PROTOPLAST PRODUCTION AND LOCALIZATION OF CELLULASE IN SPECIES OF ASPERGILLUS.

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DECLARATION.

While registered as a candidate for the degree of PhD with the CNAA the candidate has not been a registered candidate for any other award with the CNAA or of a University.

Signed 25th June 1981

ADVANCED STUDIES, COURSES AND CONFERENCES UNDERTAKEN AND ATTENDED IN CONNECTION WITH THE PROGRAMME OF RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF Ph.D.

1. October 1977 to June 1978 (114 h).
   A course in microbiology in the Department of Biological Sciences, Sheffield City Polytechnic.

2. Between May 1977 and September 1978.
   Trips to the Biodeterioration Centre at Aston University in Birmingham, to abstract information on extracellular enzymes from micro-organisms.

3. Between December 1977 and September 1978.
   Two visits to the University of Strathclyde to discuss aspects of fungal research with Professor J.E. Smith and observe relevant laboratory techniques.

4. October 1977 to March 1980.
   Organised a series of postgraduate research colloquia in the Department of Biological Sciences, Sheffield City Polytechnic.
   1 h per week.

5. October 1977 to March 1980.
   Attended regular research colloquia with staff in the Department of Life Sciences at Trent Polytechnic, Nottingham and the Department of Botany at Nottingham University.

In addition the following conferences have been attended ;

1. 5th - 7th April 1978.

   "Wall and Hyphal Growth", British Mycological Society, Fungal Physiology Group, Queen Elizabeth College, University of London.

2. 8th - 11th September 1978.

   "Workshop on Techniques in Mycology Teaching", British Mycological Society, University of Exeter. A communication on protoplast production was given at this meeting.

3. 18th - 20th September 1978.

   "Fungal Biotechnology", British Mycological Society, Fungal Physiology Group. University of Glasgow.

Forays attended

1. 5th - 12 September 1979.

   Annual Autumn Foray, British Mycological Society, Foray Group, University of Surrey, Guildford.

2. 16th - 22nd May 1980.

   An invitation was extended from the British Mycological Society to organise and act as local secretary for the Annual Spring Foray. This was accepted and the foray took place at Loosehill Hall, Castleton, Derbyshire.

Teaching committments.

Whilst in the Department of Biological Sciences, Sheffield City Polytechnic the following courses were taught,

1. HNC Medical Laboratory Science, Cell Biology.
2. HNC.Medical Laboratory Science, Science Methods.
3. HND Applied Biology, Cell Biology Practicals.
4. BSc, Applied Science , Cell Biology Tutorials.
5. Various talks and demonstrations were also given to a number of schools visits, illustrating modern biological techniques, with special reference to the electron microscope.
6. A number of students were also instructed in their special projects for HND and BSc courses. These projects involved some practical application of microbiology viz,
   a. Investigation into the effects of heavy metals on protoplast release from A. niger.
   b.. Investigation into the effects of sugars on the release of cellulase from A. niger.
   c. Investigation into the ultrastructure of cell wall degradation of A. niger by different enzyme treatments.
   d. Investigation into the wall structure of brewing yeasts under different growing conditions.

CONTENTS.

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ABBREVIATIONS.

A. fumigatus.	*Aspergillus fumigatus*.
A. nidulans.	*Aspergillus nidulans*.
A. niger.	*Aspergillus niger*.
A. terreus.	*Aspergillus terreus*.
BMC	Ball milled cellulose.
BSA	Bovine serum albumin.
$CaCl_2$	Calcium chloride.
$Ca(NO_3)_2$	Calcium nitrate.
CMC	Carboxymethylcellulose.
CMCase	Carboxymethylcellulase, (Endo-1,4-β-glucanase).
DNS	Dinitrosalicylic acid.
DMAB	*p*-Dimethylaminobenzaldehyde reagent.
D.p.m.	Decompositions per minute.
EDTA	Ethylene diamine tetra acetic acid (di-sodium salt).
h	Hour.
HCl	Hydrochloric acid.
HEPES	*N*-2-hydroxyethylpiperazine sulphonic acid.
I.M.I.	International Mycological Institute.
KOH	Potassium hydroxide.
L	Litre.
M	Molar
mM	Millimolar.
mg	Milligram.
$MgCl_2$	Magnesium chloride.

$MgSO_4$	Magnesium sulphate.
min	Minute.
ml	Millilitre.
mRNA	Messenger RNA.
mU	Milliunit.
NaCl	Sodium chloride.
NH_4Cl	Ammonium chloride.
nm	Nanometre.
PAHBAH	p-Hydroxybenzoic acid hydrazide.
PCGT	Phosphorylcholine-glyceride transferase.
RNA	Ribonucleic acid
SEM	Scanning electron microscopy.
TEM	Transmission electron microscopy.
TRIS	Tris(hydroxymethyl)-aminomethane.
T. viride.	Trichoderma viride.
µ	Micrometre.
U	Unit.
µg	Microgram.

ABSTRACT.

PROTOPLAST PRODUCTION AND LOCALIZATION OF CELLULASE IN SPECIES OF ASPERGILLUS. By Keith Robert Thomas.

The production of a cellulase enzyme by Aspergillus fumigatus (Fres) was investigated using a number of techniques including cell fractionation, cold osmotic shock, wall digestion and histochemical localization. The production of the enzyme by protoplasts of the fungus was also followed and investigated.

To enhance the production of protoplasts the factors affecting the release of protoplasts from A. niger and A. fumigatus were investigated. Factors which strongly affected protoplast release were pH, temperature, concentration of the osmotic stabilizer and the concentration of calcium ions. The hydrolysis of the inner, chitin wall - layer was an important factor in ensuring optimal protoplast release. For this reason chitinase was included in the digestion medium.

Carboxymethylcellulase (CMCase) was produced in the mycelium of A. fumigatus growing on ball milled cellulose (BMC) and on carboxymethylcellulose (CMC). Actively growing mycelia released the enzyme into medium containing BMC but not into medium containing CMC. Sharp peaks of enzyme synthesis and activity were evident after an initial lag period. Supplementation of the culture medium with glucose considerably reduced this lag period.

Localization procedures indicated that the enzyme occurred within the cell wall, in the periplasmic space and upon, or enclosed by, membranes. CMCase was released by the action of chitinase enzymes and by cold osmotic shock. It was also sedimented by high-speed centrifugation of cell homogenates from young mycelia. Older mycelia contained more soluble CMCase. Histochemical localization showed the enzyme to be present in cytoplasmic vesicles and in the cell wall. Triton X-100 acted to increase the release of CMCase in crude homogenates and in centrifuged fractions.

Protoplasts regenerating in CMC medium released CMCase into the medium but retained little in the cytoplasm. Cycloheximide was found to substantially reduce the production of CMCase by protoplasts and protoplasts from mycelium already producing CMCase were not predisposed to synthesise the enzyme.

The Aspergilli are a group of fungi important in many ways. Members of the genus have been exploited commercially for over 80 years and their ability to hydrolyse almost every biological macromolecule has ensured their continued investigation and use. Their rapid growth is an additional advantage in the commercial respect, particularly when coupled with a rapid production of secondary metabolites. Alternatively this and their ability to grow in a wide variety of habitats have caused concern with regard to the contamination and deterioration of foods and materials. Production of toxic metabolites is also an undesirable aspect of some members of the genus and is also receiving attention at present (Purchase, 1971 and Moss, 1977). The continued reliance on Aspergillus niger for commercial production of citric acid (Berry et al., 1977) is, however, a good indication of the value of the genus and the information available on many aspects of the group indicates their continuing potential (Raper and Fennell, 1965 and Smith and Pateman, 1977).

Aspergillus fumigatus is responsible for considerable spoilage of materials and moreover, being thermotolerant, has the status of an opportunist pathogen able to grow at mammalian body temperatures (Edwards and Al-Zubaidy, 1977). A number of reports have demonstrated that A. fumigatus is commonly found in conditions of cellulose decomposition such as straw composting (Chang and Hudson, 1967 and Mills, 1973) and in woodchip piles (Flannigan and Sagoo, 1977) as well as in sewage sludge (Millner et al., 1977) and during barley processing (Sellars et al., 1976). Little attention has, however, been given to determining the extent by which the organism is able to use cellulosic substrates or towards elucidating the methods of growth or enzyme synthesis involved in this. Information on these factors would not

only aid in the control of the organism but would also be of relevance to the purposeful use of the fungus, for example, in the rapidly growing concern regarding the recycling of cellulosic materials (Fig. 1.1).

It has been estimated that 4×10^{10} tons of cellulosic biomass are produced each year (Coughlan and Folan, 1979) and although only a small percentage of this may be recoverable for commercial use the value in terms of raw material is very large. There are two ways in which this cellulose may be used, each of which poses different attitudes towards the involvement of cellulosic micro-organisms. Firstly, cellulose may be used as a structure itself in the form of wood or as a modified derivative of this,such as paper or cellophane. The growth of a celluloytic micro- organism on these is not to be encouraged until such time as the resource is to be disposed of, whereupon active growth may be desirable. Much work is thus orientated towards discouraging growth of fungal organisms while the cellulose material is in use and encouraging growth to degrade it when it is discarded. The former process involves the development of anti-fungal agents (Kobayashi and Medoff, 1977) and an understanding of the factors preventing fungal growth, while the latter aspect requires an encouragement of growth and also knowledge of the factors which regulate growth on cellulose.

Secondly, not only may cellulose be used in its original form, but through recent developments in processing technology,it has become apparent that cellulose may be converted to valuable secondary compounds such as glucose (Wilke and Mitra, 1975), protein (Bellamy, 1974 and Gupta et al., 1976) or ethanol (Miller, 1975). From these a range of further,chemically important derivatives,such as plastics,may be obtained (Edwards, 1975 and Goldstein, 1976). Since glucose and protein may be used as food and ethanol as a combustable fuel

the potential of cellulose in these terms alone is considerable.

In order to convert cellulose to these products the cellulolytic abilities of micro-organisms are particularly relevant. Pyrolysis and acid hydrolysis will convert cellulose to energy and sugar respectively but will not provide the range or efficiency required and moreover may, as in the production of glucose, also produce unwanted decompositions (Goldstein, 1976). The advantage of using enzymes to produce a more rapid, efficient and directed reaction is therefore well appreciated.

The basic scheme of using cellulase enzyme solutions, extracted from micro-organisms, to hydrolyse cellulose has been widely discussed and investigated (Wilke et al., 1976). The possibilty of growing the organisms directly upon the substrate has also been considered (Toyama, 1976). Despite the apparent simplicity of these processes a number of limitations are nevertheless encountered and centre upon the cost of obtaining raw materials in sufficiently hydrolysable condition and the cost of producing sufficient amounts of highly active enzyme (Brandt, 1975). Sources of the former are commonly waste or by-products such as straw or other agricultural residues and in considering these it is necessary to note the limitations imposed on the hydrolysis by the molecular structure of cellulose. In its natural condition cellulose has been shown to possess a high degree of crystallinity (Cowling, 1975) and because of this has few available sites for enzyme attack. In order to increase the surface area pre-treatments are necessary and range from alkali incubations to swell the fibres to ball milling and irradiation (Millett et al., 1975 and Halliwell, 1977). Another limitation is due to the presence of subsidiary, protective residues such as lignin. Again these act to

restrict access of cellulase enzymes and for an efficient digestion must be removed by an appropriate treatment. Obviously such processes can add substantially to the cost of the cellulosic substrate in a commercial process and may even involve the use of additional enzymes, such as xylanase, to digest the subsidiary residues (Ghose and Bisaria, 1979). A further danger is that undigested residues may clog the equipment involved. However, waste products from industrial processes which have already extracted some contaminating substances are more suitable raw materials, for example bagasse from sugarcane processing.

Alternatively, the use of cellulosic fractions derived from municipal waste collections pose the additional problem of contamination with heavy metals and toxins liable to inhibit enzyme hydrolysis. Such wastes would probably be better suited to the production of fuels rather than food in case of possible carry over of these contaminants.

The need for pre-treatments may possibly be reduced by using more active cellulase enzymes or types specificelly suited for hydrolysing crystalline regions. Similarly, a combination of cellulases with other enzymes, for example ligninases, may be of great advantage. A full understanding of the modes of action of the enzymes used is, however, required if they are to be used to their optimum efficiency, particularly on more resistant substrates. The cost of the enzymes themselves is also a limiting aspect of cellulose degradation and considerable effort is being expended to increase yields from micro-organisms (Mandels and Andreotti, 1978 and Montenecourt et al., 1079) and to devise efficient processing schemes and continuous culture procedures (Wilke et al., 1979 and Ryu et al., 1979).

Efforts to increase yields of cellulase enzymes have involved screening micro-organisms for their ability to secrete cellulases

(Mandels and Weber, 1969 and Pyč et al., 1977) and also investigating the factors controlling release of the enzyme from a few, highly cellulolytic bacteria (Yamane et al., 1970a and Berg et al., 1972a) and fungi (Sternberg, 1976; Mandels and Andreotti, 1978 and Folan and Coughlan, 1979). Although not all organisms have been investigated it has been noted that an organism which readily degrades cellulose does not necessarily produce large amounts of cellulase enzymes (Rautela and Cowling, 1966 and Wabnegg et al., 1978). It has also been noted that not all cellulolytic micro-organisms produce the full range of cellulase enzymes (Mandels, 1975). A suitable micro-organism for the production of cellulase enzymes should preferably possess the ability to secrete a full range of the enzymes in high concentrations, although the possibility of using dual cultures containing two, or more, micro-organisms should not be overlooked (Somkuti, 1974 and Khan, 1977). An intensive investigation of one cellulolytic fungus, Trichoderma viride (harzianum), has produced a considerable advance in the understanding of cellulase enzymes (Mandels, 1975). However, reports on other organisms producing as much cellulase and/or with a greater growth rate may provide further information on aspects lacking in T. viride (Folan and Coughlan, 1979). Relevant additional properties would include the production of cellulase enzymes not synthesised by T. viride, enhanced growth rates and the ability to grow in different conditions and temperatures.

One difficulty in the comparison of cellulase production by micro-organisms is that many important assessments have used the activity of cellulase enzymes in the culture filtrate as a criterion of synthesis (Mandels, 1975 and Montenecourt et al., 1979). However, a low extra-cellular secretion does not necessarily imply an inability to

synthesise the enzyme. For instance recent work on cellulase release indicates that some cellulase enzymes are intracellular, for example β-glucosidase (Deshpande et al., 1978 and Vaheri et al., 1979) while other cellulase enzymes are only secreted in response to an appropriately stimulating medium such as solid cellulose (Yamane et al., 1970a and Berg, 1975). Moreover, even if the enzymes are secreted their affinity for solid cellulose substrates may be very high resulting in considerable adsorption (Halliwell and Griffin, 1978). This is particularly so for highly active cellulase enzymes or for certain types of the enzyme (Bisaria and Ghose, 1977). An assessment of culture filtrates may therefore readily omit the presence of such enzymes within the filtered residues and thus underestimate the ability of the organism to produce the enzyme.

Nevertheless,considering cellulase synthesis as a whole, a number of limitations relevant to industrial extraction have been identified and recent investigations have begun to provide information on these (Mandels and Andreotti, 1978 and Montenecourt et al., 1979). From a commercial point of view it is desirable to obtain as much recoverable cellulase within the shortest possible time of growth. Factors which affect these yields include the late release of enzyme from the growing organism, the effect of substrate type and concentration, adsorption of the enzyme onto the substrate, effects of inducers and repressors and the controls governing intracellular synthesis and subsequent secretion.

A considerable amount of information is available regarding the adsorption of cellulase enzymes onto solid cellulose and this process would appear to be a necessary consequence of enzyme action since the most active enzymes are the most strongly adsorbed (Ghose and Bisaria,

1979). Some workers have further proposed that this process is the first requisite for enzyme action (Wood and McCrae, 1977). The implications of this are that best yields will result from extractions after the cellulose has been degraded and that culture filtrates extracted before this will contain less active enzyme. Unfortunately growth on pure cellulose is generally slow, particularly in commercial terms, and procedures to release the adsorbed enzymes from the cellulose may need to be developed (Bisaria and Ghose, 1977). It has also been observed that the enzyme may adsorb to the hyphal walls of the organism (Berg and Pettersson, 1977). Procedures to release cellulase enzymes bound to or within the cell wall have been developed and include the use of detergents (Reese and Maguire, 1969 and Sternberg 1976) and osmotic shock (Heppel, 1967 and Ramasamy and Verachtert, 1980). Such a localization of cellulase enzymes within the cell wall may well be advantageous to the organism in that it allows release of the digestion products at a position where they may be readily taken up by the cytoplasm.

Another factor relevant to the secretion of cellulase enzymes is the influence of substrate type and concentration. A number of studies have noted that solid celluloses induce high levels of cellulase enzymes (Suzuki et al., 1969 and Berg et al., 1972a). However the actual induction of the enzyme appears to be due to one of the hydrolysis products of the reaction, possibly the di-glucose sugar cellobiose (Mandels and Reese, 1960 and Ericksson and Hamp, 1978). Similar compounds also act as inducers, for example lactose (Lobanok and Pavlovskaya, 1977 and Ryu et al., 1979) and the β-1,2-disaccharide sugar sorphorose (Mandels et al., 1962). The use of inducers to stimulate enzyme production is particularly promising since they may

be used in the absence of the substrate itself (Vaheri et al., 1979). In contrast, glucose, the final substrate of cellulose hydrolysis, has been found in many cases to inhibit cellulase synthesis (Sternberg, 1976). Consequently in conditions of cellulose hydrolysis care must be taken to keep glucose concentrations as low as possible.

The concentration of cellulose substrate has also been studied and reports have shown that a high level can induce a high synthesis of cellulase enzymes (Targonski and Szajer, 1979 and Nystrom and Diluca, 1977). Substrate concentration may even be increased to the point of having a solid process such as that employed in the Koji system in the Orient. Here Aspergillus strains are inoculated onto a mixture of beans and rice to produce soy sauce by fermentation in a solid system (Wood, 1977). Control of production and subsequent extraction of enzymes from these conditions, however, becomes difficult.

The regulation of enzyme release from the mycelium according to the growth state of the mycelium may in some cases be an influential factor and it has been noted that for T. viride only low levels of cellulase enzymes are evident in the culture medium until most of the substrate has been digested (Mandels and Andreotti, 1978). Since growth of most organisms on cellulose is slow there is a strong possibility of this late release being consistent with the autolysis of the organism, particularly since extensive autolysis has been observed in T. viride (Berg and Hofsten, 1976) and in other organisms (Berg et al.,1972b) growing on cellulose. Procedures to avoid autolysis and to encourage an earlier secretion of enzyme involve the addition of supplementing nutrients which may be more readily utilised to provide an early bulk of mycelium (Sternberg, 1976 and Trivedi and Rao, 1980). Alternatively more rapidly growing organisms, such as

the thermophiles, may be an advantage (Bellamy, 1974; Lee and Blackburn, 1975 and Romanelli et al., 1975).

More regular growth forms may also solve this and other problems particularly as the development of the hyphal form segregates the organism into actively growing regions and older areas with more degenerative contents. The use of spherical forms, such as yeasts, allows a more uniform cell structure although few cellulolytic fungi appear to possess yeast morphologies. This may in fact reflect the advantage of a hyphal form in penetrating a solid substrate. The slow growth of bacteria on cellulose would be in agreement with this (Berg et al., 1972b).

For ease of manipulation spherical forms would be most desirable and investigations into modifying cellulolytic fungi would aid this aim. Some advance has been made with the discovery of giant, spherical cells of A. niger (Anderson and Smith, 1971; 1972) although the potential of these has yet to be realised. Alternatively techniques are readily available to obtain protoplasts from many fungi (Peberdy and Isaac, 1976) and the use of these, particularly as they lack a cell wall, has been shown in previous studies to be of great value in the production of extracellular enzymes (Andres and Peberdy, 1974) cellular enzymes (Archer, 1977) and metabolites (Dutton and Anderson, 1978).

Detailed studies of protoplast release (Peberdy et al., 1976) indicate that individual fungi may differ greatly in their response to protoplasting techniques and also that a range of culture conditions and incubation parameters may affect the quantity and quality of protoplasts released. In order to ensure an efficient production of protoplasts it is desirable that the particular species in question

is investigated for its response to lytic digestion and that the appropriate parameters are optimised. Considerable work has been conducted to determine these for A. niger (Musilkova and Fencl, 1966; 1968 and Davis et al., 1977) but only brief notes have been reported for A. fumigatus (Archer, 1977; Ferenczy et al., 1977 and Hearn et al., 1980). Similarly, little information is available on the details of cellulase synthesis in these two species.

The difficulty of obtaining high yields of cellular extracts is one problem in this respect since many fractionation procedures for fungi require considerable force to rupture cell walls and these consequently lyse cell organelles. The use of protoplasts obtained by enzymatic digestion of cell walls is one way in which cell fractions may be obtained without undue disruption of cell organelles. In many cases, however, low yields may result unless optimal conditions are employed.

As well as investigating the factors determining synthesis and release of cellulase enzymes much effort has been directed towards the selection of strains which hyperproduce the enzyme (Gallo, 1978 and Montenecourt et al., 1979). These studies have resulted in strains of T. viride able to produce many times more enzyme than the original wild type (Nevalainen et al., 1980). A full understanding of the genetic controls of cellulase synthesis, secretion and expression are necessary for such work to be successful and once the genes are identified there is the further possibility of using recent developments in genetic engineering to allow a much more efficient and controlled production of the enzyme.

It should also be noted that this need not be restricted to the consideration of just one organism and while the pathogenic qualities

of some thermophilic fungi are undesirable their rapid growth features (Lee and Blackburn, 1975; Loginova and Tashpulatov, 1965 and Folan and Coughlan, 1979) and the high thermostability of their enzymes (Singleton and Amelunxen, 1973) would be desirable features to include in any ultimate genetic combination.

An investigation of the cellulolytic abilities of A. fumigatus and the characteristics of the cellulase enzymes produced relates well to these aims. Further, the increasing importance of protoplasts in cellular and metabolic studies may be particularly valuable in this as they allow an easier access to the cytoplasm as well as providing a system free from the restraining cell wall.

To determine more information on the aspects outlined above this thesis aims to

i) investigate certain aspects relating to the maximum production of osmotically stable protoplasts from two cellulolytic species of Aspergillus;

ii) investigate the synthesis and intracellular localization of cellulase in A. fumigatus using both protoplasts and the filamentous form.

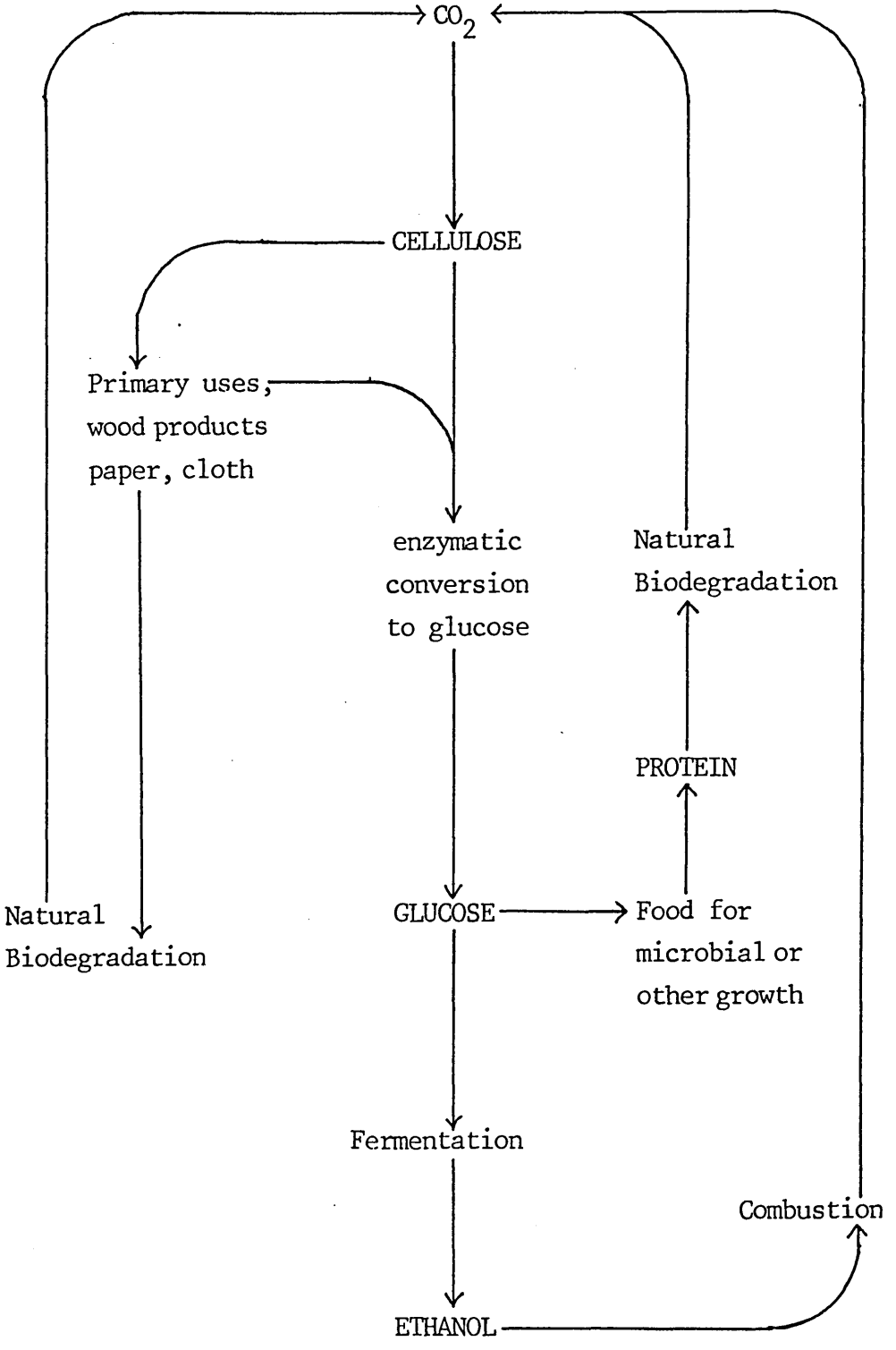


FIGURE 1.1. The carbon cycle according to cellulose.

2.1. ORGANISMS.

a) Aspergillus niger, University of Strathclyde strain, was provided by Dr B Davis, Department of Biological Sciences, Sheffield City Polytechnic.

b) Aspergillus fumigatus, (Fres. IMI 143864), a white, cellulolytic mutant, was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England.

c) Aspergillus fumigatus, (Fres. IMI 226070), was a strongly cellulolytic strain isolated from soil by plating out a soil suspension onto Eggins and Pugh (1962) cellulose medium (Table 2.1). Plates were incubated for 2 days at 48°C to selectively encourage thermophilic growth and spores of a suitably vigorous colony were aseptically transferred to fresh plates. A spore suspension, in distilled water, was obtained from the growth on these plates after the same incubation procedure and was diluted in sterile 0.9% (w/v) NaCl to cover the range 1×10^{3} to 1×10^{12} spores per ml.

Duplicate 0.5 ml samples of each dilution were spread on fresh plates of the same medium and incubated as above. The dilution which produced a single, fast growing colony was selected and used for inoculation onto the same medium contained in 1 oz McCartney bottles. These were maintained as stock cultures of the strain. The Commonwealth Mycological Institute identified the strain as A. fumigatus and numbered it IMI 226070.

d) Trichoderma viride (harzianum), was provided by Dr B Davis, Department of Biological Sciences, Sheffield City Polytechnic.

2.2. MEDIA AND CULTURE CONDITIONS.

Stock cultures of A. niger were produced by growing the fungus in 1 oz McCartney bottles at 30°C for 5 days on Eggins and Pugh cellulose medium (Table 2.1). Stock cultures of both A. fumigatus strains were produced as described previously. After the growth period, wherein a dense sporulation was evident, the bottles were stored at 4°C until required for use. The cultures were sub-cultured by aseptic transfer every 8 - 10 weeks and reserve samples of spores were freeze dried and also stored at 4°C in sealed ampoules.

Conidia from all Aspergillus strains were obtained for inoculation of liquid cultures by growing the fungi on bread. Five to ten g of homemade bread were cut into small cubes and placed into 250 ml Erlenmeyer flasks and autoclaved for 15 min at 121°C and 15 Ib per sq in. Spores of the appropriate strain were obtained by washing slopes with 5 ml of sterile distilled water. These suspensions were added to the bread and the flasks incubated at 30°C for 10 to 14 days. Conidia were harvested by covering the bread with sterile distilled water, shaking vigorously and filtering the suspension through glass wool to remove particles of bread and hyphae. The conidia were washed three times in sterile distilled water, shaken on a Griffin flask shaker to break up clumps of spores, counted on a haemocytometer and stored at 4°C until required for use.

