment which indicated that chitin was bound with protein and found to be the innermost layer of the wall. The reported structure for the conidium wall of C. adiposa is shown in Fig. 75 from Fig. 69

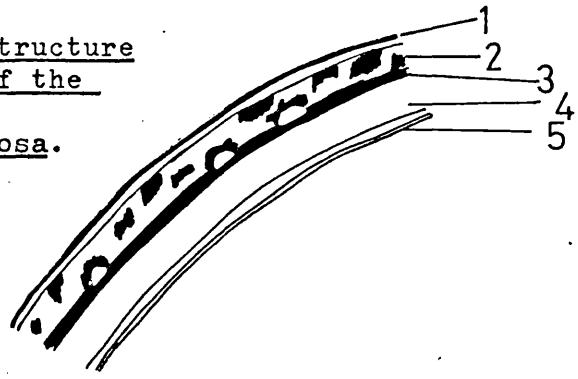


Fig. 75 Structure of the conidium wall of Ceratocystis adiposa.

Key (from Hawes,1979).

1. sheath, possibly membrane which holds conidia together as shown in Fig.
2. α -glucans and melanin.
3. β -glucans and melanin.
4. chitin microfibrils, β-glucan, protein.
5. plasma membrane

The hyphal tip of C. adiposa was shown to contain a Woronin body at the tip. The cytoplasm was dense and contained many organelles including a nucleus 1.5 μm diameter. Unlike Grove & Bracker (1970), who reported that the hyphal apex of Pythium was devoid of organelles, the apex of C. adiposa

contained a Woronin body and nuclei within the apical 5 µm of the hyphae. The cell membrane at the apex was not smooth but invaginated possibly as the result of the fusion of apical vesicles.

The hyphal tip in Fig. 47 which appeared swollen may be the tip of a conidiogenous cell with a spore initial emerging at the top as the first conidium as described by Hawes & Beckett (1977).

There appeared to be a slight bulging of the cell in the area of a septum (Fig. 43). The septum was found to be continuous with the two innermost layers of the cell wall.

In some sections there appeared to be a simple pore in the centre of the septum which may be blocked by a Woronin body. (Moore & McAlear, 1962).

Reichle & Alexander (1965) found four Woronin bodies 200 nm diameter associated with each septum of Fusarium sp and Collinge et al (1978) of Penicillium chrysogenum. C. adiposa may have two Woronin bodies on each side of a septum but these were not always in the plane of section. (Fig. 41)

There may have been strands passing through the septal pore of C. adiposa (Fig. 41). Although the section is thick and only shows 3 Woronin bodies, it could be interpreted as Fig. 45 If this is the case, then the structure of the septum appears very similar to that described by Thibaut (1969) for Sporothrix schenckii although the "disc" are not very clear.

There is evidence for a relationship between Sporothrix schenckii and some species of the genus Ceratocystis. Mariat (1978) has reviewed the evidence to date.

At the ultrastructural level, the hyphal cells of the mycelial phases of Ceratocystis stenoceras and Sporothrix schenckii are quite similar and the events occurring during M - Y transformation, direct budding and the production of oidia inside

the hyphal cells, proceeded in a similar manner. (Garrison et al, 1976). de Hoog (1974) reported that it appeared to be impossible to find steady morphological differences between S. schenckii and the conidial states of Ophiostoma stenoceras and O. tetropii although Ophiostoma colonies tended to be more extended, floccose and the conidiogenous cells are somewhat wider and more regular.

Various chemical comparisons have been done between S. schenckii and Ceratocystis stenoceras (? Ophiostoma). Two polysaccharides produced by these fungi were compared for chemical composition, proton magnetic resonance and antigenic value. The principal sugars were mannose and rhamnose with some galactose in one polysaccharide and traces of glucose and hexosamines in similar proportions. The pmr spectra were practically superimposable between themselves and those for other Ceratocystis polysaccharides reported in the literature. (Toriello & Mariat,1974). Cross reactions with antigenic tests also confirmed similarity between the polysaccharides and also the fungi.

Rhamnomannans from S. schenckii and Ceratocystis species were compared and no major differences were found between the carbohydrate composition of polysaccharides from either species. The pmr spectrum for S. schenckii was very similar to those of C. clavata and C. ambrosia but not identical. (Travassos et al 1973).

The base composition of DNA from C. stenoceras and S. schenckii was different (Victeria et al 1970). Guanine + cytosine accounted for 49.5% of S. schenckii DNA and 52% of Ceratocystis DNA.