To obtain mycelia for protoplast experiments spores were grown in 30 ml of liquid medium (Table 2.2) contained in 100 ml Erlenmeyer flasks. These were inoculated with 1×10^6 conidia per ml of culture medium and incubated at 30°C on an orbital shaker at 200 rpm.

TABLE 2.1

EGGINS AND PUGH CELLULOSE MEDIUM

Ammonium sulphate	0.5 g
L-Asparagine	1.0 g
Potassium dihydrogen phosphate	1.0 g
Potassium chloride	0.5 g
Magnesium sulphate	0.2 g
Calcium chloride	0.1 g
Difco yeast extract	0.5 g
Cellulose (CMC or BMC)	10.0 g
Agar	20.0 g
Distilled Water	1.0 L

The above components were mixed and autoclaved at 15 Ib per sq in and 121^{o}C for 15 min after adjusting the pH to 6.2 with HCl or KOH. Glucose or other carbon substrates were substituted for the cellulose as required.

Liquid medium for the induction of cellulase enzymes was that of Eggins and Pugh (1962) as described in Table 2.1 but without agar. Glucose or other sugars were added as required at a concentration of 0.2% (w/v) to enhance the initial rate of growth in standard experiments. Flasks were inoculated as described previously and incubated at 30 or 48°C for the times indicated.

T. viride was maintained as for the Aspergillus strains in 1 oz McCartney bottles, with medium as described in Table 2.3. Cultures were grown for 5 days at 25°C and then, to induce sporulation, for 5 days at 30°C in an illuminated incubator. Liquid medium for the production of lytic enzymes for protoplast production was as described in Table 2.3 but without agar and the glucose was replaced with an equal weight of dried, autoclaved, hyphal walls of A. fumigatus grown in protoplast (Table 2.2) or cellulose (Table 2.1) medium. Cell walls were prepared by sonicating autoclaved hyphae until extensive fragmentation was evident and then washing the fragments until no cytoplasmic remains were evident with methylene blue stain.

Medium for the comparison of cellulolytic activity of fungal strains was that of Eggins and Pugh (1962) with ball milled cellulose and the addition of Rose Bengal at a concentration of 0.035 g per L to aid resolution of the clearing fronts. Fungal strains from stock cultures were inoculated onto the solid medium in 25 ml glass test tubes and incubated at 30 or 48°C for 21 or 10 days respectively.

TABLE 2.2

CULTURE MEDIUM FOR ASPERGILLUS NIGER AND ASPERGILLUS FUMIGATUS.

Ammonium nitrate	3.0 g
Glucose	25.0 g
Potassium dihydrogen phosphate	2.0 g
Magnesium sulphate	0.5 g
Stock salt solution	1.0 ml
Distilled water	1.0 L

Stock salt solution

Ferrous sulphate	1.2 g
Zinc sulphate	1.6 g
Copper sulphate	0.2 g
Manganese chloride	0.5 g
Calcium chloride	2.0 g
Distilled water	100.0 ml

The above components were mixed and the pH adjusted to 4.5 with HCl. The mixture was autoclaved at 15 Lb per sq in for 15 min at 121^{o}C. The stock salt solution was stable for 6 months at 4^{o}C.

TABLE 2.3.

CULTURE MEDIUM FOR TRICHODERMA VIRIDE (HARZIANUM).

Glucose	0.3 g
Ammonium sulphate	1.0 g
Potassium dihydrogen phosphate	1.4 g
Potassium chloride	1.0 g
Magnesium sulphate	0.2 g
Calcium chloride	0.1 g
Mycological peptone	1.0 g
Glycerol	6.0 ml
Agar	20.0 g
Distilled water	1.0 L

The above components were mixed and the pH adjusted to 4.5 with HCl. The mixture was autoclaved at 15 Lb per sq in for 15 min at 121^{o}C. When necessary the glucose was omitted and subsituted with fungal cell walls or other polymers as described in the text.

2.3. SUGAR ASSAYS.

a) Total reducing sugar.

Total reducing sugars, released from hyphal walls during enzyme digestions for protoplast release, were quantitatively assessed by the dinitrosalicylic acid (DNS) procedure of Miller *et al.* (1960). The reagents used are listed in Table 2.4. To 3.0 ml of the reagent were added 0.1 to 0.5 ml of the sample and the mixture boiled for 10 min in a water bath, allowed to cool to 20°C and the absorbance read at 550 nm against a water blank. The calibration standard (Fig. 2.1) was conducted in incubation medium used for protoplast release (page 41) so as to avoid discrepancies which may be caused by buffer interference of colour development (Eveleigh, 1967).

b) N-Acetylglucosamine sugar.

N-Acetylglucosamine sugars were assessed by the method of Reissig *et al.* (1955) using p-dimethylaminobenzaldehyde reagent (DMAB). Stock solutions of this reagent were prepared as indicated in Table 2.5 and for immediate use this was diluted to 1 in 10 with glacial acetic acid. For development 0.1 to 0.5 ml of the samples were first boiled for exactly 3 min with 0.1 ml of 0.8 M potassium tetraborate, pH 9.2. After cooling to 20°C,3.0 ml of diluted DMAB reagent were added and the mixture incubated at 37°C for 20 min. The samples were read immediately at 585 nm. N-Acetyl-glucosamine in the range 0.2 to 1.2 mg dissolved in protoplast incubation medium was used as standard (Fig. 2.2).

TABLE 2.4

PREPARATION OF DNS REAGENT

3,5-Dinitrosalicylic acid	10.6 g
Sodium hydroxide	19.8 g
Distilled water	1416.0 ml

These components were mixed until dissolved and the following added.

Sodium potassium tartrate	306.0 g
Phenol	7.6 ml
Sodium metabisulphite	8.3 g

TABLE 2.5

PREPARATION OF DMAB REAGENT

p-Dimethylaminobenzaldehyde	10.0 g
Glacial acetic acid	90.0 ml
Concentrated hydrochloric acid	10.0 ml

This stock was diluted 1 in 10 with glacial acetic acid immediately before use.

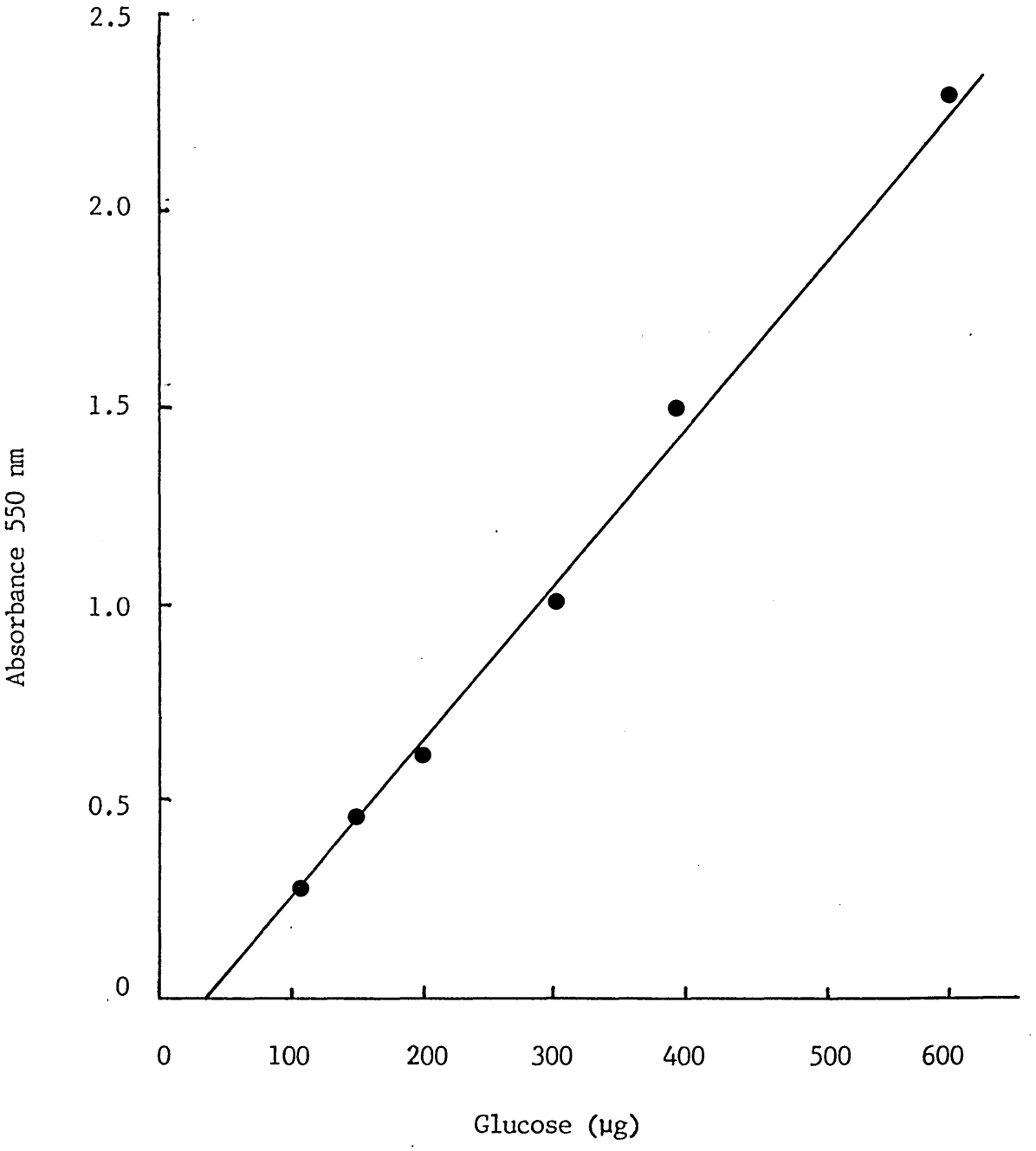


FIGURE 2.1. Standard calibration graph for reducing sugars measured with the DNS reagent. Glucose samples, in 0.1 ml protoplast incubation buffer, were processed as described in Materials and Methods.

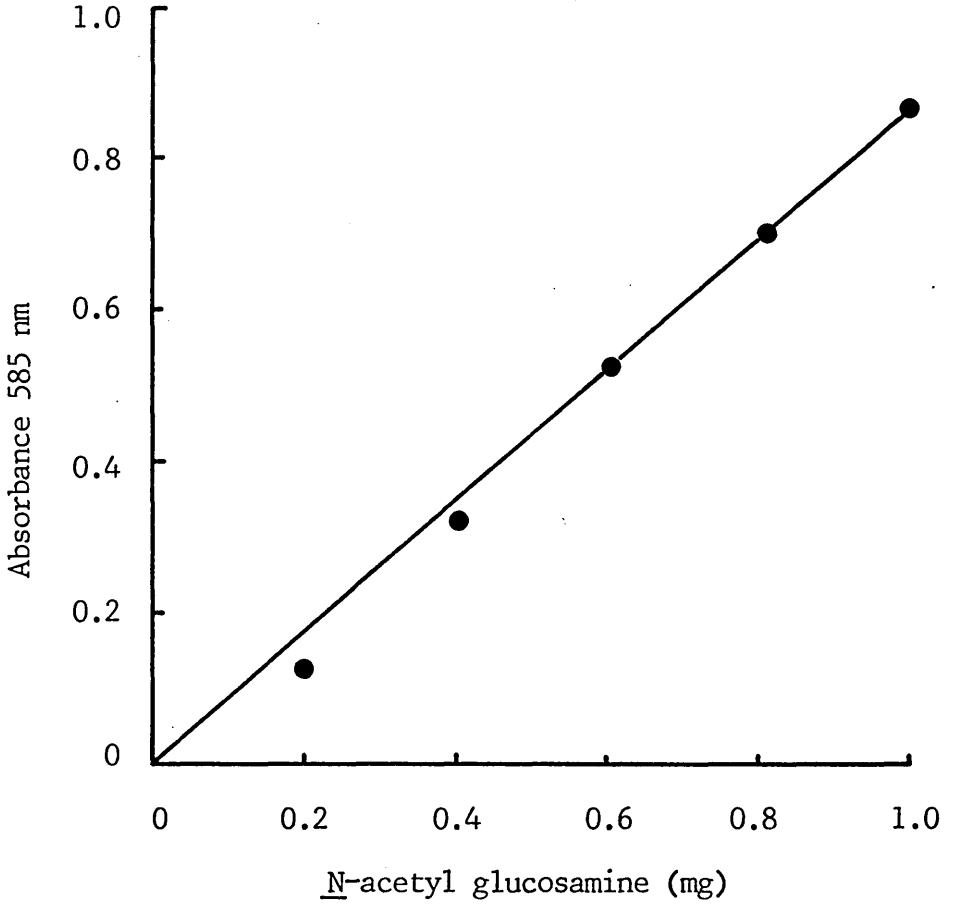


FIGURE 2.2. Standard calibration graph for N-acetyl sugars measured with the DMAB reagent. N-acetyl glucosamine samples, in 0.1 ml protoplast incubation buffer, were processed as described in Materials and Methods.

c) Reducing sugar groups released by cellulase activity.

p-Hydroxybenzoic acid hydrazide (PAHBAH) reagent was used to assess the release of reducing sugars from cellulosic substrates since it has a wide range of sensitivity and is not subject to interference from high levels of protein (Lever, 1973). The procedure followed was a modification of Hurst et al. (1977). PAHBAH reagent was prepared fresh before use as described in Table 2.6.

Aliquots of the sample, 0.1 to 0.5 ml, were mixed with 5 ml PAHBAH reagent and the mixture boiled for 5 min. After cooling to 20°C the samples were read at 420 nm against a water blank.

As indicated by Lever (1973) the development of product at 420 nm was strongly affected by the concentration of calcium ions. Initial developments using 0.02 M $CaCl_2$ regularly produced dense precipitates on boiling and a range of calcium concentrations were investigated to determine a suitable optimum. A final concentration of 0.018 M was found to give a good colour development without precipitation and this concentration was adopted as standard.

Standard curves were produced using D-glucose in the range 5 to 100 µg dissolved in 0.1 M sodium acetate buffer, pH 5.0. To determine whether any of the buffer inclusions used in the experiments affected colour development a comparison was made with a stock solution of D-glucose, 4 mg per ml, diluted 1 in 10 with each alternative buffer (Table 2.7). Samples, 0.1 ml, were developed as described above.

TABLE 2.6

PREPARATION OF PAHBAH REAGENT.

Sodium sulphide,	1.0 M.	5.0 ml
Calcium chloride,	0.2 M.	5.0 ml
Trisodium citrate,	0.5 M.	5.0 ml
Sodium hydroxide,	5.0 M.	5.0 ml

When mixed, 1.0 g of p-hydroxybenzoic acid hydrazide was dissolved in the solution. The solution was then diluted to 100 ml.

The reagent was prepared immediately before use.

TABLE 2.7

Buffer Additive	Absorbance 420 nm
Control	0.839 ± 0.114
0.2% Triton X-100	0.903 ± 0.036
0.005 M EDTA	0.849 ± 0.139
0.2% Triton X-100 + 0.005 M EDTA	0.896 ± 0.104
0.3 M Mannitol	0.922 ± 0.098
0.8 M Mannitol + 0.02 M EDTA	0.898 ± 0.077

Effect of buffer additives on PAHBAH development. Control buffer; 0.1 M sodium acetate, pH 5.0. Glucose in all developments = 40 µg.

Values in all tables are means of experimental results ± 1 standard deviation.

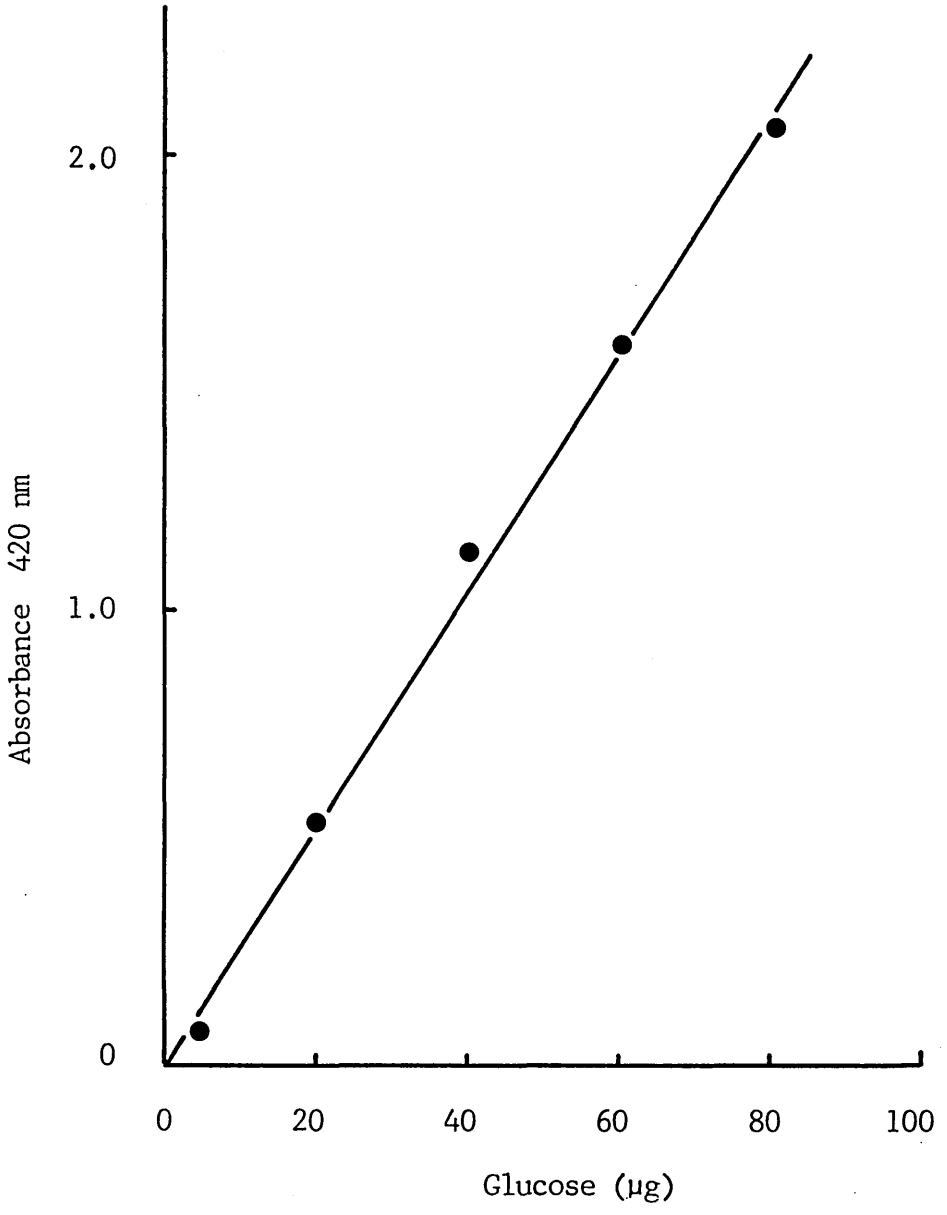


FIGURE 2.3. Standard calibration graph for sugar measured with the PAHBAH reagent. Glucose samples, in 0.1 ml protoplast incubation buffer, were processed as described in Materials and Methods.

2.4. ENZYME ASSAYS.

a) Cellulase. (Endo-1,4-β-glucanase;carboxymethylcellulase. E.C. 3.2.1.4.).

Cellulase activity was measured as release of reducing sugars from carboxymethylcellulose (CMC) according to the method described by Hurst et al. (1977). CMC was dissolved in 0.1 M sodium acetate buffer, pH 5.0, at a concentration of 1.0% (w/v). In the standard assay 0.5 ml of CMC substrate was added to 0.1 ml of suitably diluted enzyme or cell extract and the mixture incubated at 50°C for 60 min or as indicated. The reducing sugars released were determined by the p-hydroxybenzoic acid hydrazide procedure. Control samples, identically composed, were incubated for 0 min and were always included to provide assessment of reducing sugars present before enzyme activity.

One unit of activity was defined as the amount of enzyme capable of producing an increase in absorbance, at 420 nm, of 0.1 per min.

The possibility of buffer inclusions, such as Triton-X 100, affecting the activity of the enzyme and producing anomalous results was investigated and the results detailed in the Appendix.

b) Chitinase. (Poly-β-1,4-D-glucoside glycanohydrolase. E.C. 3.2.1.14.)

Chitinase was assessed by the colorimetric measurement of N-acetyl amino sugars released from chitin. Commercial chitin was purified by dissolving 20.0 g of crab chitin in 200 ml of concentrated HCl at 4°C, filtering the residue through glass wool and precipitating the dissolved chitin with 200 ml of absolute ethanol. The

precipitate was washed by centrifugation until of neutral pH and dried. Commercial chitinase was purified by dissolving 20 mg of the enzyme in 5.0 ml of distilled water and dialysing this against several changes of 0.1 M ethylene-diamine-tetra-acetic acid, di sodium salt (EDTA), for 96 h at 4°C. This procedure removed all excess calcium ions from the enzyme. The resulting solution was stored at 4°C and used within 2 days.

Purified chitin was suspended in protoplast incubation medium (page 41), pH 5.5 at a concentration of 10 mg per ml. Chitinase was assayed by incubating 0.5 ml of this stock suspension with 0.1 ml of the enzyme or cell extract at 30°C for 60 min, or as indicated. The reaction was terminated by the addition of 0.1 ml of sodium tetraborate and N-acetyl sugars estimated as described previously.

c) Fumarase. (L-malate hydroxylyase. E.C. 4.2.1.2.).
Fumarase was assayed spectrophotometrically according to the method of Massey (1955) by following the extinction of absorbance at 250 nm resulting from the conversion of L-malic acid to fumaric acid. The reaction mixture contained 100 µM potassium phosphate buffer, pH 7.4, 100µM L-malic acid and cell extract in a total volume of 3.0 ml. The reaction was started by the addition of 1.0 ml of 0.1 M L-malic acid.

d) Phosphorylcholine-glyceride transferase. (E.C. 2.7.8.2.).
Phosphorylcholine-glyceride transferase was assayed as described by Lord et al. (1972). Cell extract, suitably diluted in 0.1 ml was added to 0.5 ml of 0.1 M TRIS. HCl, pH 7.0 containing 5.0 µM

1,2-dipalmitin and 0.1 μCi (methyl-^{14}C) CDP choline (52 Ci per mole). The mixture was shaken for 60 min in an orbital incubator at 30°C and 75 rpm. The reaction was terminated by the addition of 2.0 ml of absolute ethanol. After 15 min the precipitated protein was removed by centrifugation and the pellet re-extracted with a further 2.0 ml of absolute ethanol. The ethanol phases were combined and mixed with 3.0 ml of choloroform. The organic phase was washed twice with 5.0 ml portions of 2.0 M KCl and twice with 5.0 ml portions of distilled water. Residual chloroform was evaporated in scintillation vials and the samples counted for 60 min after the addition of 5.0 ml dioxane scintillant.

2.5. PROTEIN ASSAY.

Protein was determined quantitatively according to the procedure of Ohnishi and Barr (1978). This involves a combination of the method of Lowry et al, (1951) and the Biuret method and allows determination of samples in the range 50 to 600 µg of protein. The advantages of combining these procedures are that a more stable colour development is assured. This enables many samples to be developed at once and aids reproduceability.

A standard volume, 3.8 ml, of stock BS7 reagent (Biuret solution diluted 1 in 7 with 2.3% (v/v) sodium carbonate) was added to 0.1 to 0.6 ml of the sample and the mixture incubated at room temperature for 10 min. After this time 0.1 ml of Folin and Ciocalteau phenol reagent was added and the samples incubated for a further 20 min at room temperature to allow colour development. Samples were read at 600 nm against a water blank. Stock BS7 reagent was stable when stored at 20°C for several months.

Standard curves were produced using bovine serum albumin (BSA, fraction V) dissolved in 0.1 M sodium acetate buffer, pH 5.0. Since a number of buffer additives were found to affect colour development these were investigated using a stock solution of BSA dissolved in distilled water to a final concentration of 3.0 mg per ml. Equal volumes, 0.1 ml, of the stock solution were diluted with 0.5 ml of the appropriate buffer for each assay. The results are shown in Table 2.8.

FIGURE 2.4. Standard calibration graph for protein measured by the procedure of Ohnishi and Barr, (1978). BSA samples, in 0.1 ml, 0.1 M sodium acetate buffer, pH 5.0, were processed as described in Materials and Methods.

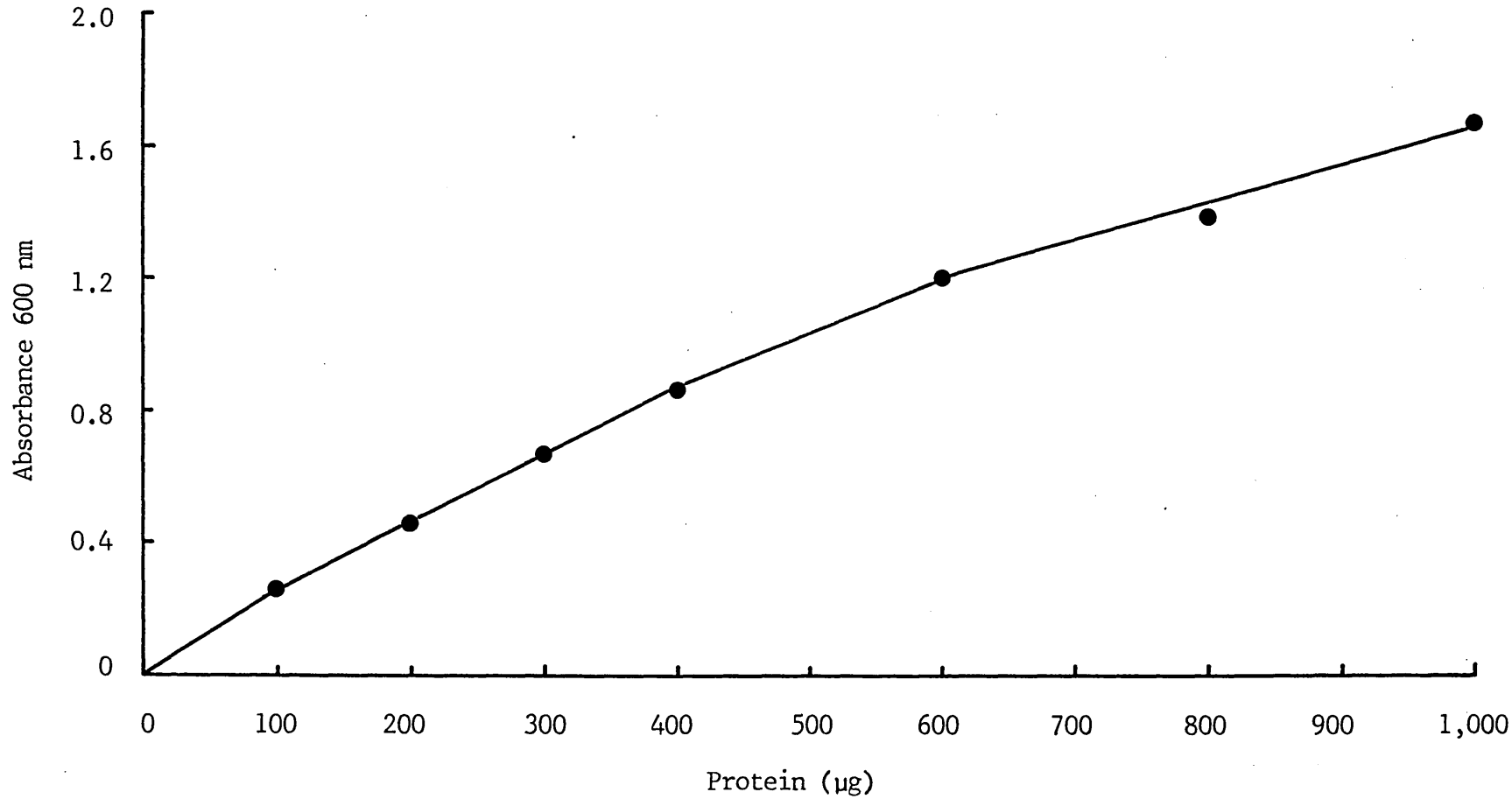

2.0
1.6
1.2
0.8
0.4
0
Absorbance 600 nm
0
100
200
300
400
500
600
700
800
900
1,000
Protein (µg)

TABLE 2.8.

Buffer additive	Absorbance 600 nm
Control	0.732 ± 0.048
0.2% Triton X-100	0.731 ± 0.018
0.005 M EDTA	0.658 ± 0.013
0.2% Triton X-100 + 0.005 M EDTA	0.706 ± 0.140
0.3 M Mannitol	0.677 ± 0.017
0.8 M Mannitol + 0.02 M EDTA	0.352 ± 0.014

Effect of buffer additives on protein determinations. Control buffer; 0.1 M sodium acetate, pH 5.0. Protein in all determinations = 300 µg.

2.6. $^{45}CaCl_2$ ASSAY.