On classification of long-chain fatty acids by Dart et al (1976) Sporothrix was closest to C. Stenoceras. Taylor (1970)

reported similarities between these two fungi in serological and physiological tests.

The induction of paramorphs, dimorphic forms or rhythmic growth may be considered as the result of a switch in the direction of cell wall synthesis from apical insertion of material in elongation to insertion of material all over the cell wall in yeast development or to the initiation of branches in paramorphs or rhythmic growth.

In paramorphs, the products of synthesis are directed away from apical growth to branching. Sorbose may induce this by inhibition of the enzymes involved in cell wall glucan synthesis, reflected in the lower values of the glucan fractions 1, 11 and 111 of the cell wall of Ceratocystis adiposa. As the lytic enzymes were not inhibited, the lytic activity of the cell wall was increased so the wall was softened. Evidence for a balance between synthesis and lysis in cell wall growth has been presented by Bartnicki-Garcia and Lippman (1972) and Johnson (1968) and for unbalanced growth caused by 2-deoxyglucose by Biely et al (1971).

Another reason for the reduced Kr may have been the inhibition of glucose uptake by sorbose although this would have reduced α_s as well as Kr because less glucose was available for metabolism. However, as C. adiposa was able to use sorbose as a carbon source, the fungus must have a mechanism to overcome the inhibitory action of sorbose.

This effect of sorbose was not very marked in C. adiposa and the water potential effect was dominant at concentrations used to reduce Kr by half. PEG 200 and higher concentrations of glucose and sorbose induced low water potential in the medium and made it more difficult for the fungus to maintain turgor. Therefore, new cell wall material produced at the apex was not pushed forward by the cytoplasm and the tip became occluded. When this

happened, the branches formed in the area behind the apex where the wall was weaker. In extreme conditions, the apex was frequently occluded and sub apical branching caused tortuous growth.

Sodium deoxycholate disrupted the cell membrane possibly causing unbalanced release of lytic and synthetic enzymes from vesicles. However, the effect on morphology was similar to that of the other paramorphogens, although less alteration in cell wall chemistry was observed.

All the paramorphogens altered the composition of the cell wall. Glucose increased the levels of Fractions 1, 11 and 111 while sorbose decreased these Fractions. This finding confirmed the hypothesis that the cell wall is of major importance in determining morphology. In fact, it may be that changes in the glucan components alone were sufficient to induce morphological change as Polyoxin D, an inhibitor of chitin synthesis was reported (Endo and Misato 1969) not to alter morphology and in Neurospora paramorphs, the level of chitin remained constant. (Mahadevan & Tatum 1965).

Cell wall protein may have a role in morphogenesis as increased activity of proteases was found in the Neurospora mutant spco 1 (Wrathal and Tatum, 1973).

There are obvious parallels between dimorphism and paramorphism in that both are influenced by glucose metabolism especially glucan synthesis of cell wall and the enzymes involved.

The source of carbon, sugar, amino acid and osmotic pressure caused changes in the phase of growth of dimorphic fungi. High levels of hexoses stimulate yeast development while a less readily metabolisable carbon source favours mycelial growth. (Nickerson and Mankowski, 1953 ; Bartnicki-Garcia, 1968; Friedenthal et al, 1974).

The composition of the cell wall varied but generally the

yeast form contained a higher level of chitin or amino sugar. (Table 9). Other generalizations are difficult to make, but changes in composition reflected enzyme alterations.

Changes from Y - M form in Candida were caused by a breakdown in intracellular - SH reaction which effected cell division not cell growth. (Nickerson & Chung,1954).

The relation between carbohydrate metabolism and morphogenesis of Podospora anserina was investigated by Lysek & Esser, (1971) who maintained that a single mutation which altered the morphology of a colony like "zonata" could not effect only one metabolic pathway. Investigation of phosphorylated intermediates by Bornefeld & Lysek (1972) supported the conclusion that carbohydrate degradation, respiration, anabolic reactions, particularly polysaccharide synthesis, may have been involved in the formation of the zoned growth pattern.

The ultrastructure of the cell wall appeared not to be affected despite changes in chemical composition. Sections of hyphae in the immediate area of a septum show an extra cell wall layer (Fig.43) and Woronin bodies associated with the septum. More careful investigation may have revealed a similar structure to that reported by Thibaut (1969) for Sporothrix schenckii. This would have provided another line of evidence for the relationship between S. schenckii and Ceratocystis spp.

The effect of sugar alcohols on perithecial production by Neurospora was reported by Viswanath - Reddy and Turian (1975) when the paramorphogenetic compounds were incorporated at concentrations which reduced Kr by half into SSM, those cultures on high sugar media produced fewer perithecia. PEG appeared to enhance the effect of temperature in that no perithecia were produced on mycelium which had been grown at 30°C for two days, although mycelium grown on other paramorphogenetic substances did

produce sparse perithecia.

As a colony must develope from a germinating spore, the effect of paramorphogenetic substances on germination and germ tube growth was investigated. Glucose and sorbose did not inhibit germination (0.25M) and some conidia germinated at 1.0M. Conidia in 1 M PEG did not germinate until removed from this solution and plated onto either SSM or 2% Malt agar. Glucose and sorbose may have been taken into the cell to establish osmotic equilibrium while PEG was not taken up and so conidia were unable to establish equilibrium (as confirmed by shrinkage of conidia in Coulter analysis) and obtain water for germination.

Sodium deoxycholate inhibited germination at low concentrations and the loss of amino acids from conidia may have been partly responsible. When conidia germinated, the germ tube wall was continuous with the inner layer of the conidium wall. Lipid droplets which were visible in ungerminated spores disappeared and more cell organelles were present. It is possible that the lipid was used in forming the membranes for the organelles or in respiration.

Nuclear division by conidia during germination was observed but as in the Coulter Counter work, the conidia which germinated clumped together and were not observed, only those which were beginning to germinate. Nuclear division appeared to be slower in PEG treatments while Na deoxycholate appeared to proceed at a similar rate to the control.

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TABLE 31.

Mean ± standard error for values of Kr of Ceratocystis adiposa in mm/day (n = 30. p = 0.05).

Conc. (Moles).	Glucose	Paramorphogen Sorbose	Xylose
0	8.90 ± 0.18	9.02 ± 0.14	9.50 ± 0.25
.01	9.10 ± 0.19	9.20 ± 0.14	9.60 ± 0.29
.02	9.28 ± 0.23	9.10 ± 0.11	9.93 ± 0.34
.03	9.60 ± 0.16	9.32 ± 0.14	10.25 ± 0.24
.04	10.20 ± 0.22	8.53 ± 0.15	-
.05	10.03 ± 0.20	8.42 ± 0.21	10.12 ± 0.23
.10	9.55 ± 0.31	7.90 ± 0.15	7.95 ± 0.25
.25	5.87 ± 0.17	5.85 ± 0.12	-
.50	2.23 ± 0.08	2.76 ± 0.08	2.67 ± 0.09
.75	1.02 ± 0.06	1.53 ± 0.05	-
1.00	0.73 ± 0.07	1.07 ± 0.03	0.73 ± 0.04

	Polyethylene glycol mol. wt.		
	200	400	1500
0	10.06 ± 0.35	10.89 ± 0.42	9.05 ± 0.35
.0010	10.96 ± 0.30	11.38 ± 0.33	8.40 ± 0.38
.0025	10.64 ± 0.38	11.68 ± 0.29	8.17 ± 0.37
.0050	10.85 ± 0.37	11.37 ± 0.34	8.38 ± 0.45
.0075	10.82 ± 0.53	11.16 ± 0.31	6.27 ± 0.45
.0100	11.03 ± 0.36	11.12 ± 0.45	8.25 ± 0.39
.0250	10.33 ± 0.39	11.45 ± 0.36	6.30 ± 0.36
.0500	10.78 ± 0.28	11.07 ± 0.50	2.07 ± 0.12
.0750	10.50 ± 0.32	9.64 ± 0.41	1.29 ± 0.08
.1000	10.60 ± 0.38	7.98 ± 0.40	0.64 ± 0.06

Conc. moles	Polyethylene glycol. Mol. wt. 4000	6000
.0	9.05 ± 0.35	9.77 ± 0.38
.0010	8.42 ± 0.42	7.90 ± 0.33
.0025	8.63 ± 0.37	8.27 ± 0.38
.0050	7.82 ± 0.31	-
.0075	7.89 ± 0.46	3.98 ± 0.44
.0100	6.87 ± 0.27	-
.0250	1.38 ± 0.13	0.87 ± 0.14

Kr (mm/day) of C. adiposa.