Uptake of radioactive $^{45}CaCl_2$ was determined by adding $^{45}CaCl_2$ to 10 ml of previously washed mycelium suspensions in 0.05 M HEPES buffer, pH 5.5, to give a final specific activity of 800 mCi per M $^{45}CaCl_2$. The final concentration of $CaCl_2$ was made to 0.05 M with cold $CaCl_2$.

Mycelial suspensions for short term uptake studies were shaken in an orbital incubator at 30°C and samples harvested on Millipore filters after 1 to 20 min. Samples were washed three times with 0.05 M HEPES buffer and counted, when dry, in 10 ml of dioxane scintillant on a Nuclear Enterprises NE 8312 automatic scintillation counter.

Studies on ^{45}Ca localization in cell fractions obtained from hyphal digestions were conducted by incubating 1.0 ml of hyphal suspension with 0.1 ml of β-glucuronidase and 1 unit of chitinase at 30°C in an orbital incubator for 2.0 h. After incubation the mixture was centrifuged at 1,500 x g for 5.0 min and the supernatant kept. The resulting pellet, containing protoplasts and cell wall remains, was washed three times in the incubation buffer and then once in water to burst the protoplasts and release the cytoplasmic contents. Finally the residue remaining after this treatment was resuspended in 1.0 ml of buffer. Replicate 0.1 ml samples of each fraction were counted with 10 ml dioxane scintillant as described above.

2.7. LYTIC ENZYME PREPARATION.

Lytic enzymes for cell wall digestion and protoplast release were obtained by growing T. viride on purified A. fumigatus cell walls.

A. fumigatus was grown for 5 days on standard Eggins and Pugh liquid medium (Table 2.1) in an orbital incubator at 30°C. The mycelium was harvested, washed in distilled water and autoclaved at 15 lbs per sq in for 15 min at 121°C. When cool the resulting suspension was washed in distilled water until microscopic examination with methylene blue indicated complete absence of cytoplasmic contents. The washed walls were dried in an oven at 80°C and stored in a dessicator until required for use.

Powdered walls were added to T. viride liquid medium (Table 2.3) in place of glucose at a concentration of 3.0 g per litre. Medium, 250 ml, contained in 500 ml Erlenmeyer flasks, was inoculated with T. viride spores, washed from a slope with 5.0 ml of sterile distilled water. The organism was grown in an orbital incubator at 30°C for 5 to 7 days. The resulting mycelium and fine polysaccharide granules were removed by filtration and the crude lytic enzymes were obtained by raising the culture filtrate to 75% saturation with ammonium sulphate. The precipitated enzyme was centrifuged at 10,000 x g and the resulting pellet resuspended in 20.0 to 50.0 ml of distilled water and dialysed against 6 changes of distilled water. Insoluble particles were removed by centrifugation at 10,000 x g and the final supernatant lyophilised and stored in a dessicator at -20°C until required for use.

2.8. PROTOPLAST PRODUCTION AND ISOLATION.

Mycelia for protoplast production were harvested on a No 3 sinter glass filter funnel, washed twice with and finally suspended in incubation buffer consisting of 0.05 M HEPES, pH 5.5, containing 0.6 M KCl as osmotic stabiliser and 0.2 M $CaCl_2$ at a final mycelium concentration of 1.0 to 5.0 mg dry weight per ml. Other stabilisers and additives were added as indicated.

Lytic enzymes were added either singly or in combination to mycelial suspensions in the ratio of 10% (v/v) β-glucuronidase, 1 unit chitinase, 20 units pronase or 8 mg induced lytic enzyme per ml of mycelium suspension. The mixture was incubated at 30°C for 1 to 3 h and protoplast yields assessed by direct counting in a haemocytometer. All observations were made by phase contrast microscopy in order to emphasise the difference between protoplasts and spores.

After an appropriate time of incubation protoplasts were isolated by gravity filtration through a No 1 sinter glass filter funnel. The residue remaining was resuspended in incubation buffer and refiltered. The filtrates were combined and centrifuged at 500 x g for 5 min and washed three times in incubation buffer. Cellulase estimations (Table 4.14) indicated that all residual lytic enzymes, as judged by cellulase activity, were removed by this treatment.

Proportional release of protoplasts from tip and distal regions was estimated by scoring the number of protoplasts observed to be in the process of release at different times of incubation. Care was taken not to confuse small side branches with distal released protoplasts. Protoplast size was determined after different times of incubation by centrifuging aliquots of lytic incubations at 500 x g

for 5 min, washing in incubation buffer twice, to remove lytic enzymes, and resuspending in fresh buffer. The protoplasts were then maintained at 30°C until all samples were ready for measuring using a calibrated microscope eyepiece graticule.

Dry weights were determined by taking 1.0 ml aliquots of mycelial suspensions before digestion and filtering onto pre - weighed Oxoid membrane discs. Samples were washed twice by filtration of distilled water through the discs and dried in an oven at 50°C to constant weight.

Protoplasts for stability studies were maintained in sealed tubes to prevent evaporation of water from concentrating the sample. For regeneration studies protoplasts were resuspended in Eggins and Pugh CMC medium (Table 2.1) with the addition of 0.6 M KCl and incubated in an orbital incubator at 30°C for up to 24 h.

2.9. CELL FRACTIONATION TECHNIQUES.

Cells were fractionated for differential centrifugation by grinding in a pestle and mortar,with acid washed sand,until ruptured as indicated by microscopical observation. Care was taken to avoid excessive grinding which tended to rupture most organelles and the process was conducted on ice.

Hyphae, 5.0 to 10.0 g wet weight, were washed twice in 0.1 M sodium acetate buffer, pH 5.0, and ground with 50% (w/w) of sand and suspended in 2.5 volumes (v/w) of the same buffer with 0.3 M mannitol and 1.0 mM of the protease inhibitor phenylmethylsulphonyl fluoride.

The resulting homogenate was centrifuged at 3,000 x g for 10 min to remove cell debris and the pellet washed twice in equal volumes of buffer. The resulting three supernatants were combined and centrifuged at 10,000 x g for 20 min to remove mitochondria and the resulting pellet washed and resuspended in 1.0 ml of buffer. The supernatant was centrifuged at 100,000 or 300,000 x g in an MSE Superspeed 65 centrifuge for 1 h and the resulting pellet washed and resuspended in 1.0 ml buffer. All operations were conducted at $4^{o}C$. An outline of the scheme is shown in Fig 2.5.

Protoplasts were fractionated by centrifuging at 500 x g and resuspending in a minimum volume of 0.1 M sodium acetate buffer, pH 5.5, without the addition of a stabilising osmoticum. Cellular residues were separated by centrifuging at 3,000 x g and resuspending the pellet in an equal volume of buffer. All operations were conducted on ice. An outline of the scheme is shown in Fig 2.6.

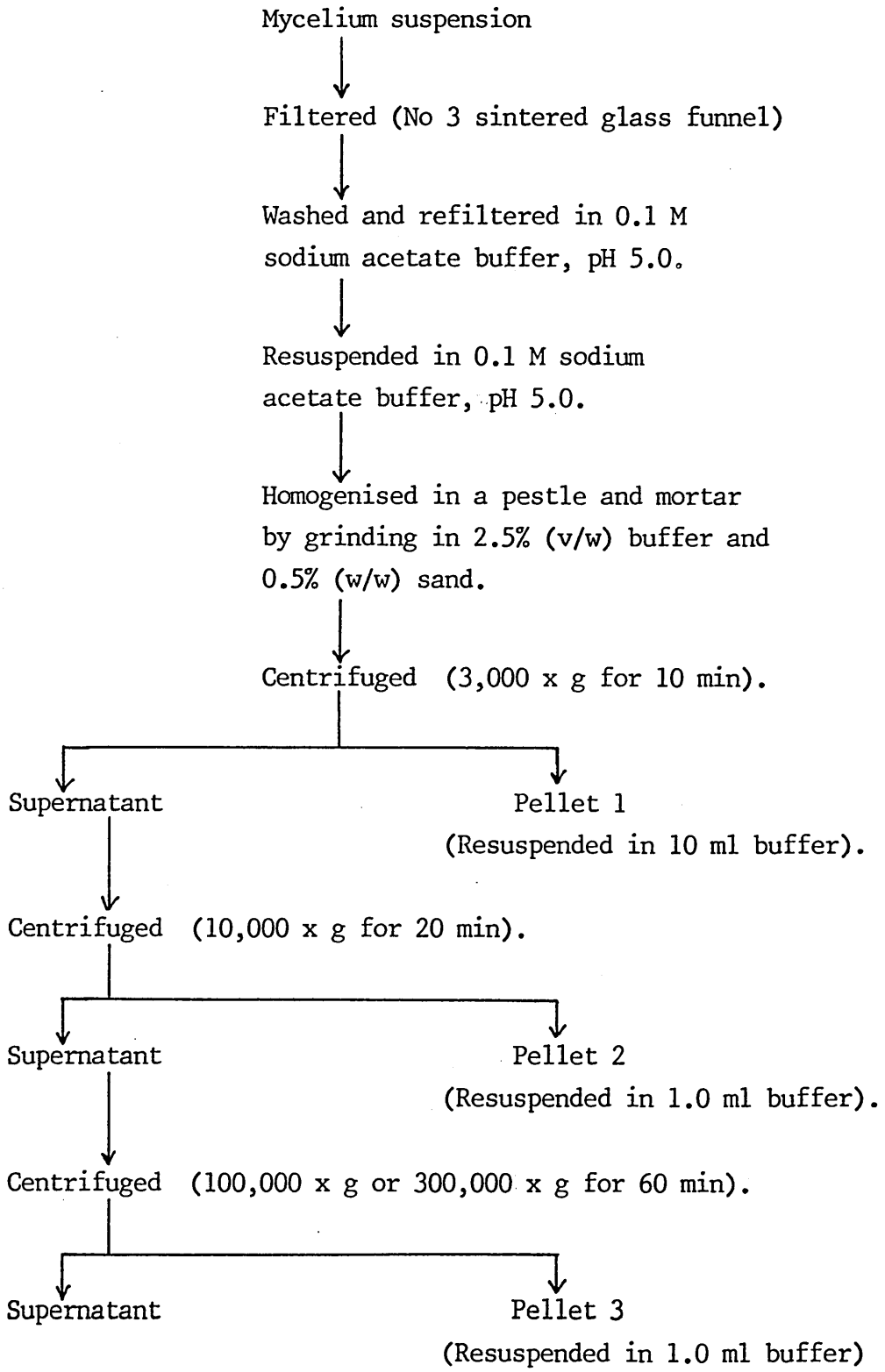


FIGURE 2.5. Flow chart for standard mycelium fractionation.

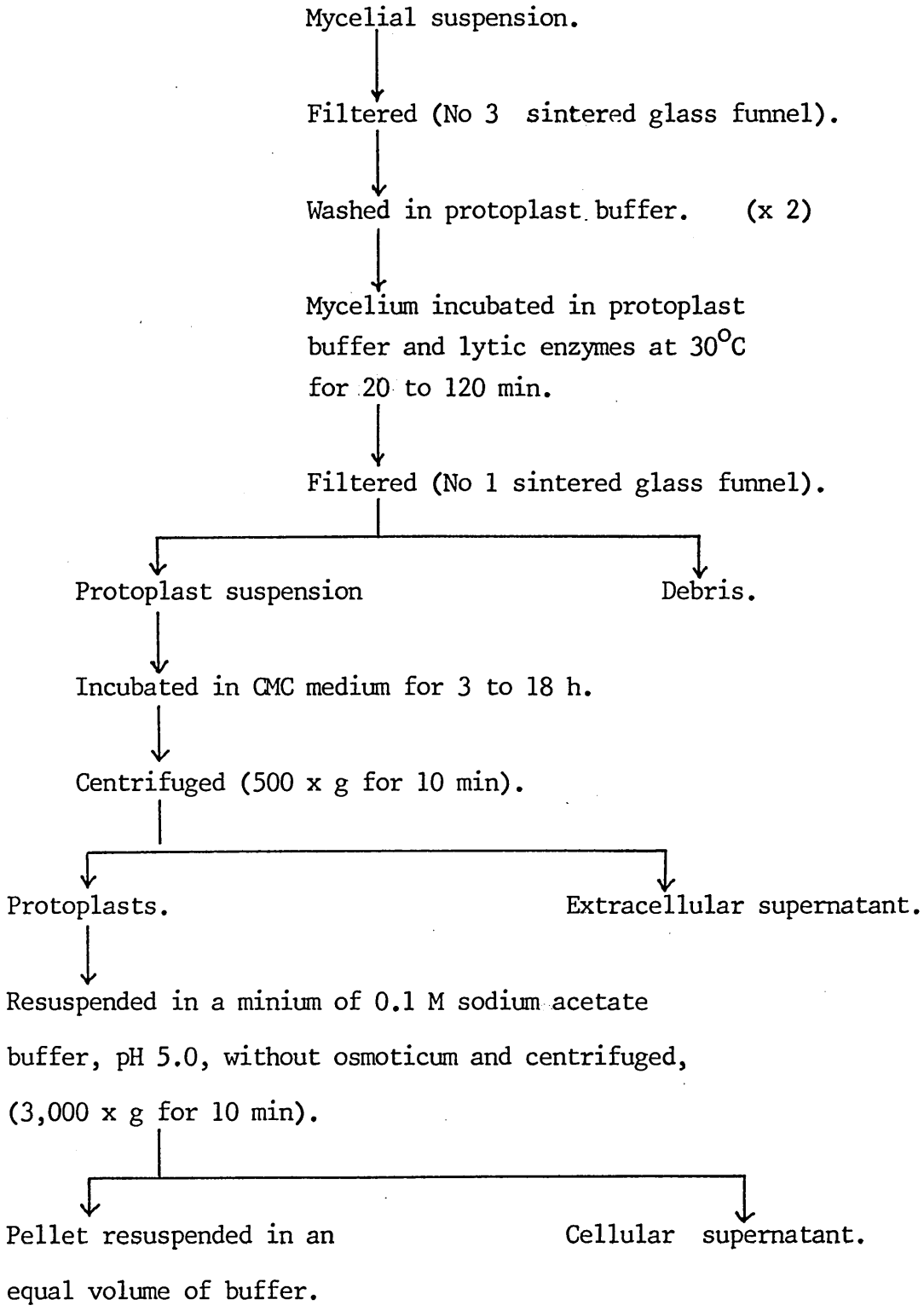


FIGURE 2.6. Flow chart for standard protoplast isolation and fractionation.

2.10. POLYACRYLAMIDE GEL ELECTROPHORESIS.

Localization of cellulase enzymes by polyacrylamide gel electrophoresis was conducted according to the method of Goren and Huberman, (1976).

Polyacrylamide gels of acrylamide concentration of 6.25% were prepared by mixing the solutions A, B, C and D listed in Table 2.9. Glass tubes 6.0 mm in internal diameter, were filled with the mixture to a depth of 75 mm. To ensure a smooth, flat surface to the gels water was layered on their surface before solidification. The tubes used were soaked in chromic acid before use and thoroughly rinsed in distilled water. Rubber caps were used to close the tubes' lower ends before filling.

At least three hours at 20°C was allowed for polymerisation of the gels and when set surface water was replaced with a 10 times dilution of the solution D to prevent dehydration. Gels were kept at 4°C and used within 48 h of preparation.

For separation of cellulase enzymes gels were held vertically between an upper reservoir containing 350 ml of electrolyte and tracking dye and a lower reservoir containing 350 ml of electrolyte only. A negative electrode was immersed in the upper tank and a positive electrode in the lower tank. The electrolysis was conducted at 4°C and the gels were pre-run in the electrophoresis buffer for 1.0 h.

Samples of cell extract or culture filtrate, 0.01 to 0.05 ml, were carefully layered onto the gels using a syringe and overlayered with electrophoresis buffer containing the tracking dye, bromophenol blue. The upper reservoir was then filled with the buffer and the tubes lowered into the lower reservoir of electrolyte. Current was applied at 2.0 mA per gel and the electrophoresis stopped when the dye reached the lower end.

When run,the gels were removed and processed to visualize the action of cellulase enzymes by incubating the gels in 0.02 M phosphate buffer pH 6.0 at 37^{o}C for 10 min. The reaction was terminated by replacing the buffer with 60% sulphuric acid for 10 min and the gels then stained with 2.0% potassium iodide for at least 2.0 h. The cellulase was localized in the clear bands on the gels. These bands correspond to the action of the enzyme in digesting the CMC in the gel.

TABLE 2.9.

PREPARATION OF ELECTROPHORESIS GELS AND BUFFERS.

1. Gels were prepared by mixing the following solutions in the proportions 2A:1B:4C:1D.

   A. 3.0 g acrylamide dissolved in 6.3 ml of water and 0.1 g N-N-methylene bisacrylamide dissolved in 6.0 ml water.

   B. 0.28% (v/v) N_4 tetramethlethylene diamine.

   C. 0.14% (w/v) ammonium persulphate.

   D. 0.725 g glycine and 0.15 g TRIS dissolved in 24.5 ml of water. To localize cellulase CMC was added to this solution at a final concentration of 0.1% of the final gel mixture.

2. Electrophoresis running buffer was prepared by diluting the following stock solution by 1 in 10 (v/v).

Stock solution	Glycine.	2.9 g
	TRIS.	0.6 g
	HCL, 1.0 M.	0.5 ml
	Water	97.5 ml

   The final pH of the running buffer was 8.1.

3. Tracking dye was added to the upper reservoir of electrophoresis buffer in the proportion of 2 drops of 0.5% bromophenol blue.

2.11. ELECTRON MICROSCOPY.

a) Fixation and embedding for thin sectioning.

Fixation of samples for observation in the transmission electron microscope was conducted by suspending the material in 0.1 M sodium cacodylate buffer, pH 7.0, containing 2.5% (v/v) gluteraldehyde. The samples were incubated for 1 h at room temperature and then for 18 h at 4°C and washed three times in 0.1 M sodium cacodylate buffer, pH 7.0. When required for protoplast stability the fixative and washing buffer contained 0.6 M KCl. After washing, the samples were suspended in 2.0% (w/v) aqueous osmium tetroxide for 2 h at 20°C and washed twice in buffer and dehydrated in an ethanol series, 30, 50, 75, 80, 90, 95 and 100%.

Embedding was in either Spurrs or E.M. Scope resin and was preceded by two changes of propylene oxide as an intermediate medium. For Spurrs resin the samples were kept overnight in 1:1 resin : propylene oxide, changed to fresh resin for three hours at 20°C and finally into 100% fresh resin for 12 h at 80°C. For E.M. Scope resin the samples were incubated in 50% resin : propylene oxide for 30 min at 37°C, 100% resin for 1 h at 37°C and finally embedded in fresh resin for 18 h at 60°C. No difference was observed on sample morphology between the two resins but E.M. Scope resin was preferred because it gave a better penetration and was less toxic.

Thin sections, 50 nm, were obtained on a Reichart microtome, collected on formvar coated copper grids and post stained with lead citrate and uranyl acetate according to the method of Reynolds (1956).

b) Shadowing techniques.

Cell walls and protoplasts were prepared for shadow casting by washing the samples twice in 0.5 M HEPES buffer, pH 5.5, containing 0.5 M KCl and twice in distilled water. They were then placed directly on grids or suspended in 4.5% (w/v) KOH and autoclaved at 15 lb per sq in for 15 min at 121°C in order to digest amorphous wall polymers. When the samples had been cooled to room temperature, after alkali digestion, they were washed three times in distilled water and resuspended in 0.5 ml of the same.

Drops of these suspensions were placed on formvar coated copper grids and left for 1.0 min to allow the particles to adhere to the surface. Excess water was removed with filter paper and the grids were shadowed to a depth of 250 nm with gold palladium at an angle of 30° in an Edwards coating unit.

c) Negative staining.

Samples of cell fractions were negatively stained with 0.2% (w/v) aqueous uranyl acetate. Formvar coated grids were placed on a drop of the sample for 1.0 min and transferred to a drop of 2.5% gluteraldehyde in fractionation buffer (page 43) for 2.0 min. The grids were then washed by placing on a drop of distilled water for 15 sec, stained by transferring to a drop of 0.2% uranyl acetate for 1.0 min and finally dried with filter paper. A control grid, immersed in the fractionation buffer was also included.

d) Histochemical localization of cellulase.

Cellulase was localized in gluteraldehyde fixed tissue by the procedure of Bal (1972). Tissue was fixed for 15 min at 20°C in 2.5% double distilled gluteraldehyde, dissolved in 0.1 M sodium cacodylate buffer, pH 7.0, and was washed overnight in the same buffer at 4°C. After two more washes at 20°C in 0.1 M sodium cacodylate buffer, pH 5.0, the samples were incubated with a 1.0% (w/v) CMC solution in this buffer for 5 to 15 min at 50°C to allow enzyme action to proceed. Subsequently the samples were centrifuged and resuspended in Benedict's solution at 75°C to allow deposition of copper oxide at sites of reducing sugar release. After 10 min the samples were washed in 0.1 M sodium acetate buffer, pH 5.0, osmicated in 1.0% aqueous osmium tetroxide, dehydrated in ethanol and embedded as described previously.

Due to the ease with which the reaction product of this procedure may be confused with non-specific deposition and osmium deposits a number of precautions were taken and appropriate controls were conducted. Firstly, a short time and low concentration of osmium tetroxide were employed to prevent excess precipitation. Secondly, a control sample of tissue, boiled for 15 min after fixation, was included to indicate how much deposition was due to non-enzyme causes. Thirdly, the type of deposition was identified as being due to the action of cellulase by processing a sample of plant tissue and observing the deposition within the middle lamellae, a location which has previously been reported to react by this technique.(Bal, 1972). Finally, fixation was deliberately kept short to prevent excessive inactivation and samples were observed and photographed without further staining.

e) Scanning electron microscopy.

Samples of hyphae and protoplasts were processed for scanning electron microscopy by fixing the tissue in 2.5% gluteraldehyde in 0.05 M HEPES buffer, pH 5.5, with the addition of 0.6 M KCl for protoplast samples for 1 h at 20°C and 16 h at 4°C. After washing in the same buffer the samples were impacted onto Millipore filters, dehydrated in ethanol and finally critically point dried using the apparatus and method of Brown (1977). When dry the samples were splutter coated with gold in a vacuum of 10^{-3} torr and observed in a Philips scanning electron microscope.

2.12. MATERIALS.

a. Gases. Carbon dioxide for critical point drying of SEM samples was supplied by British Oxygen Company, Leeds, Yorkshire, U.K.

b. Radioactive materials. ^{45}Ca calcium chloride and ^{14}C CDP choline were supplied by the Radiochemical Centre, Amersham, Buckinghamshire, U.K.

c. Enzymes and reagents.

i. β-glucuronidase, BSA, cellulase, chitinase and pronase were supplied by Sigma Chemical Company, Poole, Dorset, U.K.

ii. Osmium tetroxide, purified gluteraldehyde and other electron microscope reagents and apparatus were supplied by E.M.Scope Laboratories, Ashford, Kent, U.K.

iii. Other reagents were supplied by British Drug Houses, Poole, Dorset, U.K and were of the highest quality commercially available.

3.1. ENZYME EFFECTS ON PROTOPLAST RELEASE.

Since the cell wall is the initial limiting barrier to protoplast release much work has been directed into assessing the effects of hydrolytic enzymes on wall digestion. In most cases the enzymes used have been independently applied but there is evidence (Bartnicki-Garcia and Lippman, 1972) that enzymes within fungal hyphae may be induced to release the cell contents and that this may reflect the dynamic properties of the cell wall. Similarly in the more stagnant conditions of terminal autolysis, internal enzymes are again implicated in wall breakdown and may even be isolated for use in releasing protoplasts (Reyes and Lahoz, 1976).

Many studies on protoplast release have investigated the types of enzyme mixtures necessary to produce the maximum number of protoplasts in the shortest possible time. From these studies it is not surprising that only enzymes corresponding to the wall components present are necessary to ensure digestion although it is interesting to note that combinations of enzymes frequently act synergistically (Sietsma and Wouters, 1971; de Vries and Wessels, 1972; Anne et al., 1974 and Jeffries et al., 1977) and that some systems benefit from a number of additives, for example sulphydryl compounds (Sommer and Lewis, 1971 and Dooijewaard-Kloosterziel et al., 1973).

Fungal walls contain complex layers of carbohydrate polymers bound into matrices with protein (Bartnicki-Garcia, 1968 and Hunsley and Burnett, 1970) and to ensure protoplast release appropriate lytic enzymes must be used. Aspergillus cell walls have been shown to contain principally glucans and chitin and some protein (Johnston, 1965 and Bull, 1970a). The cell wall of A. fumigatus has apparently not

been investigated but is presumably of similar construction although there may be modifications owing to its thermotolerant characteristics.

To ensure digestion of the cell wall,a complex of lytic enzymes derived from snail gut juice, sold commercially as β-glucuronidase, was tested for its efficiency in releasing protoplasts from A. niger and A. fumigatus mycelia. This enzyme complex is well known for its ability to digest a range of fungal polysaccharides and has been used to liberate protoplasts from yeast (Deutch and Parry, 1974) and filamentous species (Musilkova and Fencl, 1966; 1968). It is also readily available and of consistent activity.

To assess the effectiveness of this on A. niger and A. fumigatus cell walls, different concentrations of mycelia, in 1.0 ml buffer (page 41), were incubated with 0.1 ml of β-glucuronidase for 1 h. After this time protoplasts and total reducing sugar release were determined (Figs. 3.1 and 3.2). As may be seen,the values of both parameters were proportionately related to the amount of mycelium added for both organisms. Concentrations as high as 20 mg dry weight per ml of buffer for both organisms were digested by 0.1 ml of enzyme resulting in a high yield of protoplasts. This yield was consistently greater for A. fumigatus than A. niger (Figs. 3.1 and 3.2). In order to prevent high concentrations of undigested mycelium obscuring the counting chambers, investigations were routinely conducted with concentrations of 2 to 4 mg dry weight of mycelium per 0.1 ml of enzyme. Although β-glucuronidase alone released protoplasts from both species of Aspergillus,and in increasing amounts for up to 2.5 h ,considerable residual mycelium remained undigested, indicating that the enzyme may not have been fully effective in digesting the walls. Microscopical observations indicated that only about 20% of the mycelium was removed

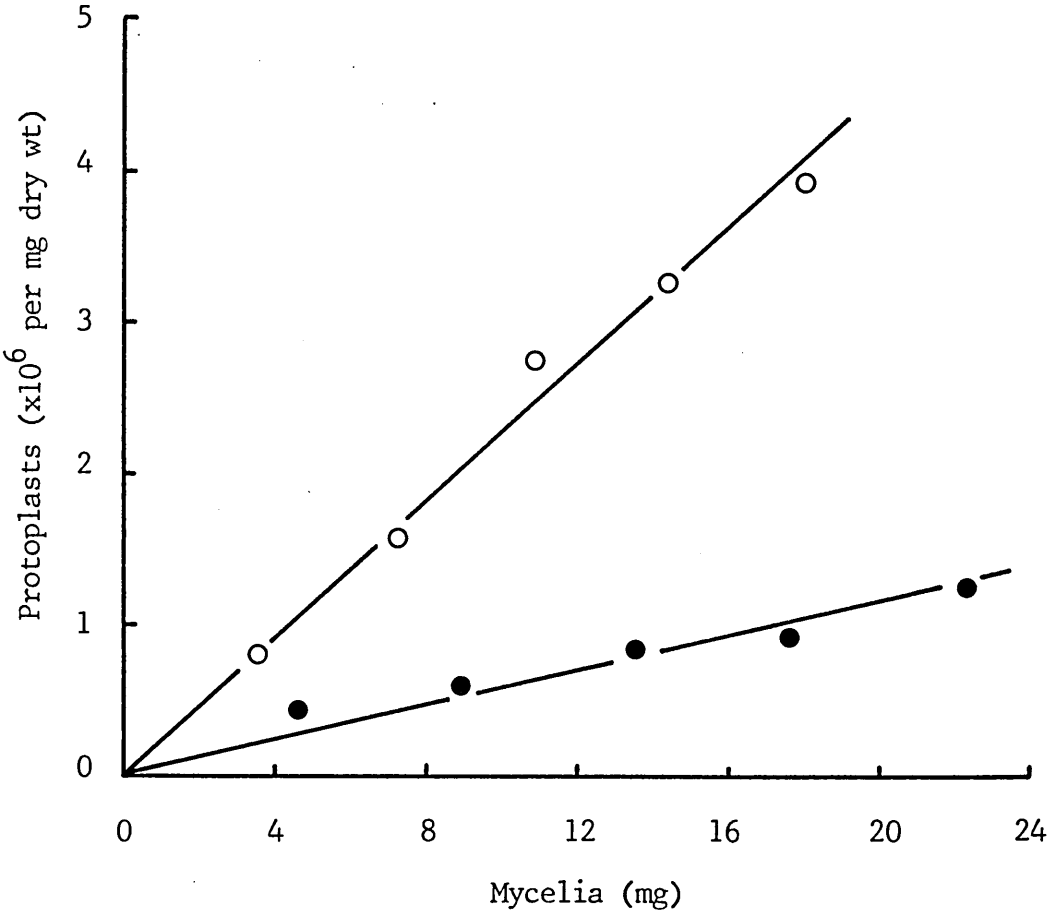


FIGURE 3.1. The effect of mycelium concentration on release of protoplasts from A. niger (●) and A. fumigatus (O). Mycelium samples, 1.0 ml, were incubated for 1.0 h, with 0.1 ml of β-glucuronidase at 30°C as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer.