Conc. moles.	Sodium deoxycholate
0	8.85 ± 0.16
10^{-5}	7.62 ± 0.12
2×10^{-5}	5.79 ± 0.11
3×10^{-5}	4.93 ± 0.24
4×10^{-5}	4.03 ± 0.07
5×10^{-5}	3.55 ± 0.08
7.5×10^{-5}	3.93 ± 0.09
10^{-4}	3.47 ± 0.08
2×10^{-4}	2.54 ± 0.07
5×10^{-4}	1.81 ± 0.08
10^{-3}	1.81 ± 0.08

TABLE 32.

Values of Kr expressed as % control for Ceratocystis adiposa.

Concentration Moles	Paramorphogen			
	Glucose	Sorbose	Xylose	Polyethylene glycol 200
0	100.0	100.0	100.0	100.0
.01	102.2	102.0	101.1	-
.02	104.3	100.9	104.5	-
.03	107.9	103.3	107.9	-
.04	114.6	94.6	-	-
.05	112.7	93.3	106.5	85.6
.10	107.3	87.6	83.7	75.9
.25	65.9	64.9	-	-
.50	25.1	30.6	28.1	13.8
.75	11.5	17.0	-	-
1.00	8.2	11.9	7.7	-

Concentration	PEG 200	PEG 400.	PEG 1500.	PEG 4000.	PEG 6000.
moles					
0	100.0	100.0	100.0	100.0	100.0
.0010	109.0	104.5	92.8	93.1	80.8
.0025	105.8	107.3	90.2	95.4	84.6
.0050	107.9	104.4	92.6	86.4	-
.0075	107.6	102.5	69.3	87.2	40.8
.0100	109.7	102.6	91.2	75.8	-
.0250	102.7	105.1	69.6	15.3	8.9
.0500	107.2	101.7	22.8	-	-
.750	104.4	88.6	14.3	-	-
.1000	105.4	73.3	7.1	-	-

TABLE 32 (Contd).

Values of Kr expressed as % control for Ceratocystis adiposa.

Concentration	Sodium deoxycholate
moles	
0	100.0
10^{-5}	86.1
2×10^{-5}	65.4
3×10^{-5}	55.7
4×10^{-5}	45.5
5×10^{-5}	40.1
7.5×10^{-5}	44.4
10^{-4}	39.2
2×10^{-4}	28.7
5×10^{-4}	20.5
10^{-3}	20.5

* Results from two experiments are very different. The second set of results on Poly-ethylene-glycol of various molecular weights were obtained from plates which had been stored for some weeks at 4°C in order that the higher molecular weight plates might set.

TABLE 33.

Mean ± standard error for values of Kr of Botrytis fabae in mm/day. (n = 30. p = 0.05).

Conc. moles.	Glucose	Paramorphogen Sorbose	Xylose
0	3.34 ± 0.17	3.34 ± 0.19	3.19 ± 0.14
.01	3.41 ± 0.16	1.00 ± 0.07	3.49 ± 0.11
.02	3.29 ± 0.21	0.94 ± 0.08	-
.03	3.43 ± 0.19	0.62 ± 0.04	3.55 ± 0.18
.04	3.42 ± 0.17	0.57 ± 0.06	-
.05	3.38 ± 0.21	0.50 ± 0.04	4.37 ± 0.22
.10	4.01 ± 0.24	0.36 ± 0.03	4.14 ± 0.19
.25		0.25 ± 0.02	
.50	3.45 ± 0.19		
.75	2.69 ± 0.16		
1.00	1.94 ± 0.12		

	Polyethylene glycol 200.
0	3.22 ± 0.17
.01	3.88 ± 0.22
.05	3.43 ± 0.19
.10	3.57 ± 0.19
.25	2.62 ± 0.12
.50	1.52 ± 0.11
1.00	0.84 ± 0.06

TABLE 33.

Conc. moles.	Paramorphogen Sodium deoxycholate.
0	3.22 ± 0.17
10^{-5}	3.33 ± 0.14
2×10^{-5}	2.83 ± 0.17
3×10^{-5}	2.28 ± 0.14
4×10^{-5}	2.05 ± 0.15
5×10^{-5}	1.53 ± 0.12
7.5×10^{-5}	1.22 ± 0.07
10^{-4}	1.33 ± 0.12
2×10^{-4}	0.95 ± 0.06
5×10^{-4}	0.44 ± 0.05
10^{-3}	$\ll$ 0.2

TABLE 34.

Values of Kr expressed as % control for Botrytis fabae.

Concentration Moles	Paramorphogen			
	Glucose	Sorbose	Xylose	Polyethylene glycol 200
0	100.0	100.0	100.0	100.0
.01	102.1	29.9	109.3	120.5
.02	98.5	28.2	-	-
.03	102.8	18.7	111.2	-
.04	102.4	17.0	-	-
.05	101.3	14.9	136.8	106.5
.10	120.1	10.6	129.8	110.9
.25	-	7.6	-	81.5
.50	103.4	-	-	47.2
.75	80.7	-	-	-
1.00	58.2	-	-	26.0

Concentration Moles	Sodium deoxycholate.
0	100.0
10^{-5}	103.4
2×10^{-5}	88.1
3×10^{-5}	70.7
4×10^{-5}	63.7
5×10^{-5}	47.6
7.5×10^{-5}	37.8
10^{-4}	41.5
2×10^{-4}	29.5
5×10^{-4}	13.7
10^{-3}	6.2

TABLE 35.

Apical cell length and standard deviation (μm) for Ceratocystis adiposa.

Treatment	Apical cell length mean of 20. μm.	Standard deviation.
Control	140.4	42.3
.05M glucose	160.4	47.4
.05M sorbose	133.9	42.0
.05M polyethylene glycol.	142.1	33.8
.25M glucose	85.4	31.1
.25M sorbose	80.7	18.8
.25M polyethylene glycol.	91.8	21.4
1.0M glucose	28.2	11.7
1.0M sorbose	30.4	12.5
1.0M polyethylene glycol.	32.1	13.6
5×10^{-5} sodium deoxycholate.	90.7	34.3
10^{-4} M "	106.4	30.1
10^{-3} M "	24.3	10.2

TABLE 36.

Radius (mm) of Aspergillus chevalieri on glucose and polyethylene glycol. (n = 20. p = 0.05).

Time hours	Glucose · 1.0 mole	2.0 moles	2.5 moles	3.0 moles
23	2.10 ± 0.38	1.83 ± 0.26	+	-
48	8.48 ± 0.37	8.80 ± 0.32	8.40 ± 0.49	5.38 ± 0.36
72	14.05 ± 0.59	16.97 ± 0.32	16.80 ± 0.54	12.60 ± 0.42
98	19.83 ± 1.00	24.92 ± 0.64	26.35 ± 0.41	20.65 ± 0.48
166	27.68 ± 1.77			> 40
190	29.92 ± 3.99			
	3.5 moles	4.0 moles	5.0 moles	
48	2.45 ± 0.26	-	-	
72	6.65 ± 0.38	1.90 ± 0.31	-	
98	13.35 ± 1.56	3.78 ± 0.50	-	
166	28.18 ± 0.47	10.53 ± 0.61	3.35 ± 0.41	
190	32.97 ± 0.68	12.69 ± 0.74	-	
242	> 40	17.56 ± 0.88	7.03 ± 0.55	
264		19.66 ± 0.86	-	
335		25.50 ± 1.19	11.82 ± 0.48	
380			14.16 ± 0.91	

Time hours.	Polyethylene glycol 200. 0.5 mole	1.0 mole	1.5 moles	2.0 moles
23	1.50 ± 0.25	-	-	-
48	5.45 ± 0.56	5.60 ± 0.41	2.40 ± 0.29	-
72	9.18 ± 0.66	9.48 ± 0.50	5.88 ± 0.46	1.65 ± 0.24
98	12.85 ± 0.88	12.78 ± 0.53	9.24 ± 0.65	2.65 ± 0.44
166	18.47 ± 1.14	19.76 ± 1.16	17.39 ± 0.86	6.13 ± 0.49
190	21.32 ± 1.54	22.77 ± 1.29	20.43 ± 1.25	-
242	25.83 ± 4.01	27.15 ± 2.68	26.63 ± 1.46	8.88 ± 0.59
335				12.87 ± 1.29
380				15.86 ± 1.67

TABLE 37.

Growth rates of Aspergillus chevalieri.

Concentration Glucose (M).	Kr (mm/day).	Lag hours.
1.0	5.9429	15
2.0	7.4667	18
2.5	8.0000	21
3.0	7.0073	28
3.5	5.1613	36
4.0	2.2430	54
5.0	1.1915	100
Polyethylene glucose.		
0.5	3.7590 (up to 98 hrs)	15
1.0	3.2471 (up to 166 hrs)	23
1.5	2.9720	28
2.0	1.1555 from graph.	48

TABLE 38.