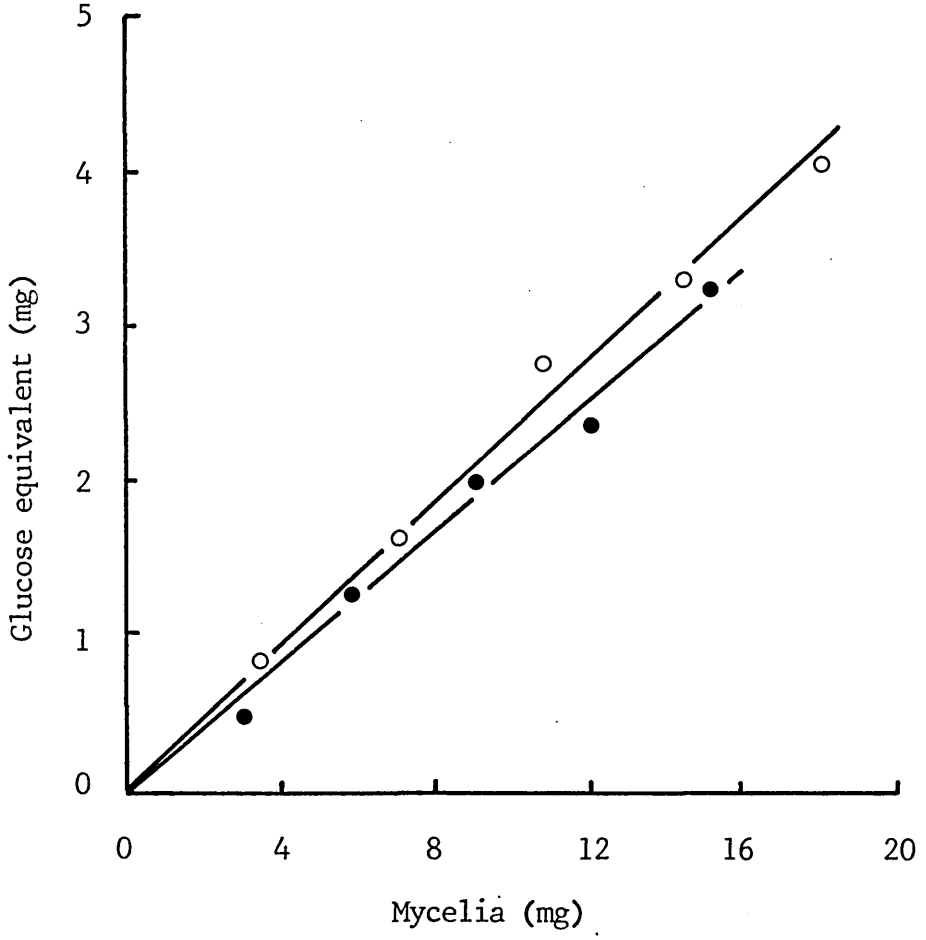


FIGURE 3.2. Effect of mycelium concentration on release of reducing sugars from A. niger (●) and A. fumigatus (○). Mycelium samples, 1.0 ml, were incubated for 1.0 h with 0.1 ml of β-glucuronidase at 30°C as described in Materials and Methods. Reducing sugars were determined using the DNS reagent.

after 3.0 h digestion with β-glucuronidase.

Recent theories on wall structure (Hunsley and Burnett, 1970; van der Valk et al, 1977 and Trinci, 1978) propose that many fungal walls consist of an inner fibril layer coated initially with protein and later with a glycoprotein reticulum. In order to release protoplasts it is possible that some wall components may be more important to hydrolyse than others. Chitin is found as a cell wall polymer in a number of fungi (Bartnicki-Garcia, 1968) and in some Ascomycetes and Basidiomycetes it is found to form the inner fibril layer (Hunsley and Burnett, 1970; Carbonell et al., 1970 and van der Valk et al., 1977). In order to investigate whether this layer is an important restraint on protoplast release from A. niger and A. fumigatus experiments were conducted using β-glucuronidase supplemented with additional chitinase.

Fig. 3.3 a and b show that the addition of only one unit of chitinase per ml of mycelium results in a three-fold increase in the number of protoplasts released from both A. niger and A. fumigatus. Further experiments indicated that this release was proportional to the amount of chitinase added (Fig. 3.4). The degree of stimulation of protoplast release obtained by chitinase addition depended upon the time of addition of chitinase during the incubation. Very little stimulation resulted from addition at 60 and 100 min but addition at 0 and 20 min produced a considerable increase in numbers (Fig. 3.5).

In order to investigate the relative behaviour of wall polymers during these digestions total reducing sugars and N-acetyl sugars were assayed and the profiles obtained compared to those of protoplast release. Total reducing sugar release, measured by the DNS method, is shown in Fig. 3.6 and shows little correlation with the differences in protoplast profiles (Fig. 3.5). Moreover, it is evident that the

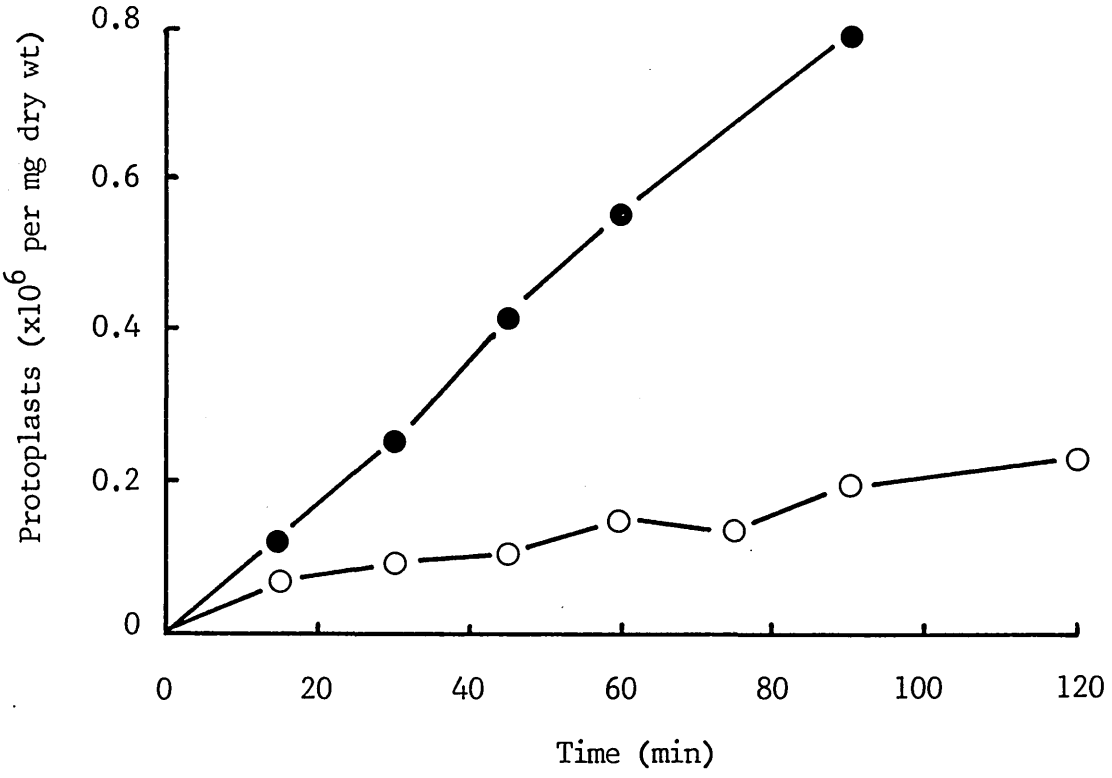


FIGURE 3.3a. Effect of chitinase on release of protoplasts from A. niger. Mycelium samples, 2.0 ml, were incubated with 0.2 ml of β-glucuronidase alone or with the inclusion of 2.0 units of chitinase at 30°C as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer. (O), β-glucuronidase, (●), β-glucuronidase and chitinase.

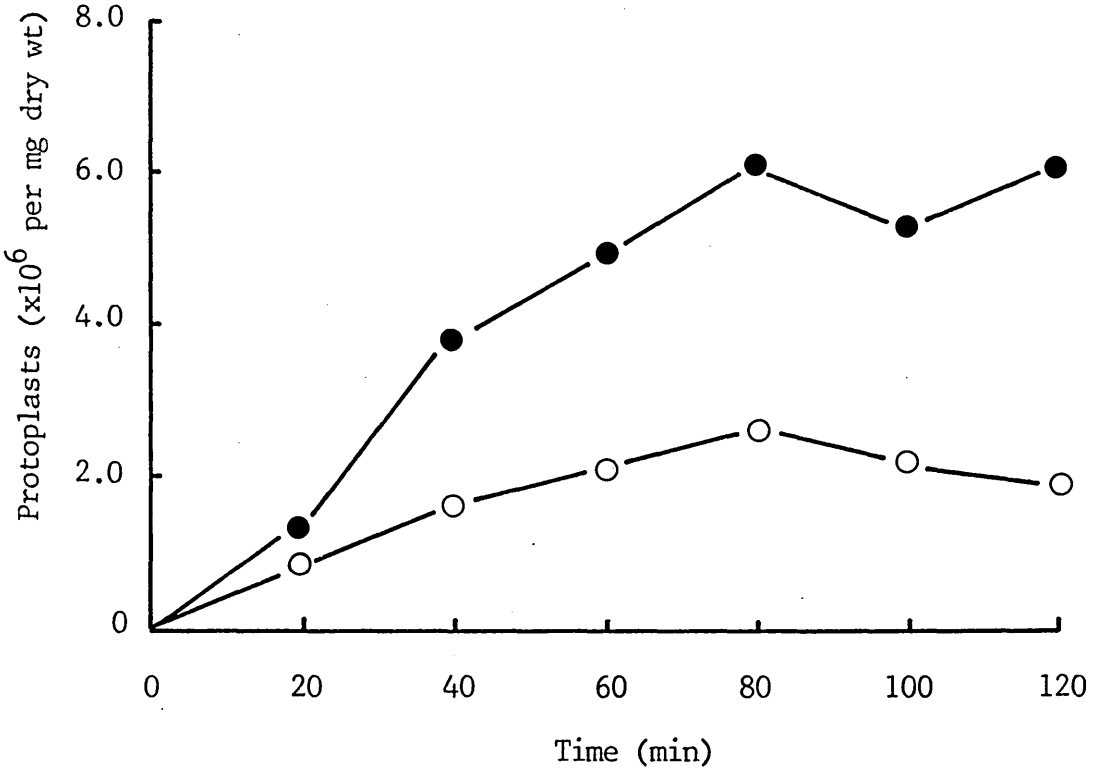


FIGURE 3.3b. Effect of chitinase on release of protoplasts from A. fumigatus. Mycelium samples, 2.0 ml, were incubated with 0.2 ml of β-glucuronidase alone or with the inclusion of 2 units of chitinase at 30°C as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer. (○), β-glucuronidase, (●), β-glucuronidase and chitinase.

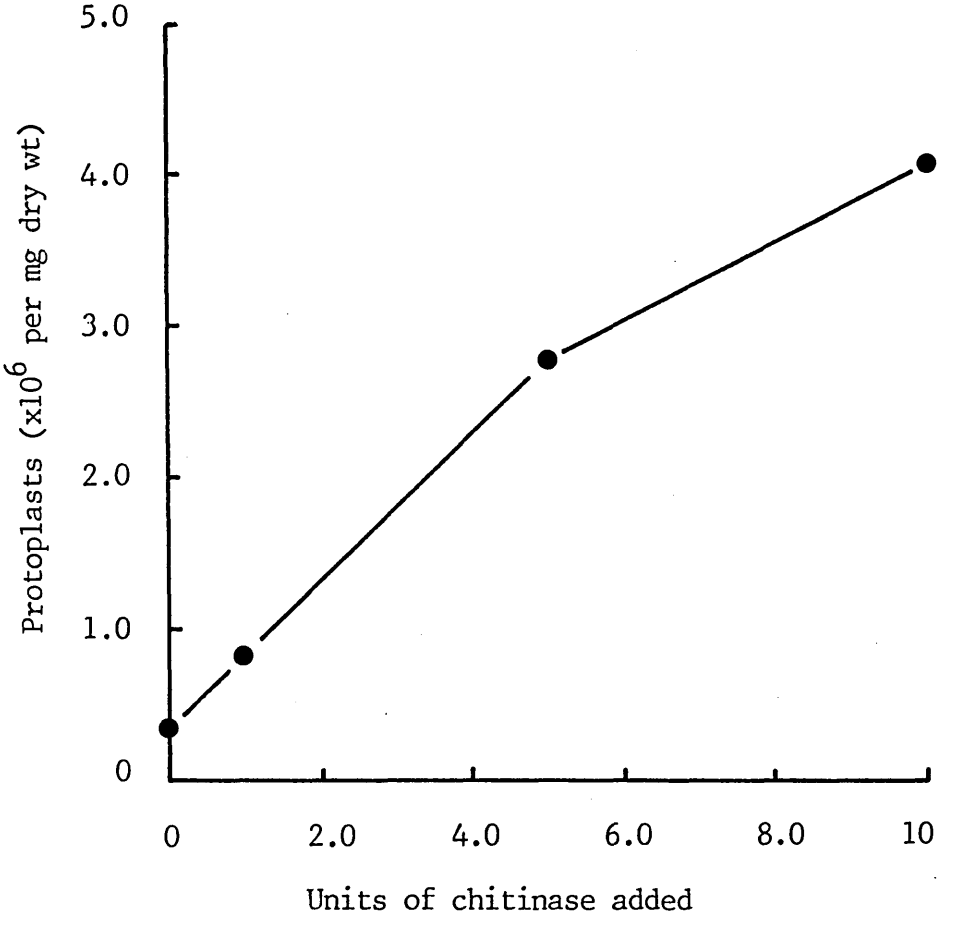


FIGURE 3.4. Effect of chitinase concentration on release of protoplasts from A. niger. Mycelium samples, 1.0 ml, were incubated for 1.0 h with 0.1 ml of β-glucuronidase and chitinase as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer.

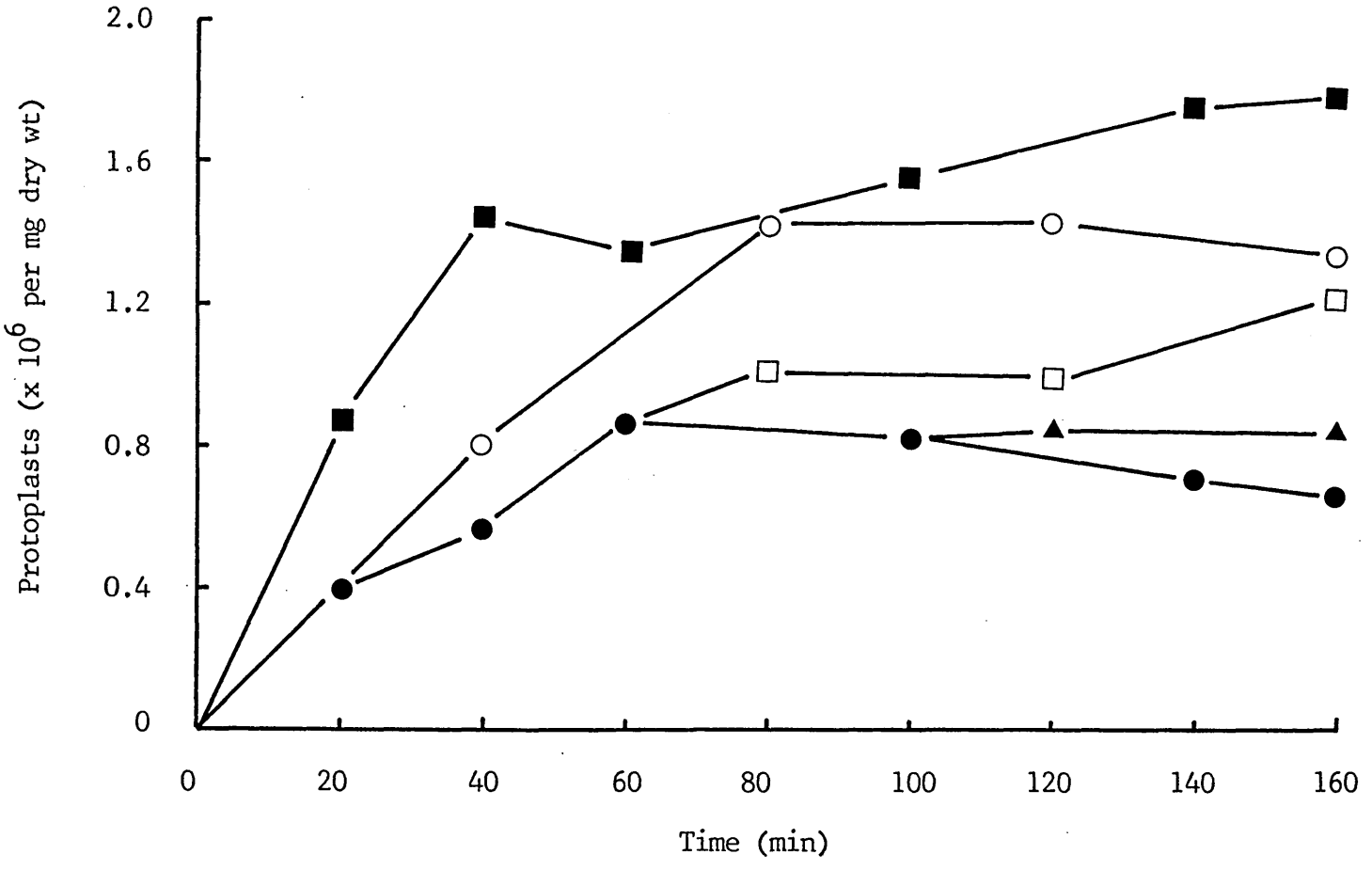
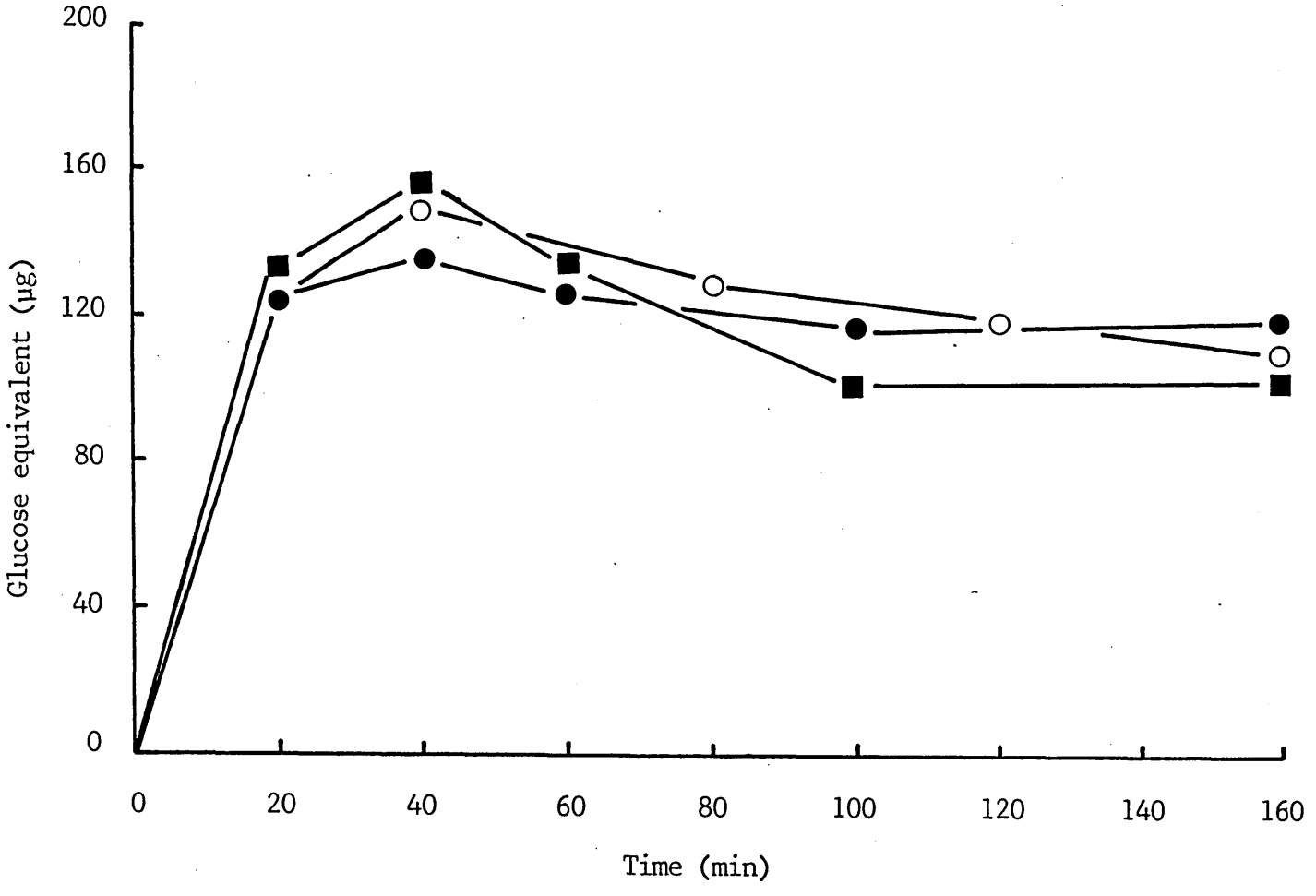


FIGURE 3.5. Effect of supplementing β-glucuronidase with chitinase at various intervals, on protoplast release from A. niger. Control (●), chitinase added at 0 min (■), 20 min (○), 60 min (□), and 100 min (▲). Mycelium samples, 5.0 ml, were incubated with 0.5 ml of β-glucuronidase at 30°C as described in Materials and Methods. Chitinase, 1.0 unit per 1.0 ml mycelium was added as indicated. Protoplast numbers were counted in a haemocytometer. Similar results were obtained with A. fumigatus.

FIGURE 3.6. Effect of supplementing β-glucuronidase with chitinase on release of reducing sugars from A. niger. β-glucuronidase (●), β-glucuronidase supplemented with 1.0 unit of chitinase per 1.0 ml of mycelium suspension at 0 min (■) and 20 min (○). Mycelium samples, 5.0 ml, were incubated with 0.5 ml of β-glucuronidase at 30°C as described in Materials and Methods. Reducing sugars were determined using the DNS method. Similar results were obtained with A. fumigatus.


200
160
120
80
40
0
Glucose equivalent (μg)
0
20
40
60
80
100
120
140
160
Time (min)

maximum release of reducing sugars occurs after only 40 min incubation whereas protoplast yield does not reach a maximum until after 80 min incubation (Figs. 3.6 and 3.5). The release of N-acetyl sugars shows a better correlation with protoplast release and also the addition of chitinase at 20 min produces a consistent stimulation in release of N-acetyl sugars (Fig. 3.7). Similarly the time course of N-acetyl sugar release does not reach a maximum until after 80 min of incubation.

In order to determine why addition of chitinase produced less stimulation at 100 min than at 20 min, experiments were conducted to assess the activity of the incubation medium after it had been used for protoplast production. Samples of media used to release protoplasts were compared to control samples incubated for the same time without mycelia. Fresh mycelia was added, after removal of protoplasts and hyphal remains, and the release of protoplasts from the fresh mycelia followed over 2 h. The results show that compared to the control those samples previously incubated with mycelia released considerably fewer protoplasts (Fig. 3.8). Analysis of sugars present after 2 h incubation indicated that release of N-acetyl sugars was reduced more than release of total reducing sugars indicating that the chitinase was being selectively inactivated (Table 3.1).

Since protein has been noted to comprise 8 to 10% of Aspergillus walls (Ruiz-Herrera, 1967 and Bull, 1970a) and has also been implicated as an important layer in wall models (Hunsley and Burnett, 1970 and Hunsley and Kay, 1976) experiments were conducted to determine whether pronase facilitated protoplast release from the two species of Aspergillus. The results show that when used alone no protoplasts were released. When used in conjunction with β-glucuronidase only a slight enhancement of protoplast release was observed (Fig. 3.9). When added to a mixture

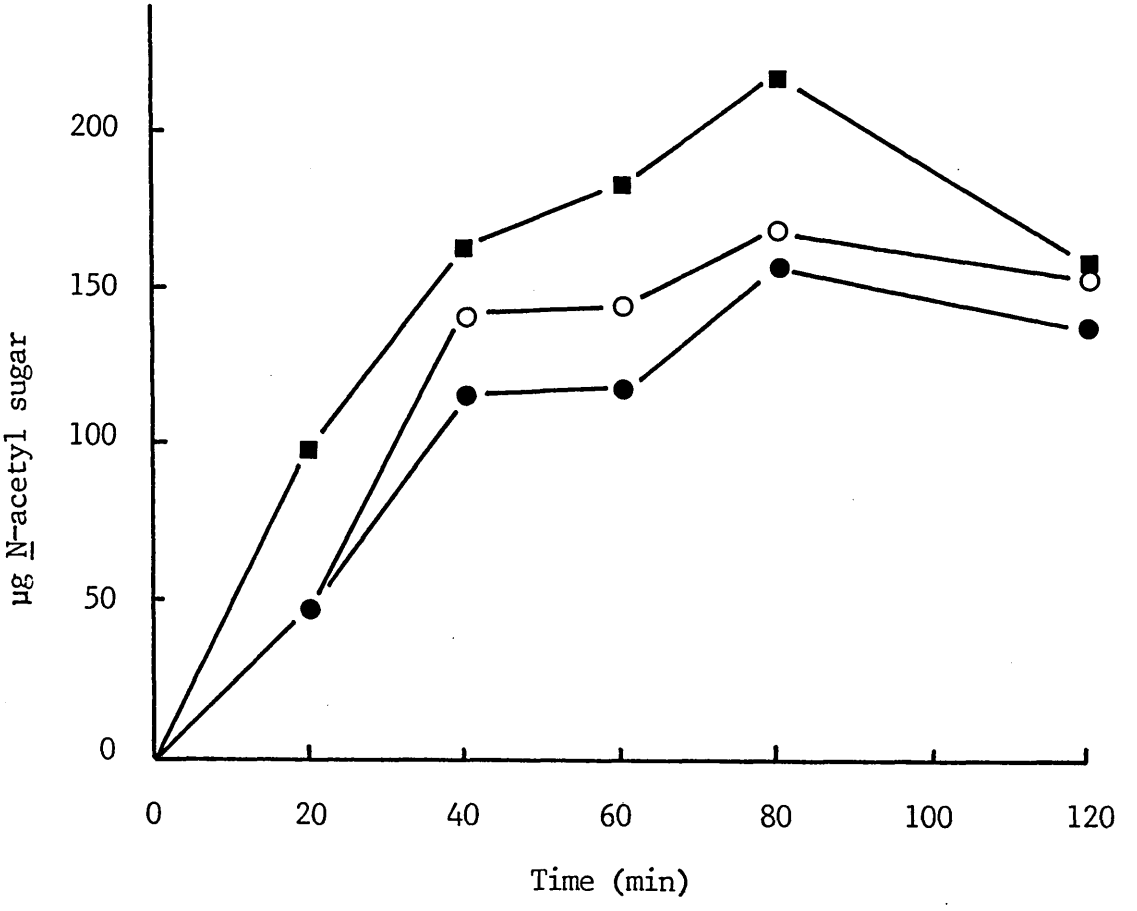


FIGURE 3.7. Effect of supplementing β-glucuronidase with chitinase on release of N-acetyl sugars from A. niger. β-glucuronidase (●), β-glucuronidase supplemented with 1.0 unit of chitinase per 1.0 ml of mycelium resuspension at 0 min (■) and 20 min (○). Mycelium samples, 5.0 ml, were incubated with 0.5 ml of β-glucuronidase at 30°C as described in Materials and Methods. N-acetyl sugars were determined using the DMAB reagent. Similar results were obtained with A. fumigatus.

FIGURE 3.8. Effect of pre-incubated enzymes on protoplast release from A. niger. β-glucuronidase control (●), β-glucuronidase, pre-incubated sample (■), β-glucuronidase and chitinase control (○), β-glucuronidase and chitinase, pre-incubated sample (□). Mycelium samples, 5.0 ml, were incubated with 0.5 ml of enzyme at 30°C as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer. Similar results were obtained with A. fumigatus.

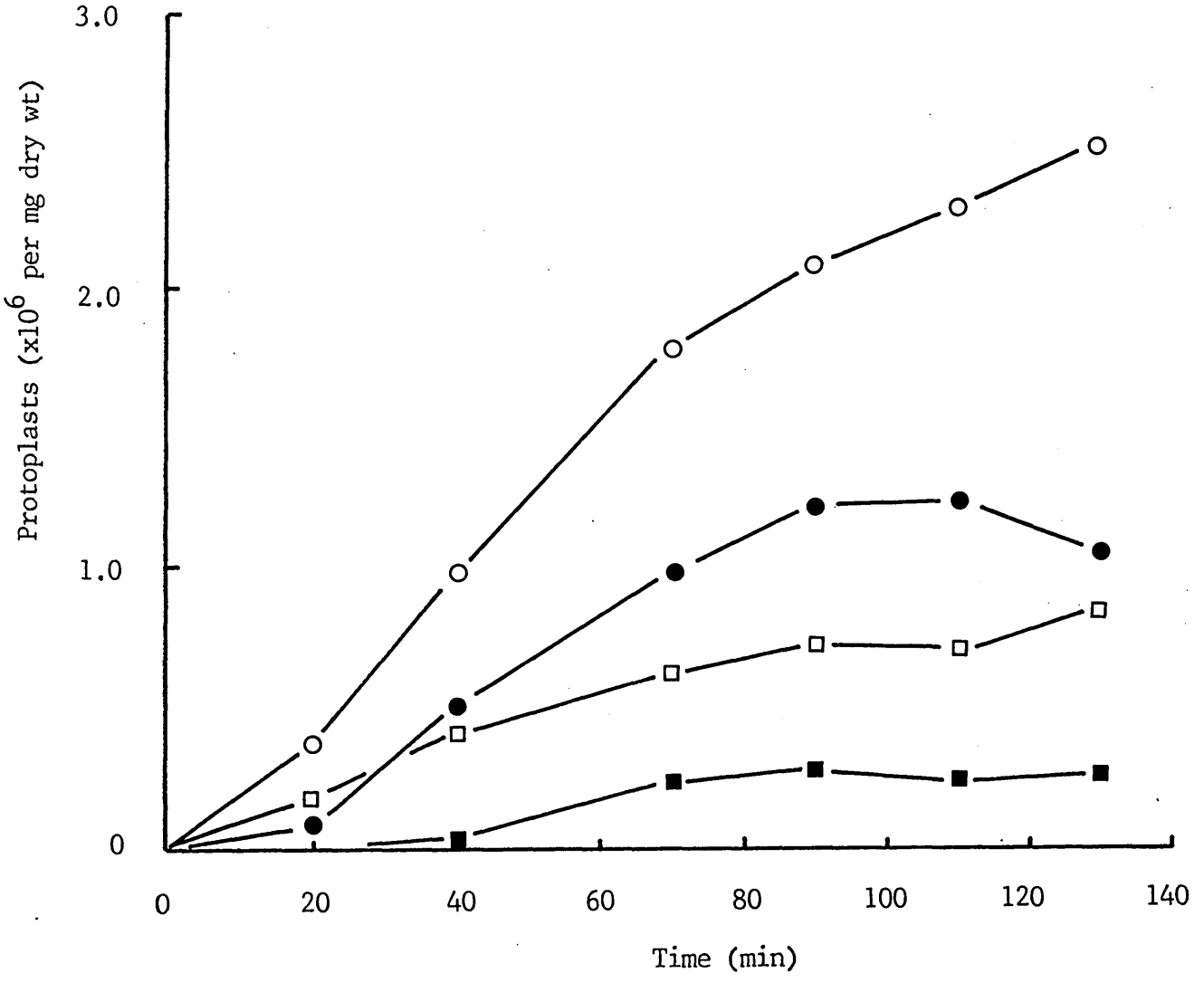

3.0
2.0
1.0
0
Protoplasts (x10^6 per mg dry wt)
0
20
40
60
80
100
120
140
Time (min)

TABLE 3.1.

	Protoplasts ($x10^6$ per mg dry wt)	% of control
Pre - incubated enzyme	1.14 ± 0.08	45
Control	2.53 ± 0.38	
	Reducing sugar*	% of control
Pre - incubated enzyme	129 ± 3.47	89
Control	145 ± 2.76	
	N-Acetyl sugars#	% of control
Pre - incubated enzyme	316 ± 39.0	59
Control	538 ± 51.6	

Comparison of protoplast and sugar production from A. niger mycelium utilising pre - incubated enzyme. (Similar results were obtained with A. fumigatus).

* µg Glucose equivalent.

\# µg N-Acetyl glucosamine...

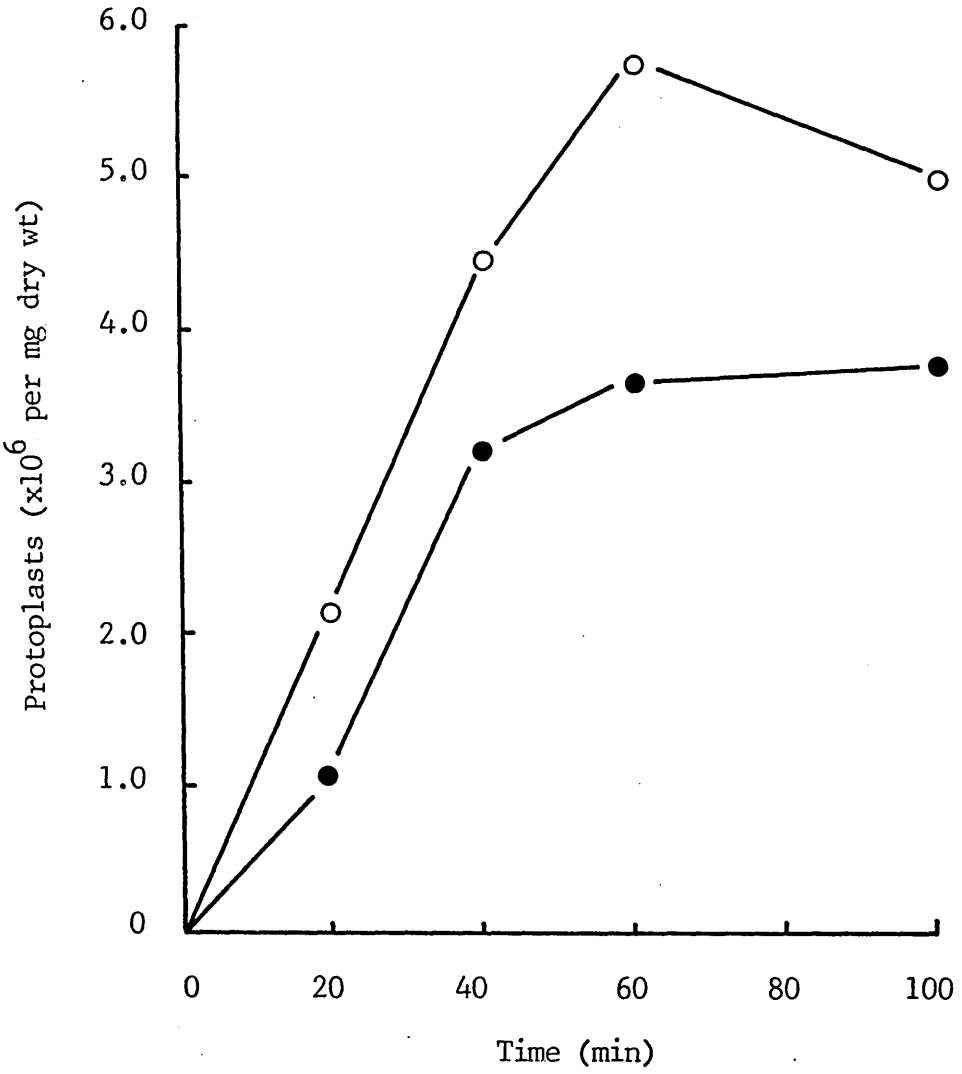


FIGURE 3.9. Effect of pronase on protoplast release from A. fumigatus. β-glucuronidase (●), β-glucuronidase supplemented with 20 units of pronase per 1.0 ml of mycelium resuspension (○). Mycelium samples, 2.0 ml were incubated for 1.0 h with 0.2 ml of enzyme. Protoplast numbers were determined by counting in a haemocytometer. Similar results were obtained with A. niger.

of β-glucuronidase and chitinase a further increase was produced (Table 3.2). However, because of possible detrimental effects on the plasma membrane of protoplasts, pronase was not included as a standard component in the medium for routine experiments.

3.2. OSMOTICA EFFECTS ON PROTOPLAST PRODUCTION.

A wide range of osmotica, including both inorganic salts and sugars, have been used to stabilise protoplasts released from fungi. Comparative assessments of these have been published (Musilkova and Fencl, 1966; Sietsma and De Boer, 1973 and Peberdy et al., 1976) and it is noticeable that some osmotica are more universal than others, particularly the salts KCl and $MgSO_4$ and the sugar mannitol.

The effect of a number of osmotica in supporting stable protoplast release from both species of Aspergillus was investigated. The results (Table 3.3) show that most salts, when used alone, supported protoplast release at a concentration of 0.6 M, except for $CaCl_2$ and $Ca(NO_3)_2$. NH_4Cl and $MgSO_4$ were the most effective stabilisers when considered in terms of number of protoplasts released per mg of mycelium. However, the size of protoplasts varied considerably between and within osmotica treatments (Table 3.3).

Another important factor affecting protoplast release is the concentration of osmoticum used. Previous reports have stated that the optimum value for fungal systems is between 0.4 and 0.6 M (Bartnicki-Garcia and Lippman, 1966; de Vries and Wessels, 1972 and Peberdy et al., 1976) and experiments with A. niger and A. fumigatus indicate that similar values apply for these fungi (Fig. 3.10).

A final factor investigated with regard to osmotica was the effect

TABLE 3.2.

Enzyme	Protoplasts ($x10^6$ per mg dry wt)
β-glucuronidase.	1.10 ± 0.29
β-glucuronidase + chitinase.	1.99 ± 0.41
β-glucuronidase + pronase.	1.41 ± 0.28
β-glucuronidase + chitinase + pronase.	2.53 ± 0.46

Comparison of protoplast release from A. niger mycelium using different enzyme treatments. (Similar results were obtained with A. fumigatus). Enzyme concentrations used were

β-glucuronidase	0.1 ml per 1.0 ml mycelium resuspension.
Chitinase	1.0 unit per 1.0 ml mycelium resuspension.
Pronase	20 units per 1.0 ml mycelium resuspension.

TABLE 3.3.

Osmoticum	Protoplasts ($\times 10^6$ per mg dry wt)
0.6 M KCl	1.60 ± 0.46
0.6 M $CaCl_2$	0.43 ± 0.17
0.6 M $Ca(NO_3)_2$	0.73 ± 0.30
0.6 M NH_4Cl	2.71 ± 0.26
0.6 M $MgSO_4$	1.17 ± 0.17
0.6 M $MgCl_2$	2.24 ± 0.23
0.6 M KCl + 0.2 M $CaCl_2$	2.34 ± 0.29
0.6 M KCl + 0.2 M $Ca(NO_3)_2$	2.27 ± 0.71

Effects of different osmotica on release of protoplasts from A. fumigatus. (Similar results were obrtained with A. niger).

1.0 hour incubation with β-glucuronidase and chitinase, 30°C.

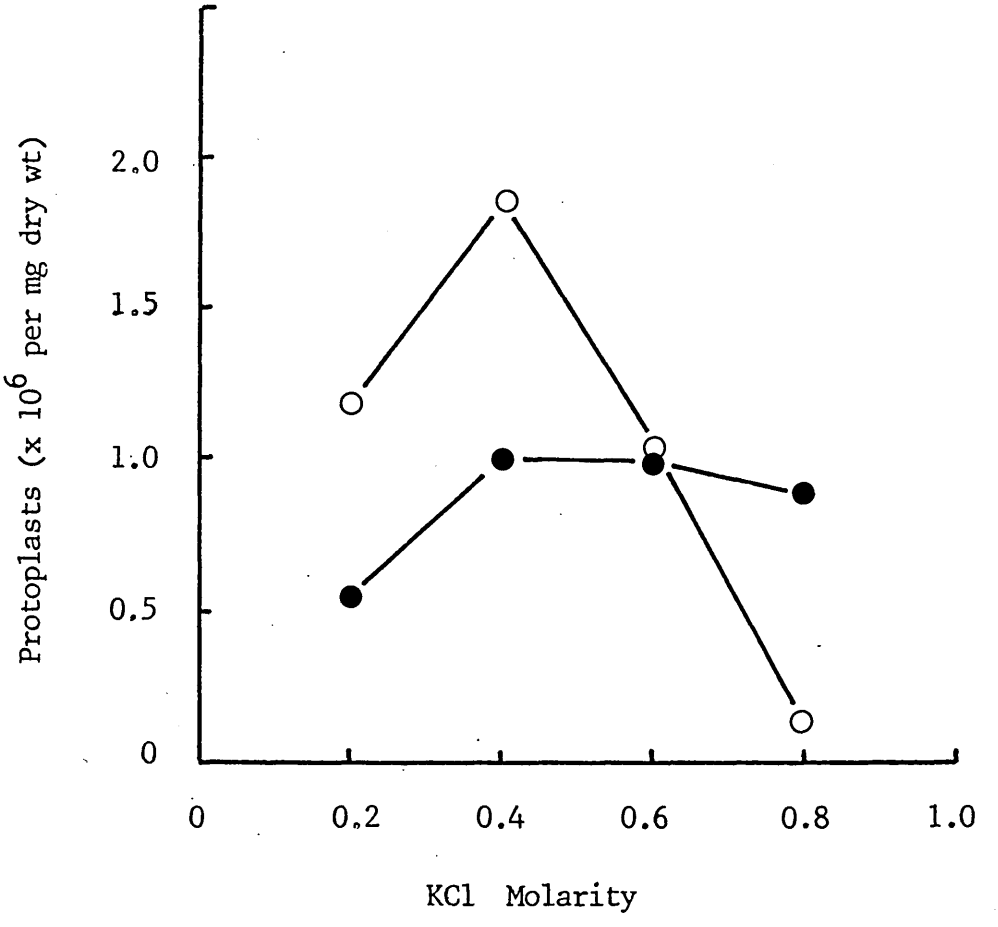


FIGURE 3.10. Effect of osmoticum concentration on release of protoplasts from A. niger (●) and A. fumigatus (○). Mycelium samples, 1.0 ml, were incubated for 1.0 h with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer.

of including low concentrations of calcium salts in the medium. Supplementation of 0.6 M KCl with 0.2 M $CaCl_2$ noticeably enhanced protoplast release from both species of Aspergillus (Tables 3.3 and 3.4). The effect, however, was not consistent and seemed to depend upon the strain of organism and the lytic system employed. For example, no enhancement was found using the white mutant strain of A. fumigatus or on the wild type strain with β-glucuronidase in the absence of chitinase. However, the stimulatory effect was particularly evident when using additional chitinase with the wild type strain of A. fumigatus (Table 3.4).

That the enhancement effect of $CaCl_2$ was due, in part, to the salt acting as a supplementary osmoticum is shown in Table 3.5. The inclusion of 0.2 M $CaCl_2$ had a greater effect at low rather than high concentrations of KCl. This greater enhancement at low concentrations could be due to increasing the osmotic potential above a necessary minimum required to stabilise the protoplasts. However, as the concentration of KCl was increased, protoplast release did not increase proportionately to the increase in osmotic potential afforded by the additional calcium salt. Moreover, addition of concentrations of $CaCl_2$ above 0.2 M to KCl (0.2 M) resulted in a decrease in yield (Table 3.5). These results, the dependence of release on the organism and enzyme system and the inability of 0.6 M $CaCl_2$ and $Ca(NO_3)_2$ used alone to support protoplast release, indicated that additional effects of the salts were involved.

The addition of $CaCl_2$ to the lytic enzyme system did not alter the pH of the medium and the effect did not appear to be connected with the chloride ion since the addition of $Ca(NO_3)_2$ also enhanced release from both A. niger and A. fumigatus. When EDTA was added to the incubation medium containing $CaCl_2$, protoplast release was severely depressed, a

TABLE 3.4

	A. niger (University of Strathclyde strain).	
	β-glucuronidase	β-glucuronidase + chitinase
0.6 M KCl	0.325 ± 0.064	0.990 ± 0.59
0.6 M KCl + 0.2 M $CaCl_2$	0.475 ± 0.092	1.400 ± 0.51
	A. fumigatus White mutant (143864).	
	β-glucuronidase	β-glucuronidase + chitinase
0.6 M KCl	0.480 ± 0.047	0.633 ± 0.068
0.6 M KCl + 0.2 M $CaCl_2$	0.280 ± 0.017	0.207 ± 0.099
	A. fumigatus Wild type (226070).	
	β-glucuronidase	β-glucuronidase + chitinase
0.6 M KCl	3.547 ± 2.560	1.354 ± 0.560
0.6 M KCl + 0.2 M $CaCl_2$	2.087 ± 1.540	4.702 ± 1.660

Efect of $CaCl_2$ on release of protoplasts from A. niger and A. fumigatus. Figures represent protoplasts $x10^6$ per mg dry weight.

TABLE 3.5.

Concentration	Osmotic * Potential	Protoplasts (x 10^6 per mg dry wt)
0.2 M KCl	0.372	0.046 ± 0.004
0.2 M KCl + 0.2 M $CaCl_2$	0.902	0.818 ± 0.118
0.2 M KCl + 0.4 M $CaCl_2$	1.432	0.109 ± 0.052
0.6 M KCl	1.120	0.684 ± 0.096
0.6 M KCl + 0.2 M $CaCl_2$	1.650	0.838 ± 0.076
0.8 M KCl	1.507	0.999 ± 0.067
0.8 M KCl + 0.2 M $CaCl_2$	2.370	0.822 ± 0.049
0.6 M $CaCl_2$	1.803	0.185 ± 0.074

Effect of $CaCl_2$ on protoplast production in A. niger at different molarities of KCl.

* Calculated from CRC Handbook of Chemistry and Physics, 58th Edition, CRC Press Inc. Ed. R.C. Weast.

result to be expected if the calcium ion played an active role in release (Table 3.6).

Experiments were also conducted to assess whether the mycelium of both species of Aspergillus was particularly permeable to Ca^{2+}. Studies by Naccache et al, (1977) on rabbit leukocytes and by Chandler and Williams (1978) on mouse pancreas systems have indicated that calcium may be instrumental in the fusion of cytoplasmic vesicles with the plasma membrane and release of their contents. Similarly, calcium is also implicated in the secretions of nerve cells (Baker and Knight, 1978) and in the release of histamine from mast cells (Foreman et al., 1973). Since filamentous fungi are reported to possess many cytoplasmic vesicles and some evidence suggests that these contain hydrolytic enzymes (Meyer et al., 1976) it is conceivable that calcium stimulation may operate by inducing vesicles to fuse with the plasma membrane and release their contents to act upon the cell wall.

If this is so then Ca^{2+} may be expected to be taken up into the mycelium and could be located by radiochemical methods. Experiments were conducted with radioactive $^{45}CaCl_2$ added to the lytic enzyme medium to test this hypothesis. After 2.0 h incubation with β-glucuronidase and chitinase, an insignificant amount of label was recovered from the cytoplasm of protoplasts. A larger amount of label was found to be associated with the cell residues but was easily removed by buffer washes indicating little involvement with the cell and/or its components (Table 3.7). As protoplasts and residual cell walls were not separated in these experiments it is not possible to allocate this initial label to any particular component. However, it was noticeable that little label was released by bursting the protoplasts with distilled water indicating little penetration of the ion into the cytoplasm.

TABLE 3.6.

Osmoticum	Protoplasts ($x10^6$ per mg dry wt)	
	A. niger	A. fumigatus
0.6 M KCl	0.76 ± 0.01	5.05 ± 0.26
0.6 M KCl + 0.2 M $CaCl_2$	1.26 ± 0.03	6.08 ± 0.31
0.6 M KCl + 0.2 M $CaCl_2$ + 0.25 M EDTA	0.48 ± 0.02	1.15 ± 0.39

Effect of $CaCl_2$ and EDTA on protoplast production from A. niger and A. fumigatus (wild type strain).

1.0 h incubation with β-glucuronidase and chitinase.

TABLE 3.7.

Fraction	d.p.m.	
	A. niger	A. fumigatus
120 min suspension	632291 ± 91192	971897 ± 165223
1st buffer wash supernatant	139765 ± 55906	58916 ± 2794
2nd buffer wash supernatant	8893 ± 640	3820 ± 376
H_2O wash supernatant (protoplast contents)	357 ± 56	480 ± 80
Resuspended pellet (inc. cell wall remains)	488 ± 73	299 ± 51

Uptake of ^{45}Ca into cell fractions of A. niger and A. fumigatus. $^{45}CaCl_2$ added before digestion with β-glucuronidase and chitinase as described in Materials and Methods.

The possibility of a short term uptake of Ca^{2+} was also investigated in case the effect occurred over a brief initial period, as might be expected if a trigger mechanism was operative. Replicate, 10 ml , samples of fresh mycelia were incubated in the protoplast release medium with the inclusion of $^{45}CaCl_2$ as described in Materials and Methods, and aliquots filtered at 1.0 min intervals. These experiments also failed to localise any radioactivity within the cell (Table 3.8).

As Ca^{2+} had little or no uptake into the cell during protoplast release a series of experiments was set up to test the influence of Ca^{2+} on lytic enzyme activity. Protoplast release from A. niger and A. fumigatus is greatly enhanced by 0.2 M $CaCl_2$ in the presence of β-glucuronidase and chitinase when used in combination but not with β-glucuronidase alone (Table 3.4). It would appear, therefore, that Ca^{2+} may actually stimulate the chitinase enzyme. This possibility was tested by incubating chitin with purified chitinase in the presence different amounts of $CaCl_2$. From these results (Table 3.9) it is evident that a greater hydrolysis of the substrate occurred in the presence of 0.05 M $CaCl_2$ than without $CaCl_2$ and moreover in the presence of concentrations of $CaCl_2$ greater than 0.2 M a decrease in hydrolysis was evident. These results are in close agreement with the profile obtained for protoplast release with differing concentrations of $CaCl_2$. (Fig 3.11). The optimum concentration of $CaCl_2$ for protoplast release was also 0.05 M while concentrations greater than 0.2 M decreased yield. This latter observation could explain why 0.6 M $CaCl_2$ and $Ca(NO_3)_2$ used alone produced poor yields of protoplasts.

TABLE 3.8.

Time after addition of $^{45}CaCl_2$ (min).	d.p.m.
1.0	8530 ± 164
2.0	9673 ± 198
3.0	7328 ± 117
4.0	5908 ± 80
5.0	9701 ± 186
6.0	9159 ± 148
7.0	6440 ± 113
8.0	8226 ± 143
9.0	7684 ± 129
12	7446 ± 107
15	8197 ± 145
20	5752 ± 92

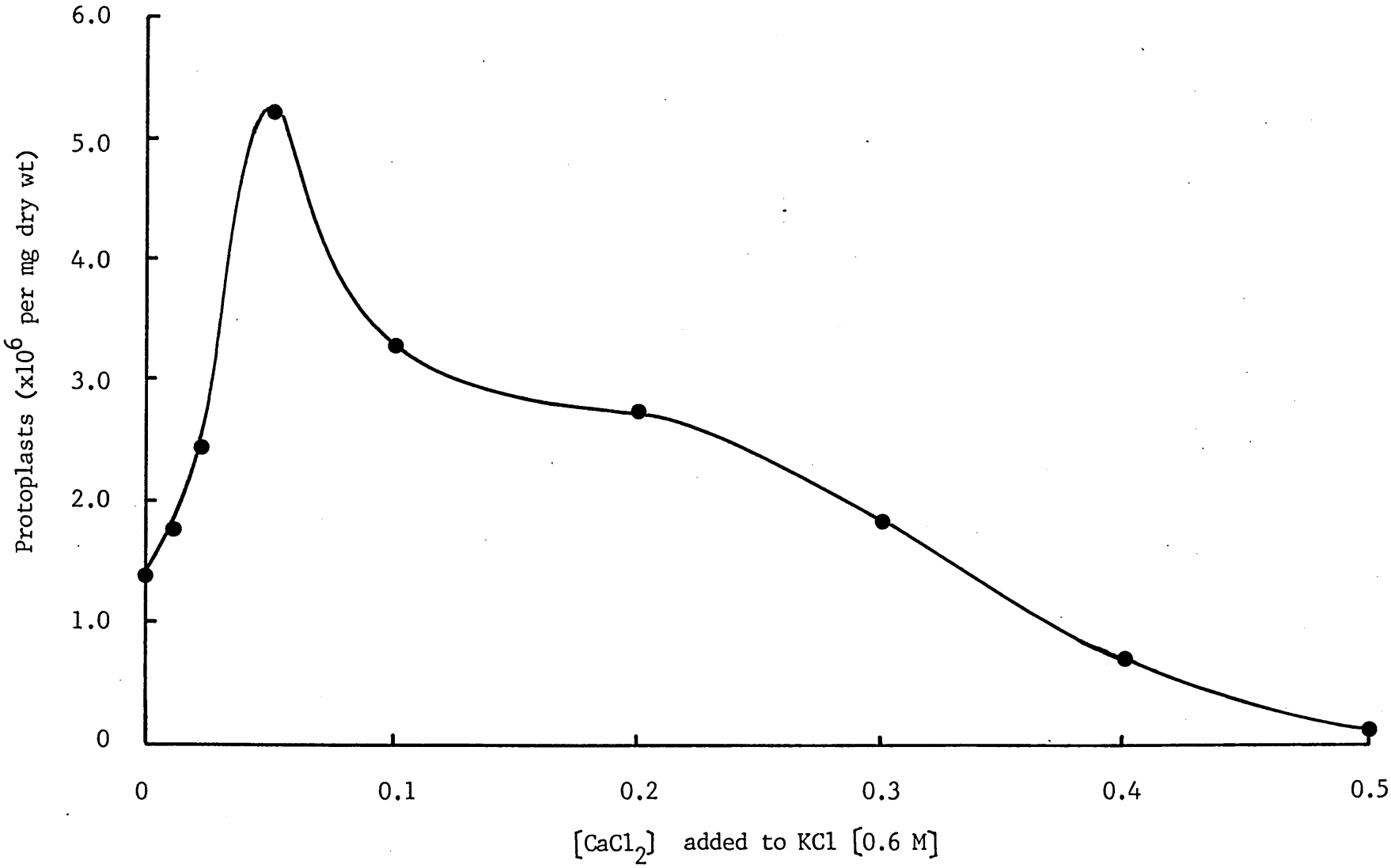
Uptake of ^{45}Ca by A. fumigatus mycelium incubated in protoplast buffer as described in Materials and Methods.

TABLE 3.9.

$CaCl_2$ concentration	µg N-acetyl glucosamine
0.00 M	440 ± 64
0.05 M	507 ± 13
0.20 M	364 ± 16
0.5 M	293 ± 38

Effect of $CaCl_2$ concentration on release of N-acetyl sugars from purified chitin by digestion with purified, calcium-free chitinase.

FIGURE 3.11. Effect of 0.6 M KCl supplemented with different concentrations of $CaCl_2$, on protoplast release from A. fumigatus. Mycelium samples, 1.0 ml, were incubated for 1.0 h with 0.1 ml β-glucuronidase and 1.0 unit chitinase at 30°C as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer.


6.0
5.0
4.0
3.0
2.0
1.0
0
Protoplasts (x10^6 per mg dry wt)
0
0.1
0.2
0.3
0.4
0.5
[CaCl2] added to KCl [0.6 M]

3.3. ADDITIONAL FACTORS INFLUENCING PROTOPLAST RELEASE.

Further factors affecting protoplast release have been noted to include pH, temperature and culture conditions. Using the standard enzyme combination of 0.1 ml β-glucuronidase supplemented with 1.0 unit of chitinase per 1.0 ml mycelium suspension each of these factors was investigated.

The temperature optimum for release of protoplasts from A. niger was found to be 30°C and for A. fumigatus 35°C. Temperatures higher than 35°C caused a significant decrease in the number of protoplasts released from both species (Fig. 3.12). Very few protoplasts were released at temperatures higher than 40°C. Protoplasts which were released from mycelium incubated at 30°C were stable for up to 18 h when maintained at 30 or 50°C, suggesting that the lack of protoplast production at the higher temperatures is due to enzyme inactivation. This is also supported by the observation that extensive mycelium remains undigested at these temperatures.