Dry weight mycelium per flask (mgm) at stated times since inoculation.

Ceratocystis adiposa.

Time hours.	Dry wt. ± 95% confidence limits.		
	Control	Glucose	Sorbose
19	10.52 ± 2.12	8.04 ± 8.01	7.40 ± 7.87
30	66.58 ± 24.56	65.12 ± 35.41	48.42 ± 26.34
43	163.02 ± 75.85	141.54 ± 58.55	143.46 ± 26.19
	Control	Polyethylene glycol	Sodium deoxycholate.
13	7.36 ± 2.07	7.22 ± 1.79	6.26 ± 0.87
21	53.98 ± 7.63	37.14 ± 9.58	41.22 ± 2.89
37	117.52 ± 7.59	77.98 ± 14.69	82.26 ± 6.47

Botrytis fabae.

	Control	Sorbose	Sodium deoxycholate
91	19.76 ± 5.62	13.64 ± 6.11	15.44 ± 4.55
115	27.14 ± 10.14	15.26 ± 9.41	26.96 ± 4.45
140	40.54 ± 10.13	30.68 ± 15.55	35.90 ± 15.49
164	57.86 ± 19.61	39.28 ± 9.09	43.76 ± 11.02

TABLE 39.

% germination and mean of 3 experiments using Ceratocystis adiposa on various concentrations of sodium deoxycholate in SSM.

Concentration of Sodium deoxycholate. (Moles).	A.	B.	C.	Mean.
0	100	100	100	100
10^{-4}	114.7	107.1	107.9	109.9
5×10^{-4}	55.6	91.0	91.4	79.3
7×10^{-4}	67.3	53.2	71.0	63.8
8×10^{-4}	18.0	4.5	21.6	14.7
9×10^{-4}	8.2	3.8	7.4	6.5
10^{-3}	3.3	1.1	2.9	2.4

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Control 12·25

3

COULTER-BA MODEL: DUAL THRESHOLD. SAMPLE: 0.5ml DATE: 1/3/77

GAIN CONTROL: 3 MATCHING SWITCH: ELECTROLYTE: 0.5% NaCl COINCIDENCE FACTOR: 2·5

APERTURE DIAMETER 100μm MANOMETER VOL: 0·5ml T: CALIBRATION FACTOR (K): 4·6

TABLE 40

T_L	T_U	I	A	Δn - RAW COUNTS				Σ Δn	Coin.	·	DIAM. RANGE μm	VOLUME μm x 10^{-3}	Δn	%	Σ Δnv.		
40		1		149	149			149		149	15·6	2·0					
31		1		197	218			206		206	14·5	1·6	57	0·8			
55		2		329	319			324		324	13·9	1·4	118	1·8			
47		2		440	405			423		423	13·2	1·2	99	1·5			
39		2		604	607			606		606	12·4	1·0	183	2·7			
31		2		906	885			895		895	11·5	0·3	289	4·3			
23		2		1535	1570			1553	15	1568	10·5	0·6	673	10·0			
37		3		2466	2460			2463	38	2501	9·8	0·5	933	13·9			
31		3		3641	3496			3568	80	3648	9·1	0·4	1147	17·1			
23		3		5224	5052			5152	166	5318	8·3	0·3	1670	24·8			
15		3		6562	6509			6535	267	6802	7·2	0·2	1484	22·1			
5		3		6541	6661			6601	275	6874	5·7	0·1	72	1·0			
20		3									2·7	·0041					

TABLE 41.

ln length of spore + germ tube (μm) with time.

Time hr.	Time min.	Values. (ln length μm).			
0		.	3.750	.	
0	15	.	3.807	3.359	2.931
0	30	3.314	3.912	3.481	3.168
0	45	3.481	4.052	3.624	3.219
1	00	3.624	4.174	3.778	3.219
1	15	3.750	4.334	3.984	3.401
1	30	3.861	4.541	4.193	3.555
1	45	3.984	4.723	4.317	3.624
2	00	4.135	4.877	4.541	3.807
2	30	4.398	5.207	4.798	3.912
3	00	4.828	5.521	5.051	4.230
3.	30	5.091	5.745	5.359	4.472
4	00	5.388	6.022	5.521	4.666

 Table 42.