The optimum pH for protoplast release from A. niger was pH 5.5 (Fig. 3.13). A. fumigatus showed a much broader pH range with the optimum at pH 6.5 (Fig. 3.13). However, for convenience, all protoplast experiments on A. fumigatus were conducted at pH 5.5. At extreme pH values protoplast yield was markedly reduced, and little digestion of mycelium was evident, indicating low enzyme activity. Fig 3.14 shows that the release of reducing sugars from isolated A. niger cell walls digested by β-glucuronidase and chitinase has a pH optimum between 5.0 and 5.5 and supports the above inference However, it is also evident that the optimum does not correlate exactly with that of protoplast release indicating that a wall polymer

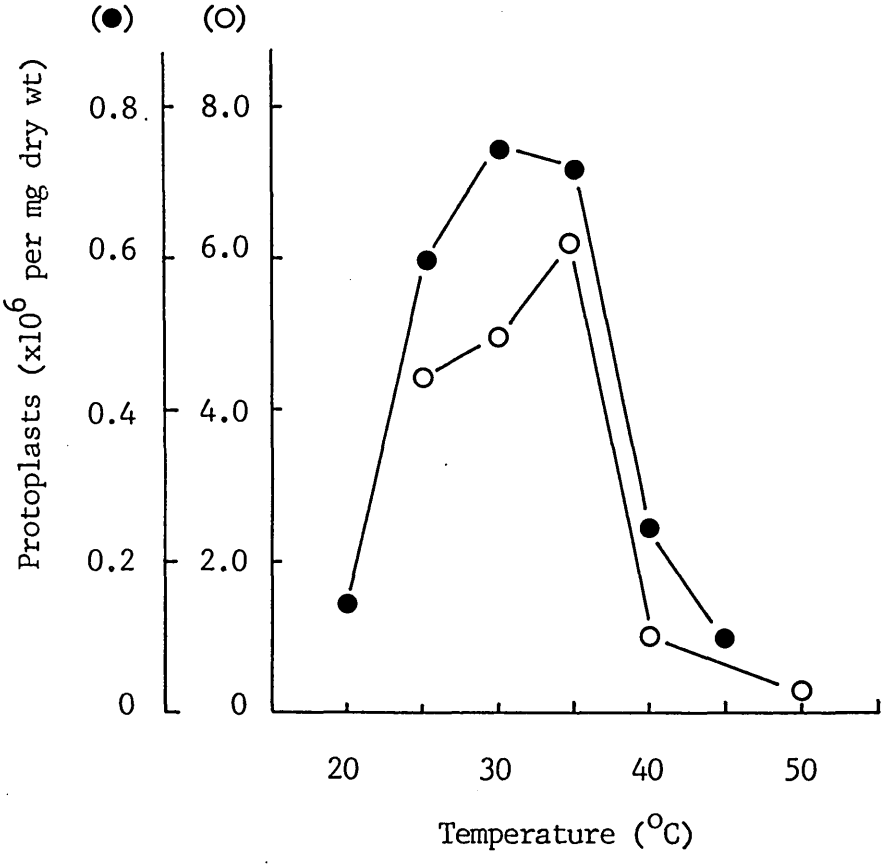


FIGURE 3.12. Effect of incubation temperature on protoplast release from A. niger (●) and A. fumigatus (○). Mycelium samples, 1.0 ml, were incubated for 1.0 h with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer.

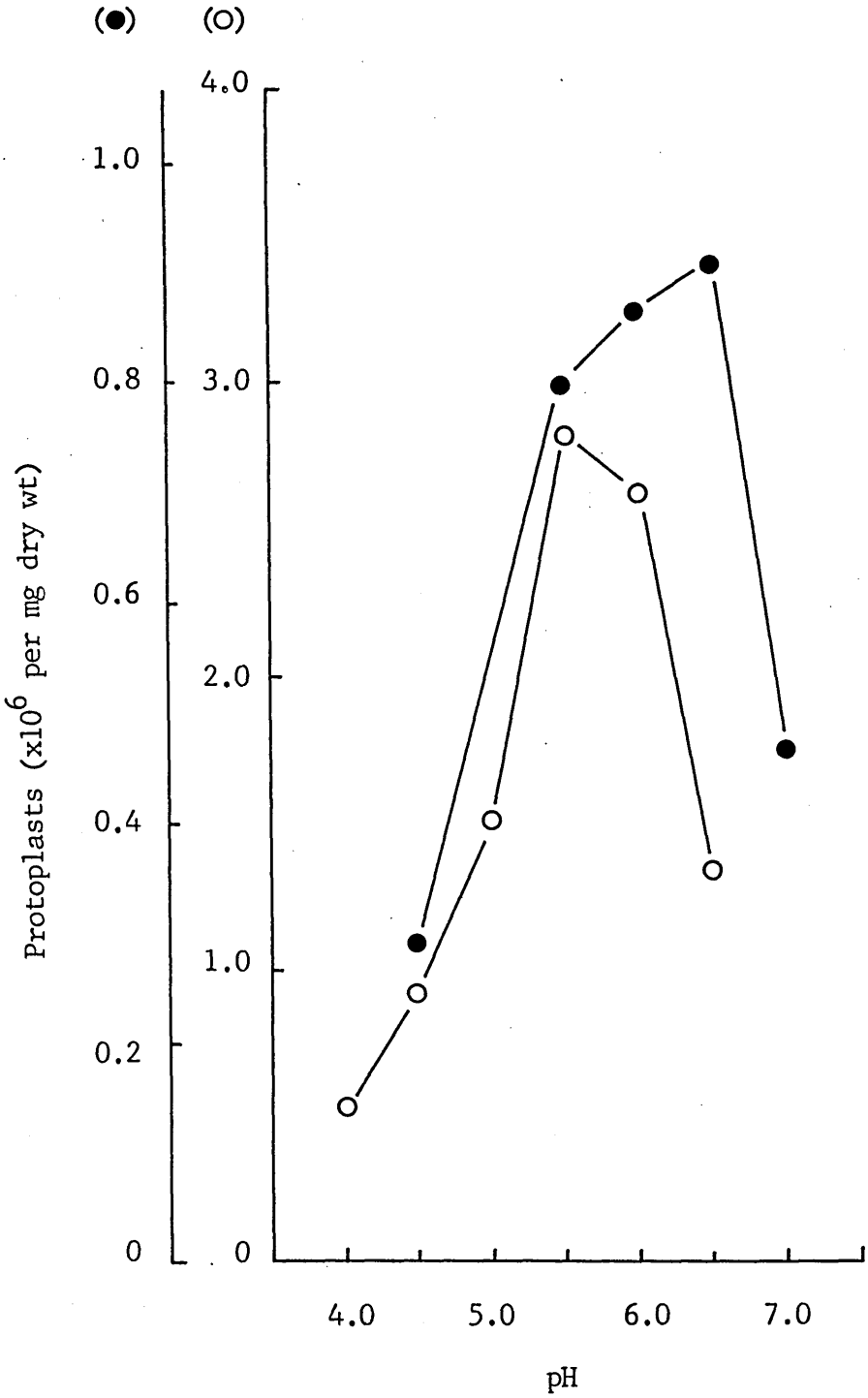


FIGURE 3.13. Effect of pH on protoplast release from A. niger (●) and A. fumigatus (○). Mycelium samples, 1.0 ml, were incubated for 1.0 h with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase as described in Materials and Methods. Protoplast numbers were determined by counting in a haemocytometer.

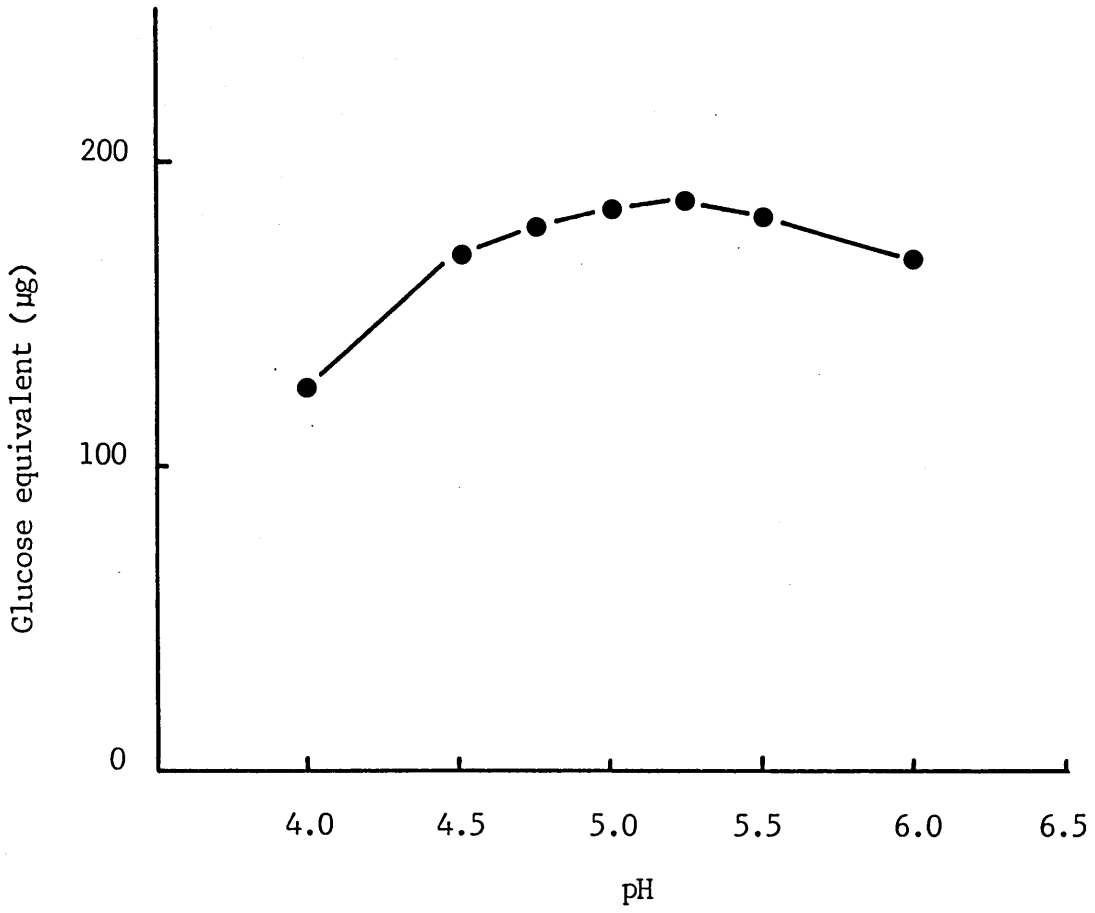


FIGURE 3.14. Effect of pH on release of reducing sugars from isolated A. niger cell walls. Wall samples, suspended in 1.0 ml of protoplast incubation buffer at a concentration of 0.5% (w/v), were incubated with 0.1 ml of β-glucuronidase and chitinase for 1.0 h at 30°C as described in Materials and Methods. Reducing sugars were determined using the DNS reagent.

other than those digested primarily by β-glucuronidase may restrain protoplast release. To test the hypothesis that this could be chitin a pH profile of pure chitinase activity acting on chitin was conducted and from Fig. 3.15 it can be seen that the optimum of chitin digestion is between 5.5 and 6.0 which is similar to that for protoplast release.

Culture conditions also play an important part in protoplast release and include a number of variables ranging from growth media and growth temperature to mycelial age and growth states. The effect of mycelial age on protoplast release from A. niger and A. fumigatus was therefore investigated. Mycelial age is an important variable since the age of mycelia from which protoplasts are desired need not correlate with that at which protoplasts are readily formed. Mycelium from 24, 48 and 72 h cultures were incubated with the standard enzyme system of β-glucuronidase and chitinase and the protoplasts released counted after 1.0 h incubation. From this the younger hyphae were found to be twice as productive as 2 day old mycelium and three times as productive as 3 day old mycelium (Table 3.10). There were, however, no apparent differences between the morphologies of the protoplasts released from different ages of mycelium.

The effect of an elevated growth temperature on the release of protoplasts from A. fumigatus was also investigated since this organism was also studied when grown at 48°C. Release of protoplasts from mycelium grown at 48°C was found to be considerably reduced compared to release from mycelium grown at 30°C (Table 3.11). The temperature profile of release, however, has an optimum at 35°C which is similar to that for release from mycelium grown at 30°C.

Finally the effect of growth media on protoplast release was also

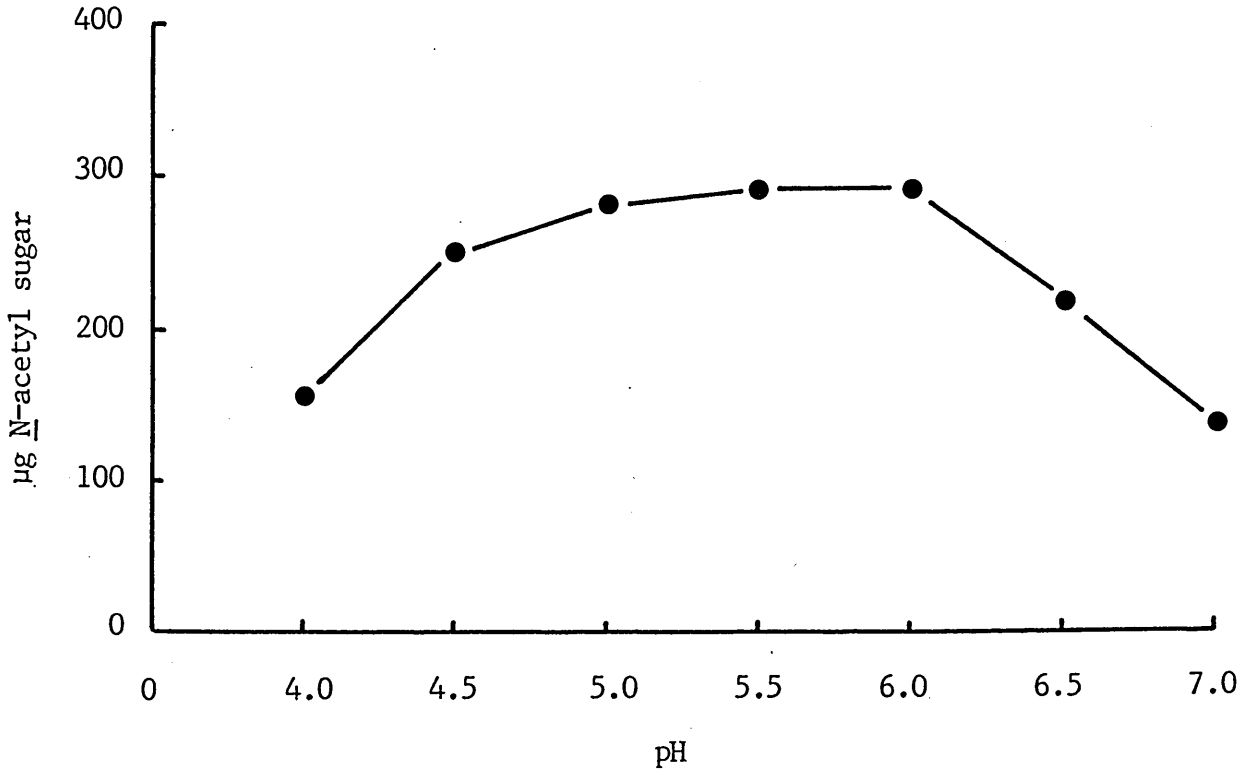


FIGURE 3.15. Effect of pH on release of N-acetyl sugars from purified chitin by chitinase. Chitin, resuspended in 1.0 ml of protoplast incubation buffer at a concentration of 0.5% (w/v), was incubated for 1.0 h with 1.0 unit chitinase at 30°C as described in Materials and Methods. N-acetyl sugars were determined using the DMAB reagent.

TABLE 3.10.

Mycelial age (Hours)	Protoplasts x 10^6 per mg dry wt
24	4.90 ± 1.03
48	2.42 ± 0.39
72	1.78 ± 0.52

Effect of mycelial age on protoplast production from A. fumigatus. 1.0 h incubation with β-glucuronidase and chitinase as described in Materials and Methods.

TABLE 3.11.

Incubation temperature ^oC	Protoplasts x 10^6 per mg dry wt	
	Growth temperature ^oC	
	48	30
25	0.021 ± 0.011	2.30 ± 0.19
30	0.030 ± 0.017	3.60 ± 0.81
35	0.047 ± 0.016	5.30 ± 1.06
40	0.014 ± 0.006	3.30 ± 0.23
50	0.013 ± 0.006	1.700 ± 0.19

Effect of growth temperature on protoplast production from A. fumigatus. 1.0 h incubation with β-glucuronidase and chitinase as described in Materials and Methods.

investigated and indicated that mycelia grown on cellulose produced more protoplasts per mg dry weight of mycelium than mycelium grown on glucose (Table 3.12).

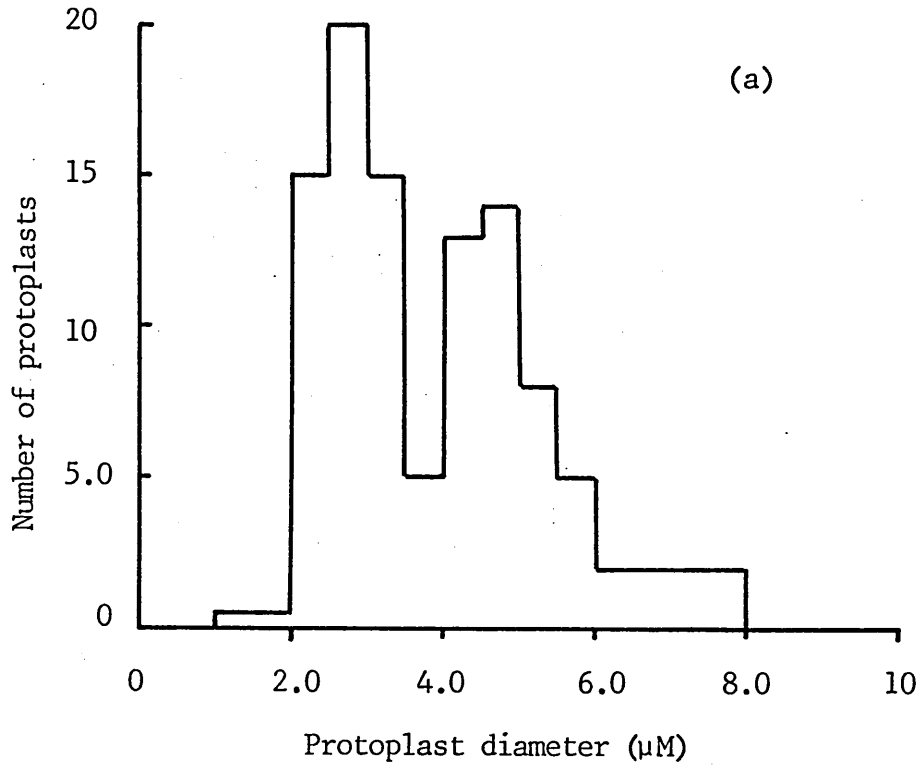
3.4. MORPHOLOGICAL OBSERVATIONS.

Samples from all protoplast experiments were observed in the light microscope to ensure that a consistent digestion and protoplast morphology were produced. In some experiments anomalous "protoplasts" appeared, particularly from poorly grown hyphae or with old stocks of enzyme. These "protoplasts" were usually small and oval or irregular in shape and appeared refractive under the phase contrast microscope indicating a different surface composition than normally formed protoplasts. Although these anomalous "protoplasts" were observed to originate from hyphae by extrusion and did increase in number in proportion with the time of digestion, they did not readily lyse on the addition of water and were not considered to be true protoplasts. Consequently, experiments in which they appeared were abandoned.

The size of protoplasts released ranged from 2.5 µm to 14.5 µm as indicated in the histogram plot of protoplasts present in the medium after 120 min of digestion (Fig 3.16). The average size depended upon the time of release and the osmoticum employed. In general larger protoplasts were evident at later stages of digestion (Table 3.13). This may be due to the possibility that hyphae in later stages of digestion possess greater areas of wall weaknesses through which larger protoplasts may emerge.

Fig.3.17 illustrates the appearance under the light microscope of a hyphal digestion after 30 min and shows both tip and distal

FIGURE 3.16. Size distribution of protoplasts released from A. niger mycelium with β-glucuronidase (a) and β-glucuronidase and chitinase (b). Mycelium samples, 1.0 ml, were incubated for 1.0 h at 30°C as described in Materials and Methods. Protoplast diameters were measured using a calibrated eyepiece graticule.


(a)
Number of protoplasts
20
15
10
5.0
0
0
2.0
4.0
6.0
8.0
10
Protoplast diameter (μM)

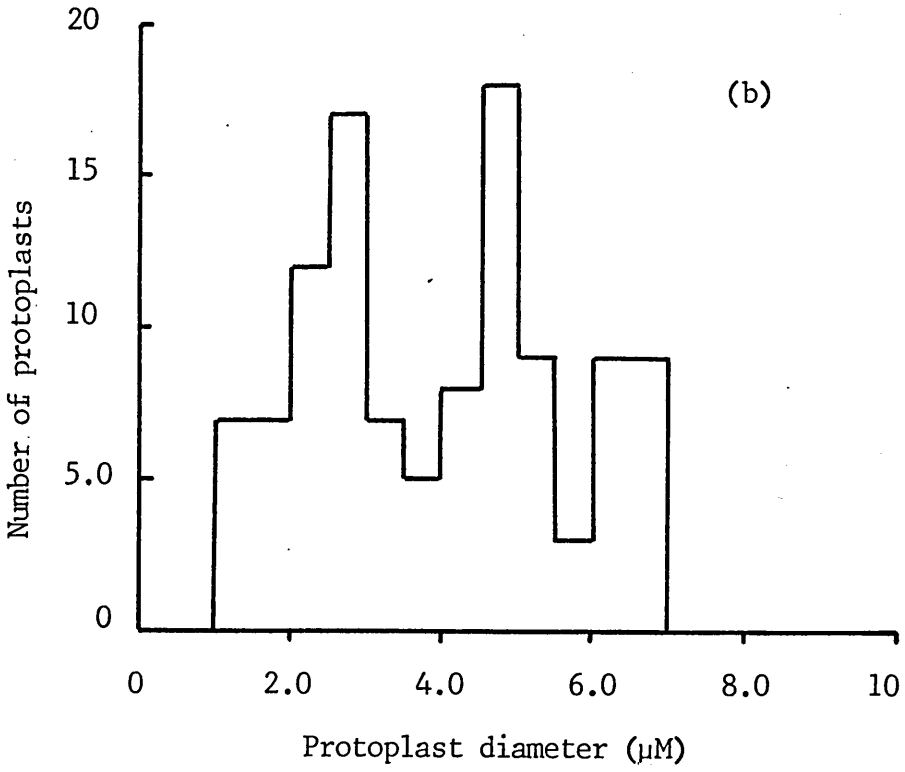

(b)
Number of protoplasts
20
15
10
5.0
0
0
2.0
4.0
6.0
8.0
10
Protoplast diameter (μM)

TABLE 3.12.

Growth medium	Protoplasts x 10^6 per mg dry wt
Glucose, 2.0%	0.135 ± 0.081
CMC + 0.2% glucose	0.271 ± 0.062

Effect of growth medium on protoplast production from A. fumigatus grown on Eggins and Pugh cellulose medium containing 2.0% glucose or 2.0% CMC + 0.2% glucose. Mycelium incubated with β-glucuronidase and chitinase for 1.0 h as described in Materials and Methods.

TABLE 3.13.

Time (mins)	β-glucurionidase µm	β-glucuronidase + chitinase µm
30	3.07 ± 0.95	2.98 ± 0.92
60	3.25 ± 0.78	2.98 ± 0.77
90	3.27 ± 0.95	3.46 ± 0.99

Size of protoplasts isolated at different times from A. niger by digestion with β-glucuronidase with and without chitinase.

TABLE 3.14.

Time (mins)	Tip	%	Distal	%
20	15.5 ± 0.54	76	5.0 ± 0.17	24
40	13.5 ± 1.91	67	6.5 ± 1.75	33
60	19.5 ± 1.97	60	13.0 ± 1.67	40

Numbers of protoplasts observed undergoing release from tip and distal regions of A. fumigatus mycelia incubated with β-glucuronidase and chitinase as described in Materials and Methods.

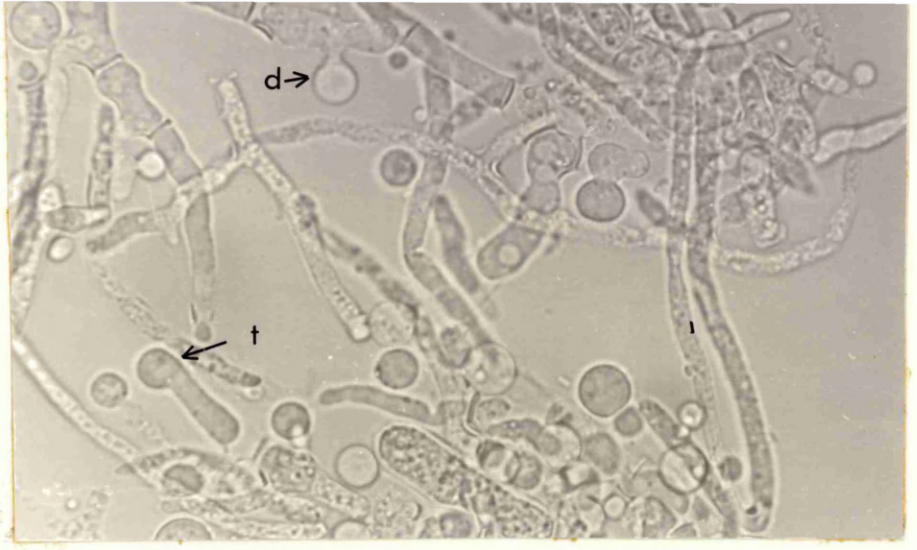


FIGURE 3.17. Hyphal digestion and protoplast release from A. niger mycelium. Mycelium samples, 0.1 ml, in protoplast incubation buffer, were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. x 800. d = distal release, t = tip release.

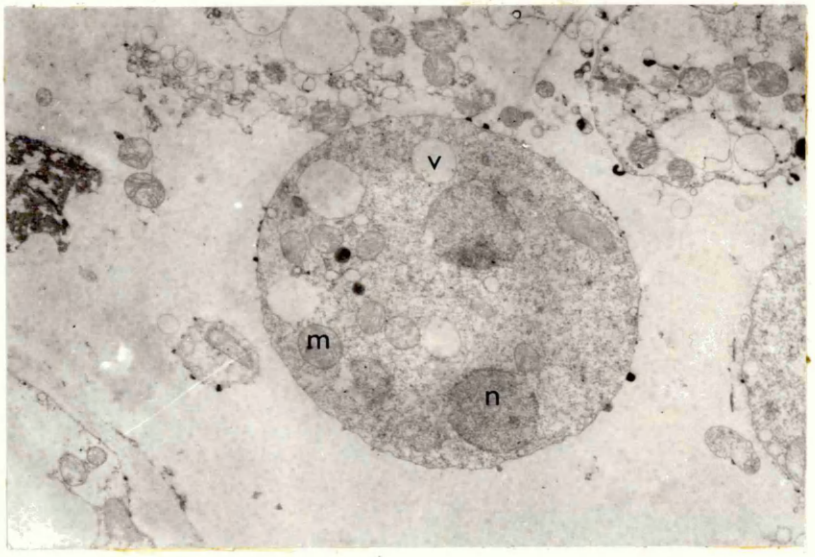


FIGURE 3.18. Protoplast from A. fumigatus. Mycelium samples, 1.0 ml, in protoplast incubation buffer, were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. x 10,000. n = nucleus, m = mitochondria, v = vacuole.

release of protoplasts. Counts made at various times of incubation indicated that the proportion of distally released protoplasts increased from 24% at 20 min to 40% at 60 min (Table 3.14).

In order to assess whether different enzyme treatments produce different sizes of protoplast, measurements were also made on protoplasts released by digestion with β-glucuronidase with and without chitinase. The results show that little difference in size is evident until the later stages of digestion when the chitinase samples produced slightly larger protoplasts (Table 3.13). However, it should be remembered that a single average value does not fully represent the distribution of protoplast size. Fig. 3.16 shows that two populations of protoplast commonly occur, and that the size profile did not differ significantly between incubations with β-glucuronidase alone or with the addition of chitinase. Osmoticum concentration did affect protoplast size (Table 3.15).

Transmission electron microscopy (TEM) was conducted on mycelium and protoplasts to determine whether any morphological details of digestion would correlate with lytic enzyme activity and more particularly to ensure that the protoplasts possessed a cytoplasmic morphology comparable to that of intact hyphae and that damage was not frequently evident. Details of the stages of hyphal digestion were also observed.

Figs 3.18 and 3.19 illustrate the characteristic appearance of protoplasts from A. fumigatus and show a full range of intact organelles to be present. Mitochondria and nuclei are readily discernible and vacuoles are particularly common near the plasma membrane. The density of the cytoplasm varies, protoplasts containing large vacuoles generally possessing a less dense cytoplasm. Granules in protoplasts with a more dense cytoplasm often occur in regular

TABLE 3.15.

KCl concentration	Protoplast diameter (μm)
0.2 M	3.06 ± 1.02
0.4 M	2.87 ± 0.94
0.6 M	2.49 ± 0.65
0.8 M	2.20 ± 0.54

Effects of osmoticum concentration on diameter of protoplasts released from A. fumigatus after 1.0 h digestion with 0.1 ml β-glucuronidase and 1.0 unit chitinase. Mycelium was resuspended in 1.0 ml of protoplast incubation medium containing KCl and 0.2 M $CaCl_2$ and incubated at 30°C.

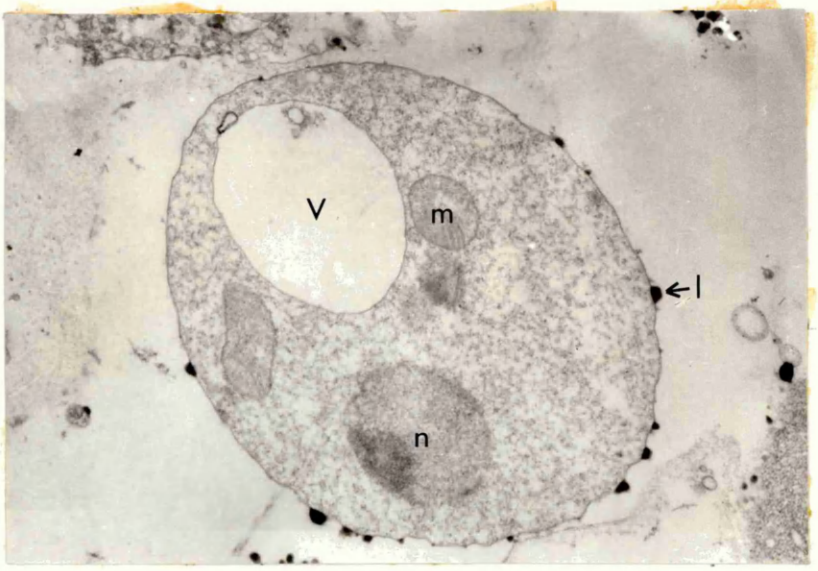


FIGURE 3.19. Protoplast from A. fumigatus. Mycelium samples, 1.0 ml, in protoplast incubation buffer, were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 90 min. x15,000.
l = lipid like droplet, m = mitochondria, n = nucleus, v = vacuole.

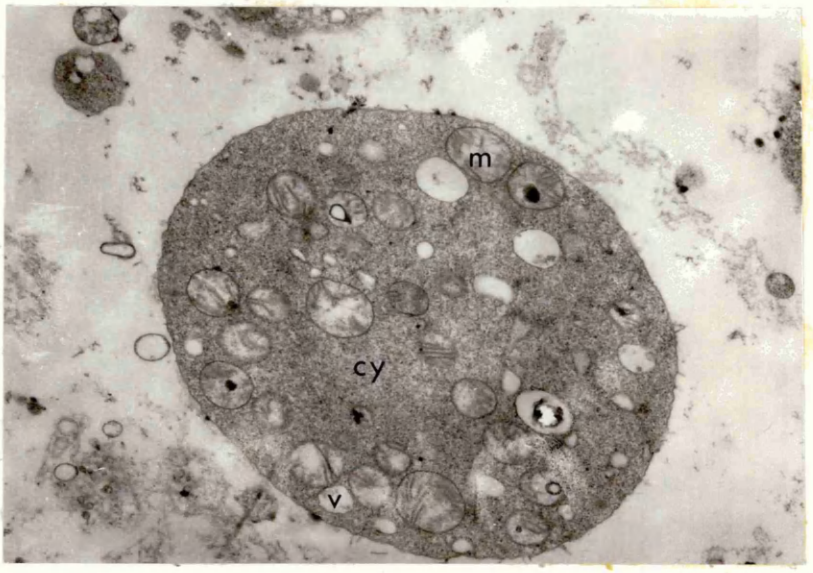


FIGURE 3.20. Protoplast from A. fumigatus. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 90 min. x 12,500.
cy = dense cytoplasm containing many ribosomes, m = mitochondria, v = vacuole.

arrangements indicating the presence of functional ribosomes (Fig. 3.20). The surface of most protoplasts, observed in thin section, was generally irregular and frequently contained areas of dense, lipid-like globules. There was, however, no evidence of cell wall layers being present (Fig. 3.20).

Some indication of the details of wall breakdown was obtained by examining thin sections of hyphae after short periods of digestion. Fig. 3.21 shows a hypha after 30 min of incubation with β-glucuronidase and chitinase and indicates that the wall has not only fragmented into sections with distinct separations but that some sections have become detached from the underlying cytoplasm. Differences, particularly with regard to staining and continuity, are apparent between some of the wall layers. Surface layers generally stained more heavily and became more fragmented than underlying layers in the early stages of digestion and occasionally rounded bodies of cytoplasm were evident with attached 'tails' of lightly staining material (Fig. 3.22).

In order to elucidate more clearly whether any layers of wall material remained on the protoplasts, after liberation, the techniques of shadowing for TEM and observation in the scanning electron microscope (SEM) were employed.

Shadowing has been used extensively to determine the characteristics and arrangements of fungal wall polymers and has indicated that many fungal walls contain an inner layer of microfibrils coated and surrounded with layers of more amorphous polymers including protein (Hunsley and Burnett, 1970; Carbonell et al., 1970 and van der Valk et al., 1977). Shadowed samples of undigested A. fumigatus hyphae showed characteristically amorphous surfaces and no details of any regular wall structure are discernible (Fig. 3.23).

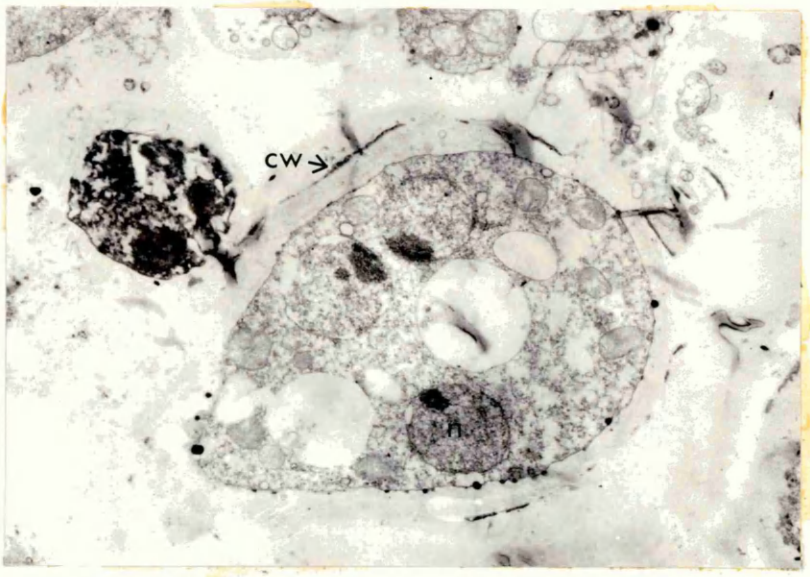


FIGURE 3.21. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer, were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. x 12,500.
cw = cell wall fragments, n = nucleus.

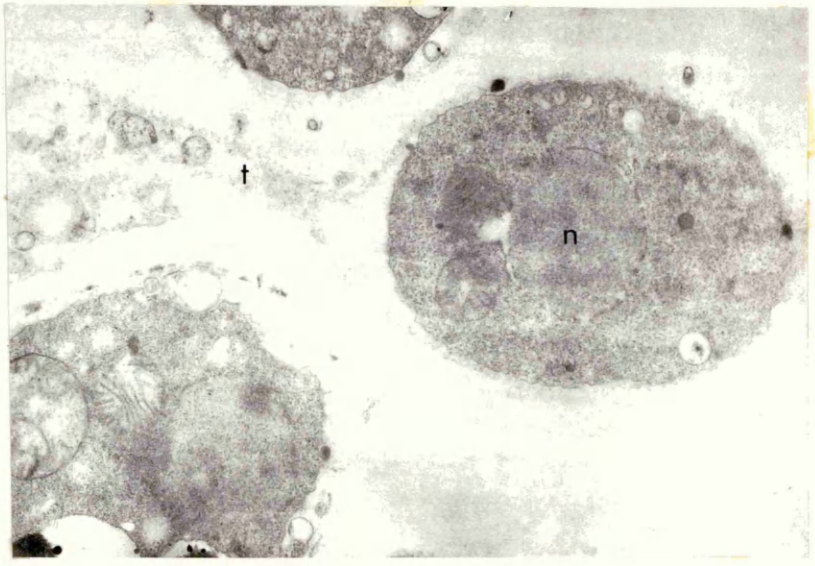


FIGURE 3.22. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer, were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. x 12,500.
n = nucleus, t = tail of residual wall material.

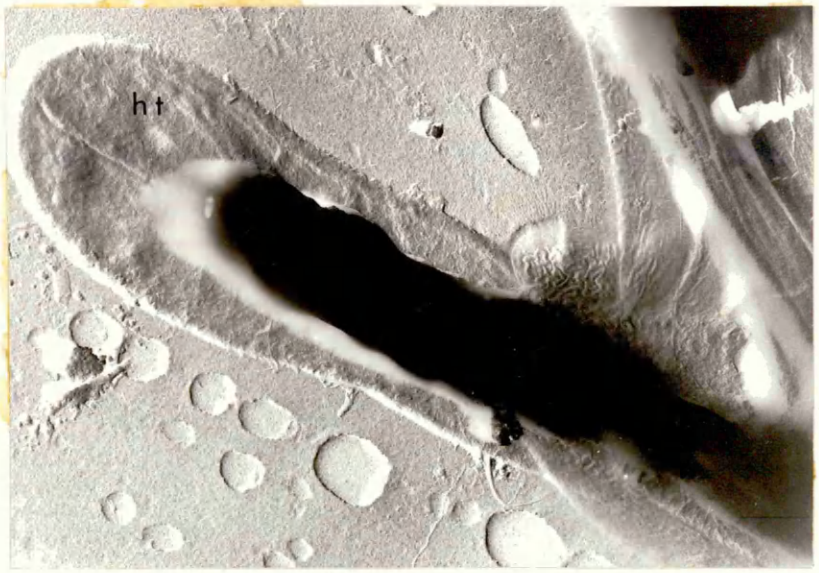


FIGURE 3.23. A. fumigatus undigested hyphae. Mycelium samples, in protoplast incubation buffer,were prepared for shadowing as described in Materials and Methods. x 18,500. ht = hyphal tip.

FIGURE 3.24 A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase for 30 min. Samples were prepared for shadowing as described in Materials and Methods. x 40,000. mf = microfibrils.

Samples digested with β-glucuronidase, however, were considerably thinner and showed some details of underlying wall microfibrils (Fig. 3.24). In some cases the remains of protoplasts could be seen emerging from the hyphae (Fig. 3.25). Free protoplasts were also visible and showed no evidence of any adhering wall polymers although vesicle like bodies were evident at the broken edges of these and in the protoplasts emerging from the hyphae.

The lack of any visible surface layers on the released protoplasts observed by this technique and by shadowing and their appearance while undergoing release suggests that wall digestion need not be complete for release to occur. Internal pressure coupled with a weakening of some of the polymers could lead to extrusion of the cytoplasm through the remaining layers. In this respect it is relevant to note the apparent absence of microfibrils around the area of protoplast release (Fig. 3.25). Shadow casting unfortunately only provides a flattened image of surface features and in order to obtain better evidence samples were processed for the SEM. With this technique a greater depth of focus is afforded by the optical system and a near three dimensional appearance produced of the surface features. Consequently the spatial relationships between these may be more readily investigated. Fig. 3.26 shows the appearance of A. fumigatus hyphae incubated in KCl buffer in the absence of lytic enzymes. The position of the spore case is easily discernible and the outline of a septum evident on one hypha. The surface features of the hyphae were rough in parts but continuous and no holes or ruptures were evident at any magnification. Generally the spore coats had characteristic sculpturing and Fig 3.27 illustrates the features of unimbibed spores.

After 30 min of incubation with β-glucuronidase and chitinase

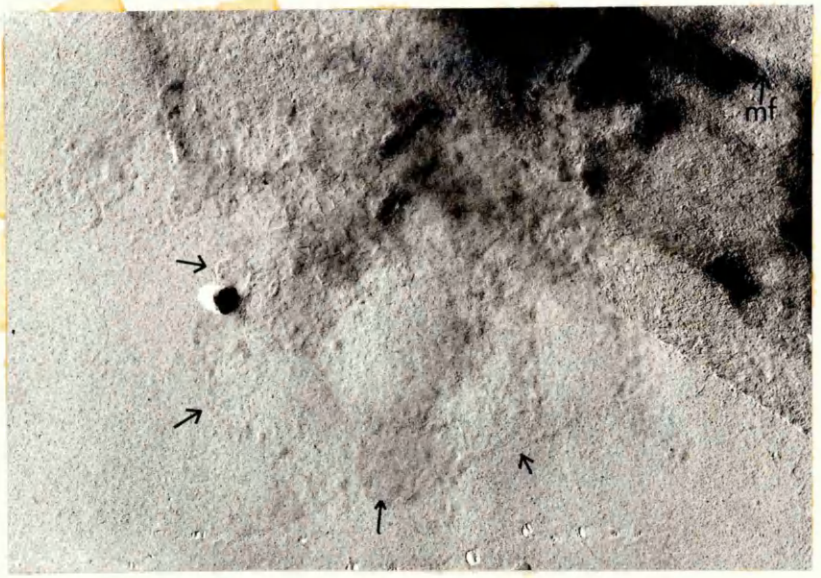


FIGURE 3.25. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase for 30 min. Samples were prepared for shadowing as described in Materials and Methods. x 25,000. mf = microfibrils, arrows show outer edge of emerging protoplast.

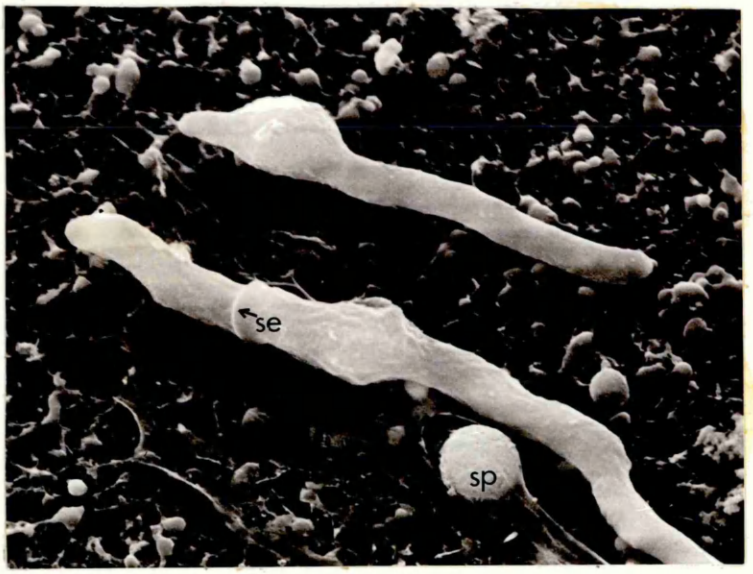


FIGURE 3.26. A. fumigatus undigested hyphae. Mycelium samples in protoplast incubation buffer were prepared for SEM as described in Materials and Methods. x 17,600. se = septum, sp = spore.

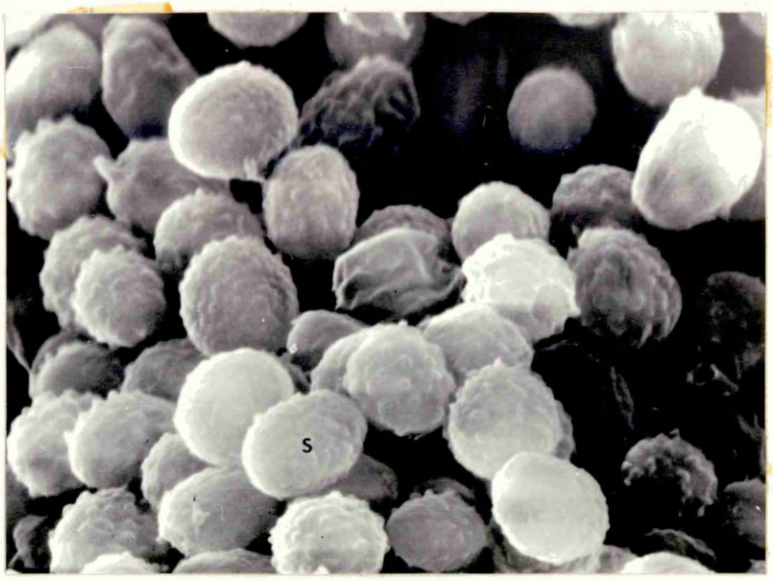


FIGURE. 3.27. A. fumigatus unimbibed spores. Conidiophores from mycelium grown on solid BMB medium were prepared for SEM as described in Materials and Methods. x 23,000. s = unimbibed spore.

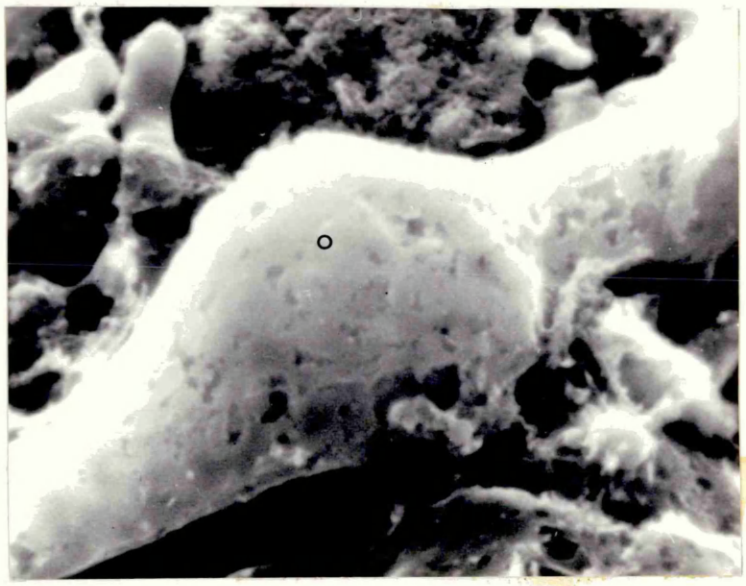


FIGURE 3.28. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. Samples were prepared for SEM as described in Materials and Methods. x 44,000. o = outer hyphal layer, p = pitting.

hyphal surfaces were extensively roughened and showed occasional areas of fracture (Fig. 3.28). After 90 min of incubation fragmentation of the hyphae was common and most pieces were extensively pitted with holes (Fig. 3.29). Observations on samples digested for 30 min indicated that pitting in these only went as deep as a loosely fitting surface layer whereas in 90 min samples the pitting was evident through the depth of the wall. By this time sheets of wall material were evident either detached from or loosely attached to the hyphae (Fig. 3.30). Protoplasts were also observed and increased in number with the time of incubation (Figs. 3.31 and 3.32). Moreover, some protoplasts could be seen in the process of release and from these it was evident that at least in distal areas, the hyphal wall was actively split apart confirming that complete wall digestion was not necessary for protoplast release (Figs. 3.33 and 3.34).

Few wall fragments were seen to be associated with the surface of released protoplasts although considerable debris was evident (Figs. 3.29 and 3.30). The surfaces of most of the released protoplasts and those undergoing release were often crenated although overall the protoplasts were spherical. Occasionally, perfectly spherical protoplasts were observed (Fig. 3.35). No details could be seen within the crenations which varied in size and complexity from protoplast to protoplast. Also, no connections were observed between protoplasts released from distal areas of hyphae and the hyphal interior or between protoplasts after release. Some protoplasts, however, did appear to maintain a narrow connection with the hyphal body in tip areas of release (Fig. 3.36). Further details of wall structure in these areas were not obtained.

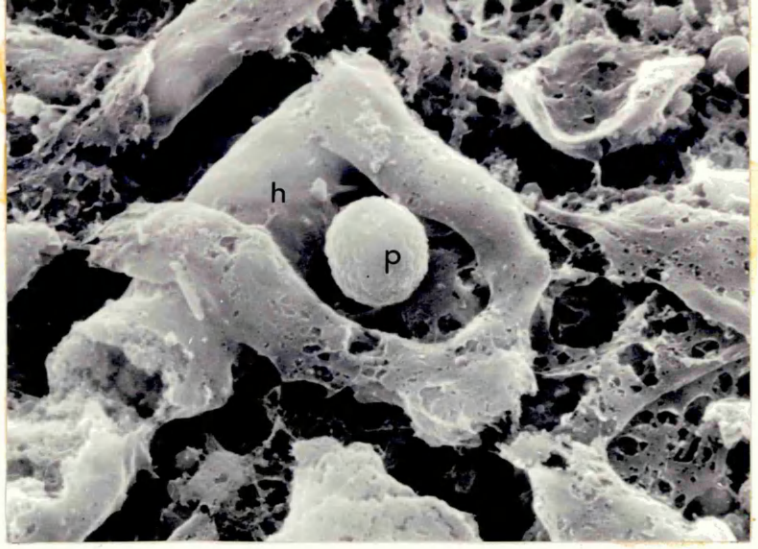


FIGURE 3.29. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 90 min. Samples were prepared for SEM as described in Materials and Methods. x 27,500. h = hyphae, p = protoplast.

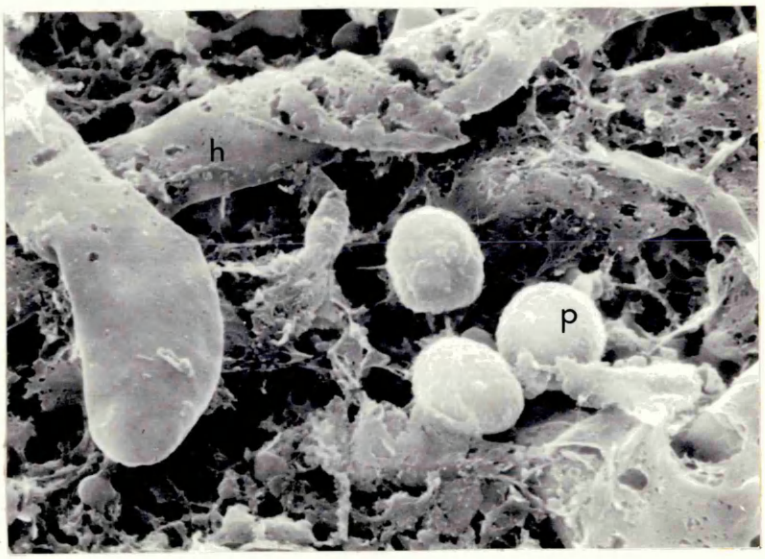


FIGURE 3.30. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 1.0 ml of β-glucuronidase and 1.0 unit of chitinase for 90 min. Samples were prepared for SEM as described in Materials and Methods. x 27,500. h = hyphae, p = protoplast.

FIGURE 3.31. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 1.0 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. Samples were prepared for SEM as described in Materials and Methods. x 7,500.
h = hyphae.

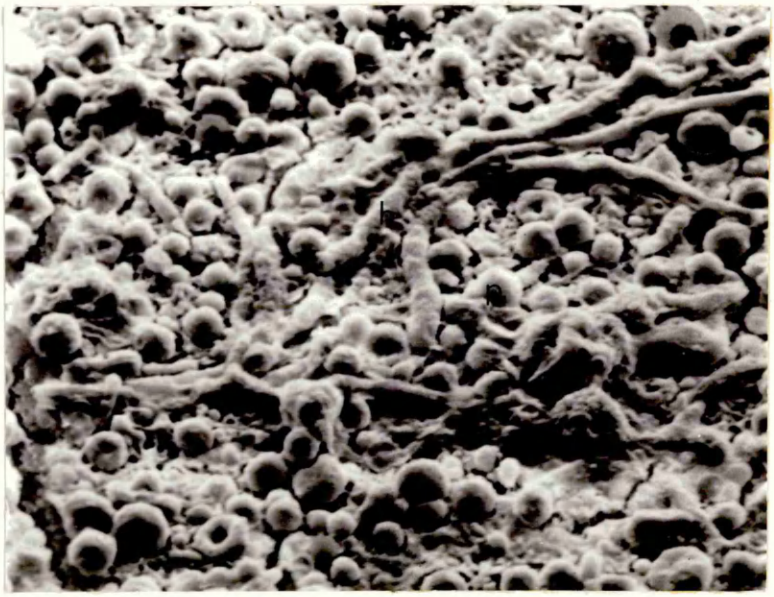


FIGURE 3.32. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 90 min. Samples were prepared for SEM as described in Materials and Methods. x 7,500.
h = hyphae, p = protoplast.

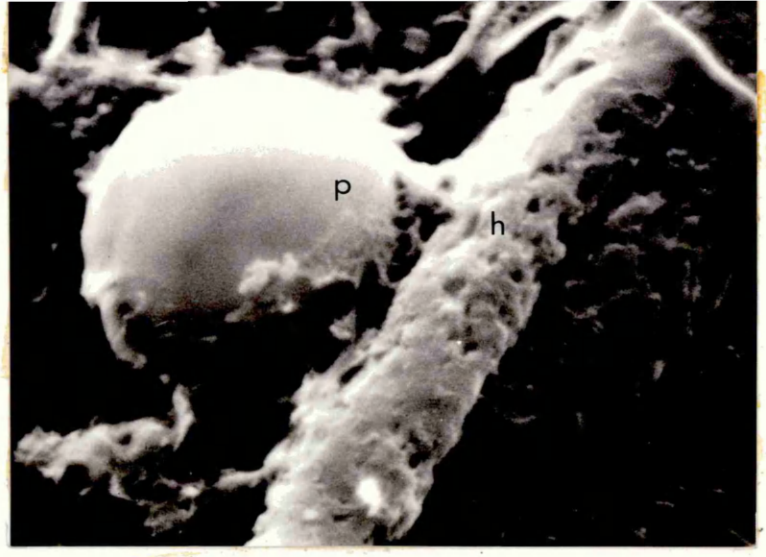


FIGURE 3.33. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. Samples were prepared for SEM as described in Materials and Methods. x 44,000. h = hyphae, p = protoplast.

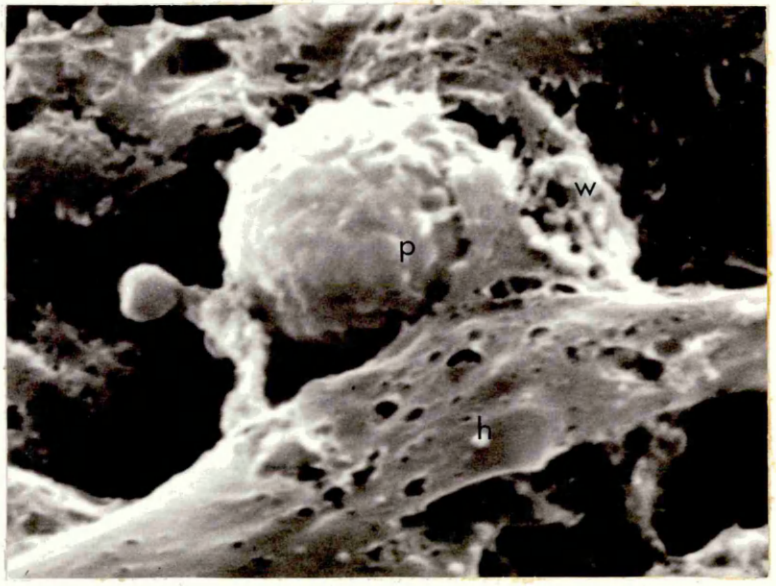


FIGURE 3.34. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 1.0 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. Samples were prepared for SEM as described in Materials and Methods. x 44,000. h = hyphae, p = protoplast, w = wall layers.

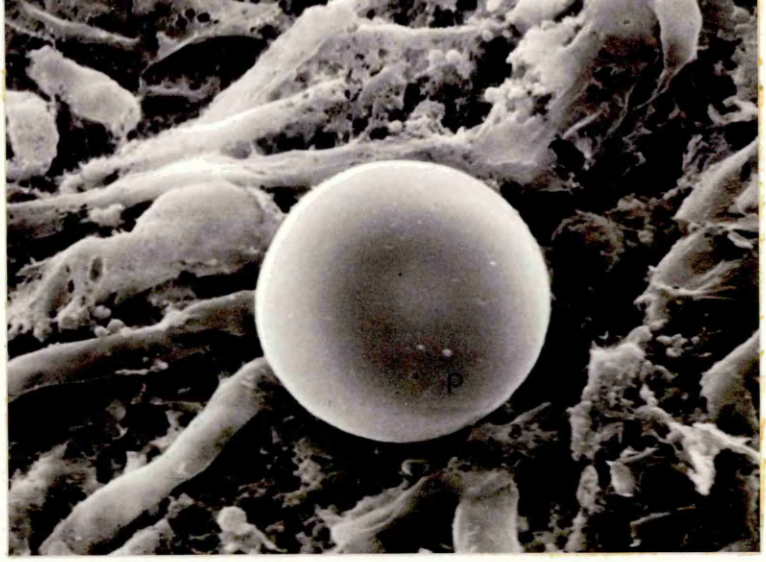


FIGURE 3.35. A. fumigatus protoplast. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 0.1 ml of β-glucuronidase and 1.0 unit of chitinase for 90 min. Samples were prepared for SEM as described in Materials and Methods. x 44,000.
p = protoplast.