Numbers of perithecia of Ceratocystis adiposa in four 5 mm fields

Medium	Temperature and Time		
Control	20°C		
	25°C		
	20°C	3 days	then 30°C
	30°C	1	20°C
	30°C	2	20°C
	30°C	3	20°C
0.25M Glucose	20°C		
	20°C	3 days	30°C
	30°C	1	20°C
	30°C	2	20°C
	30°C	3	20°C
0.25M Sorbose	20°C		
	20°C	3 days	30°C
	30°C	1	20°C
		2	
		3	
0.25M Polyethylene glycol.	20°C		
	20°C	3 days	30°C
	30°C	1	
		2	
		3	20°C
0.25 x 10^{-4} M. Sodium deoxycholate.	20°C		
	20°C	3 days	30°C
	30°C	1	20°C
	30°C	2	20°C
	30°C	3	20°C
	25°C		

grown on different media at different temperatures. (Fig 31&32)

Centre	Numbers per fields from centre.							edge
189	180	122	122	103	100	82	64	14
87	57	32	9	1	1			
94	32							
435	245	235	193	166	117	42	4	
615	216	149	195	252	191	142	25	
674	354	189	157	161	112	53	15	
41	62	32						
19								
142	30	15	11	6				
93	36	17	7					
263	38	70						
67	48	54	40	10				
47								
147	124	54	37	18	1			
230	74	29	17	14	1			
330	38	11	7					
277	141	73	64	96	94	89	79	43
3								
270	62	91	99	77	45	48	17	8
0	154	77	71	54	74	47	10	2
0	18	70	33	42	58	59	21	4
240	203	168	68	66	28	1		
84								
786	305	33	2					
605	198	25						
437	314	94	21					
42	105	22	14	17	21	5	5	

Table '43 Relationship between concentration and water potential.

PEG 200	Ψ - bars.	
.01 M	3.1	
.10 M	5.3	
.50 M	15.05	Depression of freezing point.
1.00 M	27.1	
PEG 1500		
.01 M	3.3	
.10 M	7.0	

PEG 4000 and PEG 6000 would not freeze.

PEG 4000 from Wescor Dew Point Microvoltmeter.

mM	- bars.	
0	1.6	Mexal & Reid 1973.
13.75	2.5	
32.50	5.3	
48.75	10.8	
57.50	16.7	
62.50	21.8	
62.50	23	
75.00	33	
87.50	45	
100.00	65	
106.25	80	
112.50	90	

Glucose.

0.01	M	2.39 atmos.	
0.10	M	11.55	Hand book of chemistry
1.00	M	23.8	& Physics.

Relationship between % Relative Humidity and Water Potential.

% RH	Water potential (- bars) (Leyton 1975)
99.9	1.46
98.9	14.60
89.8	146.0
34.0	1460.0

Table 44 % Germination with time of conidia of Ceratocystis Adiposa.

Fig 51.

Time (hours)	% germination.
0	0
1	0
3	22
$3\frac{1}{2}$	39
4	60
$4\frac{1}{2}$	68
5	72

After 5 hours, germ tubes tangled and it was not possible to count germinated spores in clumps.

Table 45

Dry weight, pH and titratable acid from cultures of C. adiposa.

Fig 72.

Time (hours)	Dry weight (mean of 3).		pH	titratable acid (ml N/10) (Na OH)	
2	13.0	8.6	5.80	0.91	.11
5	7.7	12.5	5.73	0.84	.16
8	8.8	4.1	5.63	0.97	.21
11	4.3	2.6	5.48	1.04	.24
24	89.3	23.0	4.10	1.27	.18
28	234.3	33.7	3.10	1.81	.10
96 (one flask)	574		2.80	2.00	

Table 46 Enzyme degradation of Ceratocystis adiposa cell wall.

Yield from 20 mgm of cell wall in mgm. Fig 35.

Enzymes	Glucose	Protein	N-acetyl glucos-amine.
None H_2O.	0.50	0.30	.03
Chitinase	4.50	0.93	.20
Pronase	3.35	1.10	.04
Cellulase	4.10	1.10	.03
Chitinase & Pronase	5.00	1.43	.14
Chitinase & Cellulase	6.25	1.25	.14
Pronase & Cellulase	6.10	1.48	.05
Chitinase, Pronase Cellulase	4.80	1.29	.09
Glucanase	2.80	1.10	.01
Glucanase & Chitinase	4.25	0.86	.12
Glucanase & Pronase	4.25	1.10	.02
Glucanase & Cellulase	6.00	0.86	.02
Glucanase, Cellulase, Chitinase & Pronase	6.00	1.48	.13

Table 47 Values of Kr of Ceratocystis adiposa for determination of Ks. Fig 26

Concentration (Molar)	Kr (µm / hour). Glucose	Sorbose
10^{-4}	320 ± 8.96	-
2×10^{-4}	330 ± 11.89	262 ± 9.31
3×10^{-4}	336 ± 10.98	255 ± 10.72
4×10^{-4}	354 ± 12.73	262 ± 8.27
5×10^{-4}	369 ± 17.48	254 ± 13.64
7.5×10^{-4}	377 ± 18.41	258 ± 12.17
10^{-3}	364 ± 11.47	239 ± 10.71
5×10^{-3}	406 ± 12.73	188 ± 12.64
10^{-2}	391 ± 11.89	136 ± 13.49
Receiprocals for graph.		
10000	3.125×10^{-3}	-
5000	3.030	3.817×10^{-3}
3333	2.976	3.922
2500	2.825	3.817
2000	2.710	3.937
1333	2.653	3.876
1000	2.747	4.184
500	2.469	5.319
100	2.558	7.353
Regression constant =	2.5276	coefficient 1.1087×10^{-4}

Coulter Counter Data for Fig. 56.

To show the effect of electrolyte on spore size distribution.

Diameter μm	% spores in each class.	
	First run	Second run
14.5	1.0	1.1
13.9	1.7	1.5
13.2	2.3	3.0
12.4	1.4	1.2
11.5	4.0	3.5
10.5	15.6	8.8
9.8	8.3	14.1
9.1	15.7	16.9
8.3	20.3	20.3
7.2	19.2	21.0
5.7	10.4	9.0

Coulter Counter data for Fig. 55.

Preliminary analysis of a 25°C spore suspension with a 200 μm aperture.

Diameter μm	Number of spores in each class.
33.6	9
26.7	95
24.2	112
21.2	481
17.9	394
14.5	1361
11.5	3008
9.1	4298
7.2	3398
5.7	1829
4.6	274

Coulter Counter Data for Fig. 57

Size distribution of spores
from different aged cultures.

Diameter µm	Age in days of culture.				
	5	7	8	11	15
14.5	0.3	1	1	1	2
13.9	0.6	2	2	1	3
13.2	0.6	2	2	1	2
12.4	0.9	2	3	2	4
11.5	1.2	4	5	3	5
10.5	3.0	7	9	7	9
9.8	6	11	13	12	13
9.1	10	11	13	15	12
8.3	19	20	17	22	19
7.2	40	25	25	23	22
5.7	19	15	10	13	8

Coulter Counter Data for Fig. 58

Germination of spores in SSM at 25° C.

Diameter µm	% after germination for				
	0	3	7	11	hours
14.5	1.0	0.8	1.0	1.1	
13.9	1.7	1.8	0.7	2.4	
13.2	2.3	1.5	0.7	1.8	
12.4	1.4	2.7	1.4	2.7	
11.5	4.0	4.3	3.2	3.8	
10.5	15.6	10.0	4.4	11.2	
9.8	8.3	13.9	9.2	16.5	
9.1	15.7	17.1	11.4	17.3	
8.3	20.3	24.8	22.5	17.8	
7.2	19.2	22.1	22.9	19.0	
5.7	10.4	1.0	22.5	6.4	

'Coulter Counter Data for Fig. 59 & 60.

Size distribution of spores after 5 hours incubation in different media at 25° C.

Diameter μm	Control	Media		Sodium deoxy-cholate.
		Glucose	PEG	
14.5				3
13.9				5
13.2	0.2	1	1	4
12.4	2	2	2	5
11.5	3	3	3	7
10.5	7	6	7	9
9.8	14	10	11	15
9.1	15	14	13	11
8.3	23	19	19	22
7.2	25	30	28	15
5.7	11	15	16	4
	After 10 hours incubation.			
14.5	1.1	1.2	0.4	1.1
13.9	2.4	1.5	1.2	1.6
13.2	1.8	1.8	0.8	1.6
12.4	2.7	2.8	1.5	3.0
11.5	3.8	3.9	2.3	5.2
10.5	11.2	10.7	5.4	9.3
9.8	16.5	15.6	6.1	15.7
9.1	17.3	17.1	9.0	15.0
8.3	17.8	22.4	18.7	19.5
7.2	19.0	20.1	29.2	19.2
5.7	6.4	2.9	25.3	8.7