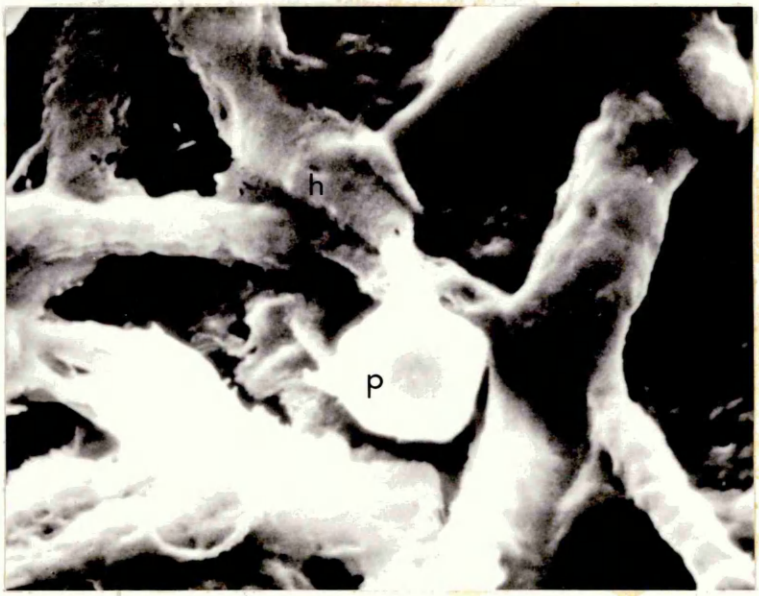


FIGURE 3.36. A. fumigatus digested hyphae. Mycelium samples, 1.0 ml, in protoplast incubation buffer were incubated at 30°C with 1.0 ml of β-glucuronidase and 1.0 unit of chitinase for 30 min. Samples were prepared for SEM as described in Materials and Methods. x 27,500.
h = hyphae, p = protoplast.

The details of protoplast release have been investigated in many species of filamentaous fungi and a number of standard procedures developed (de Vries and Wessels, 1972; Peberdy et al., 1976 and Peberdy and Isaac, 1976). While these procedures have been applied to a range of fungi it is also apparent that species vary considerably in their response (Aguirre and Villanueva, 1962: Brown, 1971 and Peberdy et al 1976).

The use of hydrolytic enzymes and various osmotica have allowed the release of protoplasts from A. niger and A. fumigatus, and from the results obtained two general conclusions may be proposed. Firstly, a range of evidence indicates that chitin is an important restraint on protoplast release and optimal conditions are necessary to ensure digestion of this polymer. Secondly, the inclusion of supplementary salts in the stabilization osmoticum may provide an enhancement of release due to their effect on the activity of the lytic enzymes.

The importance of chitin digestion in ensuring protoplast release is indicated primarily by the dramatic effect on protoplast yield of supplementing β-glucuronidase with chitinase (Figs. 3.3a and 3.3b). This would appear to be due to a synergistic combination of the enzymes involved and is probably in some way related to wall architecture since chitinase alone is unsuccessful in releasing protoplasts even after prolonged incubation (Table 3.2). Similar synergisms have been frequently observed in other systems (Sietsma and Wouters, 1971; de Vries and Wessels, 1973a; Anné et al., 1974 and Jeffries et al., 1977) and have been explained by restrictions imposed on enzyme attack by the arrangement of wall polymers (Domanski and Miller, 1968 and Hunsley and Burnett, 1970).

Further evidence indicating the importance of the chitin layer

for protoplast release from A. niger and A. fumigatus include the time course profiles of protoplast release following more closely those of N- acetyl sugar release rather than the profiles of total sugar release (Figs. 3.5, 3.6 and 3.7), and also the concentration dependence of protoplast release on added chitinase (Fig. 3.4). Further support for this theory comes from the observation that the pH optimum of protoplast release is closer to that of chitin digestion by chitinase than to that of total sugar release from hyphal walls digested by β-glucuronidase (Figs. 3.13 and 3.15). A similar difference in pH optima has been used to implicate specific lytic components in the digestion of other species (Vrsanska et al., 1977).

The presence of chitin in Aspergilli cell walls has been well documented (Horikoshi and Iida, 1964; Johnston, 1967 and Ruiz-Herrera, 1967) and the enhancement of protoplast release by chitinase has been reported in a number of systems (Skujins et al., 1965; Sietsma and Wouters, 1971 and Davis et al., 1977), indicating that chitin may fulfil a common role within many fungal walls. Current theories on wall structure in fungi (Hunsley and Burnett, 1970 ; van der Valk et al., 1977 and Trinci, 1978) propose that an inner layer of microfibrils is infused and coated with glucan and protein matrices. In older regions of the hyphae additional layers of melanin occur on the hyphal surface and a coarse reticulum develops within the wall layers (Hunsley and Kay, 1976).

β-Glucuronidase is a complex mixture of enzymes and will digest both glucan polymers and chitin (Meyers and Northcote, 1959). Consequently it is well suited for effecting protoplast release from a range of fungi. The sugar polymers digested during protoplast release from A. niger and A. fumigatus were not qualitatively investigated but

it would be advantageous to determine which are released into the medium and in what order. A comparison of the profiles of total reducing sugar release and N-acetyl sugar release (Figs 3.6 and 3.7) does indicate that the latter are released later in the digestion than the total reducing sugars. It is also noticeable that total reducing sugars are released within the first 20 min and show no significant increase in the medium after this time. Indeed a decrease is observed. This may perhaps be explained by a metabolic uptake by the protoplasts and hyphae, although no experiments were conducted to determine the fate of these sugars. The use of other techniques such as gas-liquid chromatography and radioactive labelling of wall components could be of great value in more fully elucidating the digestive sequence.

The failure of chitinase, when used alone, to effect protoplast release indicates that this polymer is probably located deep within the wall, an observation which supports the work of Carbonell et al , (1970), Domanski and Miller (1968) and Hunsley and Burnett, (1970). Observations of metal shadowed walls, before and after digestion for protoplast release, indicated that microfibrils were present in the wall (Fig 3.24) and, more significantly, appeared to be absent from areas of wall rupture (Fig.3.25) although more investigation on this is needed.

Microscopical observations have indicated that total wall digestion is not necessary for protoplast release from A. niger and A. fumigatus and close observation with scanning electron microscopy shows that, at least at distal sites of release, protoplasts are actively forced through the wall. This is further evidence that one particular component or type of bonding may be primarily responsible

for restraining the cytoplasm. The identification of microfibrils in many species as chitin (Carbonell et al., 1970; Hunsley and Burnett, 1970 and van der Valk et al., 1977) and the importance of chitin for protoplast release in the Aspergillus systems investigated here indicate that these microfibrils may be the component in question. Observations on shadowed preparations after differential enzyme digestion has provided further evidence for this identification (Mallaburn, personnal communication). However, the possibility of other, as yet undetermined, components present in small amounts should not be discounted (Villanueva, 1966).

The role of microfibrils within fungal cell walls is still under debate (Trinci, 1978) but some observations suggest that although they may not determine hyphal shape they may act to maintain this under normal conditions. For example, the dimorphic fungus Paracoccidiodes brasilensis has been cited as having different arrangements of chitin in its yeast and mycelial forms (Kanetsuna et al., 1969). Similarly the requirement of an osmotic stabilizer for the normal growth of a low chitin mutant of A. nidulans (Katz and Rosenberger, 1970) suggests a similar role for this polymer. The regeneration of protoplast walls has also been associated with a synthesis of chitin in Schizophyllum commune (de Vries and Wessels, 1975) while the absence of this polymer has been observed in aberrant hyphae produced by regenerating protoplasts of Trichoderma viride (Benitez et al., 1975). The collapse of A. niger and A. fumigatus hyphae, as seen in the SEM, after digestion with chitinase in the absence of β-glucuronidase further supports the importance of chitin.

It has, nevertheless, been suggested that the hyphal wall may not be the only contributing factor involved in maintaining the shape

of the hyphae and that turgor pressure may also play a role (Trinci, 1978). The internal osmotic potential of some hyphae has been shown to provide considerable pressure (Jennings et al., 1974) and Lopez-Belmonte et al, (1966) have reported that release of protoplasts is enhanced if the hyphae are flooded with water and attribute this to an osmotic effect. During wall digestion, protoplast release may be partially determined by this pressure from within the hyphae and also by the digestion of a certain wall layer(s) or polymer bondings which resist this pressure. The dependence of protoplast release on the concentration of osmoticum employed (Fig. 3.10) may thus be partially due to an effect on turgor pressure in the cytoplasm as well as affecting the stability of the protoplasts released. The possibility of chitin microfibrils being the restraining agent in A. niger and A. fumigatus cell walls is supported by the results obtained from both biochemical and morphological investigations.

A further general conclusion from these results is that of the importance of optimizing lytic enzyme activity to obtain high yields of protoplasts. Factors affecting this include the obvious parameters of pH and temperature as well as co-factor involvement such as associated ions and even the structural arrangement of the polymer substrates themselves.

The close correlation between the pH optima for protoplast release and for chitinase activity has been mentioned and indicates that chitin digestion is essential for release. Low pH has, however, been implicated in altering the viscosity of some wall polymers (Dow and Rubery, 1975) and this may provide an alternative explanation. Temperature could affect release through either an effect on enzyme activity or on the stability of the released protoplasts. In

distinguishing between these the use of a thermotolerant species, A. fumigatus, is particularly valuable. The use of a mesophyle alone is open to the criticism that any observed lack of release or absence of intact protoplasts at high temperatures could be due to an inherent instability of the protoplasts. Thermotolerant species should, however, possess the ability to endure these conditions. A comparison of protoplast release from A. fumigatus grown at 30°C and 48°C, however, indicated that for both temperatures the optimal range was similar, about 30 to 35°C, and provided evidence that protoplast release was correlated with enzyme activity rather than protoplast stability.

Although observations with the light microscope indicated that hyphal cytoplasm still remained after 80 min of digestion, time course profiles of protoplast release show that a saturation point occurred at approximately 80 min; few additional protoplasts were released after this time (Fig. 3.3). The correlation of maximum protoplast release with maximum N-acetyl sugar release indicates that chitinase is being inactivated during the digestion or that all the chitin has been digested. Reincubation experiments, to investigate these possibilities did show that, compared to fresh enzyme, the incubated sample was less active (Fig. 3.8). Further experiments to determine whether more protoplasts could be released from incubated hyphae by resuspending in fresh enzyme would also be valuable in this respect.

The investigation of this inhibition phenomenon in general would be worthwhile since its prevention could ensure a faster and more controlled release and perhaps selection of protoplast types. Work with other systems has indicated that the digestion products of chitin may cause inhibition of chitinase (Tracey, 1955) and that

melanin can particularly inhibit hydrolytic enzymes (Bull, 1970b).

A range of osmotica were found to support protoplast release from A. niger and A. fumigatus (Table 3.3) and it is interesting that as reported by Musilkova and Fencl, (1966), the addition of $CaCl_2$ increased protoplast yields compared to using KCl alone. This effect was more fully investigated and was found to depend upon the strain of organism and the lytic system employed. No enhancement was found with a white mutant strain of A. fumigatus or with the wild type A. fumigatus incubated with β-glucuronidase in the absence of chitinase (Table 3.4). The enhancement effect could be the result of $CaCl_2$ acting as a supplementary osmoticum. However, two observations indicate that there may be a further point of action. Firstly 0.6 M $CaCl_2$ and 0.6 M $Ca(NO_3)_2$ used alone failed to support the release of protoplasts despite not having an excessive osmotic potential (Table 3.5). Secondly, although the addition of 0.2 M $CaCl_2$ to KCl was effective in enhancing release at low molarities of KCl, the addition of greater concentrations of $CaCl_2$ decreased release (Table 3.5). Further, the dependence upon fungal strain and enzyme system indicates an effect involving these factors.

Calcium was shown to be the active factor in the enhancement effect through experiments conducted with EDTA (Table 3.6) and because the enhancement was evident with $Ca(NO_3)_2$ as well as with $CaCl_2$.

To explain the effect three possibilities were investigated, stabilization of protoplasts, disruption of normal growth mechanisms and enhancement of lytic action. The addition of $CaCl_2$ did not affect the pH of the medium, thus eliminating this simple but easily overlooked possibility.

Calcium salts are well known for providing stability in protoplast

systems (Diamond and Rose, 1970 and Ruesink, 1971) and also for inducing fusion between protoplasts (Keller and Melchers, 1973 and Ferenczy et al., 1976). This has been explained through an effect on neutralisation of acidic groups on the plasma membrane, thus allowing aggregation to occur (Grout and Coutts, 1974). In the Aspergillus species, however, the action of $CaCl_2$ did not seem to be due to membrane stabilisation since few unstable protoplasts were observed during incubation in medium lacking calcium and little decrease in numbers was observed during time course studies (results not shown). Although 0.6 M $CaCl_2$ and 0.6 M $Ca(NO_3)_2$ used alone failed to support protoplast release they will act as suitable osmotica for their maintenance after release in other osmotica. In stability studies more protoplasts remained intact in media containing calcium than in media without.

Another possibility for the effects of calcium is that the Ca^{2+} ion disrupts the normal sequence of wall growth leading to the weakening of certain polymers. Current theories of wall growth envisage that synthesis and lysis of the wall polymers are in a critical balance to allow insertion of new wall material and extension of the hyphae (Bartnicki-Garcia, 1973). The effect of low concentrations of divalent ions, including Ca^{2+}, on hyphal tips has been observed to result in swelling and occasional lysis and this has been explained as either a stimulation of lytic enzyme release (Bartnicki-Garcia and Lippman, 1972) or a disruption of polymer bonding (Dow and Rubery, 1975).

Filamentous fungi are commonly observed to contain many cytoplasmic vesicles, particularly at the hyphal tip (Grove and Bracker, 1970) and there is some evidence that these contain hydrolytic enzymes

(Meyer et al ., 1976). Similar hydrolases may be involved in spore wall digestion (Page and Stock, 1974). Further, Ca^{2+} has been noted to be instrumental in effecting release of contents from cytoplasmic vesicles in animal systems, possibly by stimulating their fusion with the plasma membrane (Naccache et al., 1977 and Chandler and Williams, 1978). Despite a wide interest in vesicle movement in fungi little information is available concerning their mechanisms and function although recent studies on the slime mould Physarium polycephalum have shown that Ca^{2+} fluxes freely across the plasma membrane (Holmes and Stewart, 1979).

If Ca^{2+} ions are involved in stimulating vesicle fusion with the plasma membrane then it would be expected that the addition of radioactive Ca^{2+} to the incubation medium would result in some accumulation of label within the cell. Labelling studies with $^{45}CaCl_2$, however, indicated that insignificant amounts of label were evident in the cytoplasm after 2 h of incubation with lytic enzymes. Moreover short term experiments with $^{45}CaCl_2$ failed to show even a transitory uptake of label indicating that vesicle release mechanisms in fungi may differ from those in other systems studied. One criticism of this is that the ion may not have been able to penetrate the cell wall. If this is so then Ca^{2+} may still be involved in vesicle release and further investigations of the process may be profitable, particularly under conditions of specific enzyme induction and release. The use of protoplasts could well be advantageous in this. Nevertheless, the absence of label in the cell after lytic digestion does indicate that the enhancement effect of Ca^{2+} is not due to mechanisms involving penetration of the ion into the cell.

The third possibility of the ion being a co-factor for the lytic

enzyme system was also investigated. the dependence of the calcium effects on the presence of chitinase (Table 3.4) suggests that this enzyme may require the Ca^{2+} ion for its full activity. As shown in Table 3.9, experiments with purified chitinase indicated that the release of N-acetyl sugars from chitin was optimal at a concentration of 0.05 M $CaCl_2$ and that high concentrations depressed activity. These results are in agreement with those obtained for protoplast release with varying concentrations of $CaCl_2$ and may explain why $CaCl_2$ and $Ca(NO_3)_2$ produced poor yields when used alone at high concentrations (Fig. 3.11). This may also help explain the fact that the enhancement effect was inconsistent in that the commercially available chitinase used contained variable amounts of calcium (Sigma Chemical Co, personal communication). A similar dependency on calcium concentration has also been observed with a purified chitinase isolated from Streptomyces (Skujins et al., 1970) and Gascon et al, (1965) have also noted that induced lytic enzymes are influenced by the presence of various ions.

It is possible that this influence may be the result of the electrokinetic potentials which exist between the enzyme and its substrate polymers in the wall. Such bonding has been shown to be important for the action of surfactants on yeast protoplasts (Tukmachev et al., 1978) and for melanin inhibition of hydrolase enzymes acting on A. nidulans wall polymers (Bull, 1970b).

The lack of Ca^{2+} stimulation on the white mutant strain of A. fumigatus might be explained on the basis of possible differences in cell wall composition. Similarly the noted differences in susceptibility to lysis as a result of hyphal age (de Vries and Wessels, 1972 and Peberdy et al., 1976) culture conditions (Musilkova and Fencl, 1968 and Schwencke et al., 1977) and growth temperature (Diamond and

Rose, 1970) could also be a result of different wall structure.

Another factor to consider in this respect is that of the positional release of protoplasts. The observation that protoplasts are preferentially released from hyphal tips during the initial stages of digestion (Gibson and Peberdy, 1972) indicates that the wall structure may differ between the tip and more distal parts of the hyphae. Ultrastructure studies have certainly shown that wall thickness and complexity increases with distance from the hyphal tip (Trinci and Collinge, 1975) and it is particularly interesting to note that in the wall of Neurospora crassa the size and density of chitin microfibrils increases similarly (Burnett, 1979). If wall structure is similar in Aspergillus species then the results in Tables 3.11 and 3.12 could be explained on this basis. The results of Ca^{2+} effects on protoplast release from the Aspergillus species studied show that chitin digestion is implicated as being one of the major requisites for ensuring release of protoplasts from the hyphae.

The second general aspect of protoplast release outlined by the results obtained is that of protoplast quality, particularly their type and condition. In connection with the previous aspect of quantitative release it is obvious that release under conditions which are detrimental to protoplast integrity, for example low osmoticum concentrations, will result in poor yields. Further factors which may also damage protoplasts are the presence of proteolytic and lipolytic enzymes in the lytic mixture and mechanical forces encountered during release.

The optimum concentration of KCl for release of A. niger and A. fumigatus protoplasts was found to be in the range of 0.4 to 0.6 M and agrees with similar values found in other systems (Bartnicki-Garcia

and Lippman, 1966 and Peberdy et al., 1976). Since low concentrations of osmotica may be expected to induce leakage of cytoplasmic contents, a concentration of 0.6 M was used as standard in most experiments.

The deleterious effect of exposure of fungi to lytic enzymes has been noted in other systems (Havelkova, 1966 and de Vries and Wessels, 1973b) and range from the damage of mitochondria (Schwencke et al, 1977) to a reduction in the endoplasmic reticulum and an alteration in general cytoplasmic structure (Manocha, 1968). For example, a decrease in the number of mitochondria and concurrent increase in autolysed structures has been observed by Havelkova, (1966) and membranes were consistently found to be swollen. However, such effects may be the result of poor fixative procedures and Partridge and Drewe (1974) found no difference in the number of mitochondria between hyphae and released protoplasts. Kobayashi et al, (1964) also found normal cell components in the protoplasts released from yeast and the commonly observed distribution of organelles within hyphae (Trinci, 1978 and Grove, 1979) is a possible cause of some protoplasts containing differing numbers of cell components. It has further been noted that many cytoplasmic structures are better defined in protoplasts than when observed within walled cells (Partridge and Drewe, 1974). A possible explanation for this result is that the fixative can penetrate more rapidly into protoplasts due to the absence of a wall. Similarly it has also been found that the presence of certain salts in the fixative medium can cause disruption of cytoplasmic contents (Thorsson and Weibull, 1958). The use of high concentrations of salts to preserve the stability of protoplasts may possibly explain such differences in protoplast morphology.

Other aspects which have been observed to differ between hyphae

and protoplasts include the uptake of amino acids (Fawcett et al., 1973) and sensitivity to antibiotics (Shockman and Lampen, 1962). However, these are functions which probably involve activities of the plasma membrane which, in protoplasts, will be in a totally different and non-comparable environment than when associated with the cell wall. Alternatively, a range of studies on fungal protoplasts has indicated that internal properties such as membrane composition (Boulton, 1965) and protein and RNA synthesis (Hutchinson and Hartwell, 1967) are retained by liberated protoplasts. The synthesis of specific enzymes (Andreas and Peberdy, 1974) and other compounds (Dutton and Anderson, 1978) have also been reported and numerous observations on the regeneration of protoplasts provide good evidence that basic cellular abilities are, if not damaged, recoverable after liberation.

Transmission electron microscopy of A. niger and A. fumigatus protoplasts (Figs. 3.18, 3.19 and 3.20), indicated that in these systems a full complement of organelles was evident within the protoplasts and no evidence of extensive autolysis was obtained. Small "mini-protoplasts" were, however, observed in the medium of some incubations and although the origin of these was not fully investigated some were observed to bud off from larger protoplasts (results not shown). Similar structures have been observed in other reports (Gibson and Peberdy, 1972 and Partridge and Drewe, 1974). In general the protoplast profiles observed are consistent with other work on filamentous protoplast ultrastructure (Manocha, 1968 and Gibson and Peberdy, 1972) and also illustrate the dichotomy between dense and vacuolated protoplasts noted in these reports. Histogram plots of protoplast diameters (Fig. 3.16) further emphasises this dichotomy and also show that there is no significant difference in size between the

populations of protoplasts released by β-glucuronidase with and without chitinase. Table 3.15 indicates that protoplasts were initially released more frequently from tip regions than from distal regions of the hyphae and, since the average protoplast diameter increased with time of incubation (Table 3.14), it is reasonable to conclude that the denser, smaller protoplasts originate from the tip regions. This is in agreement with other reports (Gibson and Peberdy, 1972) and probably relates to the observed distribution of cytoplasmic components within the hyphae (Trinci, 1978 and Grove, 1979).

The segregation of protoplasts with different cellular contents and abilities is an attractive possibility for the study of different cellular and hyphal functions. However, the purification and further separation of protoplasts is commonly hindered by the presence of contaminating hyphal fragments which often have similar sedimentation properties to protoplasts and also aggregate to each other and clog filters or disrupt density gradients. Consequently, considerable losses may be incurred during such procedures and previous cellular studies using protoplasts of A. fumigatus have been restricted through low yields (Archer, 1977). Preliminary experiments with density gradient centrifugation of A. fumigatus protoplasts indicated that this method was inapplicable to the digestion techniques used. Possibly a more concentrated enzyme mixture would leave fewer hyphal fragments, although at the risk of a more damaged protoplast. Acceptable purification of A. fumigatus protoplasts was, however, obtained by careful use of sinter filters. During incubations of protoplasts on cellulose media a control was nevertheless included to account for the activity of any residual fragments not retained by the filter.

The final aspect of the quantitative assessment of protoplast production is that involving the surface characteristics of the released protoplasts. Much discussion has been directed towards determining whether protoplasts are contaminated with wall residues adhering during release or as a result of incomplete digestion (Ottolenghi, 1966 and Darling et al., 1969). The absence of surface wall layers on protoplasts is shown by thin sectioning studies (Kobayashi et al., 1964; Manocha, 1968 and Gibson and Peberdy, 1972) but it is evident that this technique carries the criticism that not only does a single section show only a small part of the cell surface but that many fixatives are notably poor for carbohydrate polymers. Alternatively some reports do show the presence of a wall layer around sphaeroplasts from yeast cells (Darling et al., 1969) and identify this with a glucan (Darling et al., 1972).

A more extensive appearance of the cell surface is provided by the technique of freeze etching but while this has been successfully applied to some protoplasts a correct interpretation has been difficult due to confusion over the exposed fracture faces (Streiblova, 1968; Necas and Karasek, 1971 and Ramos and Garcia-Acha, 1975).

A further technique which produces a more direct and realistic image of surface features is afforded by scanning electron microscopy (SEM) which, because of an enhanced depth of focus, is able to provide a three dimentional appearance of the object. This technique has been applied to the formation of bacterial protoplasts and L-forms (Bibel and Lawson, 1972 and Kats and Glazacheva, 1977) and to yeast protoplasts (Miegeville et al., 1979) but, as yet, not to the digestion of filamentous fungi. It has, however, in conjunction with antibody labelling procedures, provided evidence of mannose residues

on the surface of yeast protoplasts (Horisberger et al., 1976).

SEM of A. niger and A. fumigatus hyphal digestions illustrated a number of factors. Firstly, as previously discussed, protoplasts were commonly observed to be released by active rupture of the hyphal wall, at least in distal regions. Few details of wall structure were obtained, however, and it was impossible to identify specific layers except for a loose surface coat (Figs. 3.28 and 3.29). Fractured walls were, however, characteristically amorphous (Fig. 3.29). The general mode of wall digestion as observed by SEM would appear to be an initial roughening followed by pitting of various dimensions until protoplast release occurs by wall rupture. Considerable fragmentation of the hyphae was evident in later stages of digestion (Fig. 3.29) and tip released protoplasts did not appear to emerge with such disruption.

Close observation of the protoplasts released indicated that few wall residues were associated with the surface despite many wall fragments being evident. Most protoplasts appeared intact although dilution with water before fixation did cause severe collapse (Fig. 3.37). Two distinct surface morphologies were evident on the protoplasts observed, smooth surfaced and crenated (Figs. 3.35 and 3.29). Although no evidence was obtained as to whether these types were released preferentially from particular hyphal locations some photographs do show that both types may arise from distal regions (Figs. 3.33 and 3.34).

The commonly observed crenated appearance of protoplasts is consistent with a number of published micrographs of protoplast thin sections (Sagara, 1969 and Gibson and Peberdy, 1972) and is unlikely to be the result of fixation procedures since smooth protoplasts were also observed in the same samples. Surface specialisations have been

noted on a number of cells and have been correlated with various functions including uptake of nutrients (Willoch, 1967), receptor site localization (Burger, 1971 and Porter et al., 1973) and growth control (Burger, 1971). Alternatively, and more probably in the case of recently released protoplasts, they may simply represent impressions of underlying organelles.

In conclusion, the results obtained for protoplast release from A. niger and A. fumigatus indicate that for optimal, quantitative yield not all the hyphal wall need be digested. Further investigation of the digestion of those wall layers found to be important in restraining release may lead to even higher yields and may prove important as protoplasts come to be increasingly used for detailed cellular studies such as demonstrated with the localization of chitin synthetase (Braun and Calderone, 1978 and Vermeulan et al., 1979).

A high quantitative yield of protoplasts is particularly essential if they are to be further fractionated to obtain cell and organelle fractions. The parameters investigated here relate directly to this consideration. Further, the microscopic observation of protoplasts released under conditions of optimal quantitative production indicated that neither surface nor cytological damage had occurred and that procedures to enhance quantitative yield may be pursued without morphological detriment.

4. CMCase PRODUCTION FROM A. FUMIGATUS

4.1. ENZYME CHARACTERISTICS.

A number of characterisation experiments were conducted, first with purified, commercial A. niger cellulase and then with culture filtrates from A. fumigatus in order to ensure that the cellulase assay chosen was representative for the variables encountered and that optimal conditions were used.

The pH profile of purified A. niger cellulase, incubated at a concentration of 100 µg per 0.1 ml with 1.0% (w/v) CMC substrate in acetate buffer is shown in Fig. 4.1. Although active at more extreme pH values the optimum is well defined at pH 4.0 to 4.5. This agrees well with the published values of Hurst et al. (1977). Since widely differing levels of cellulase activity may be encountered in cell fractions it is important that the assay used should be sensitive enough to detect the lowest levels present and yet not saturate at the higher levels. In order to determine the range of the assay, incubations of A. niger cellulase were performed with differing concentrations of enzyme incubated for differing periods of time. Fig. 4.2 shows that enzyme concentrations of 10 and 40 µg produced considerable levels of reducing sugar and that this increased linearly with time. With 40 µg of enzyme the amounts of reducing sugar detected levelled off after 30 min but this was due to a saturation of the developing reagent rather than an enzyme inhibition. Enzyme concentrations of 0.5 and 1.0 µg also produced a linear increase with time although in absolute terms these were very low indicating that adequate determinations would require a concentration greater than 1.0 µg of reducing sugar or 0.0004 units.

Concentration experiments indicated that the assay gave

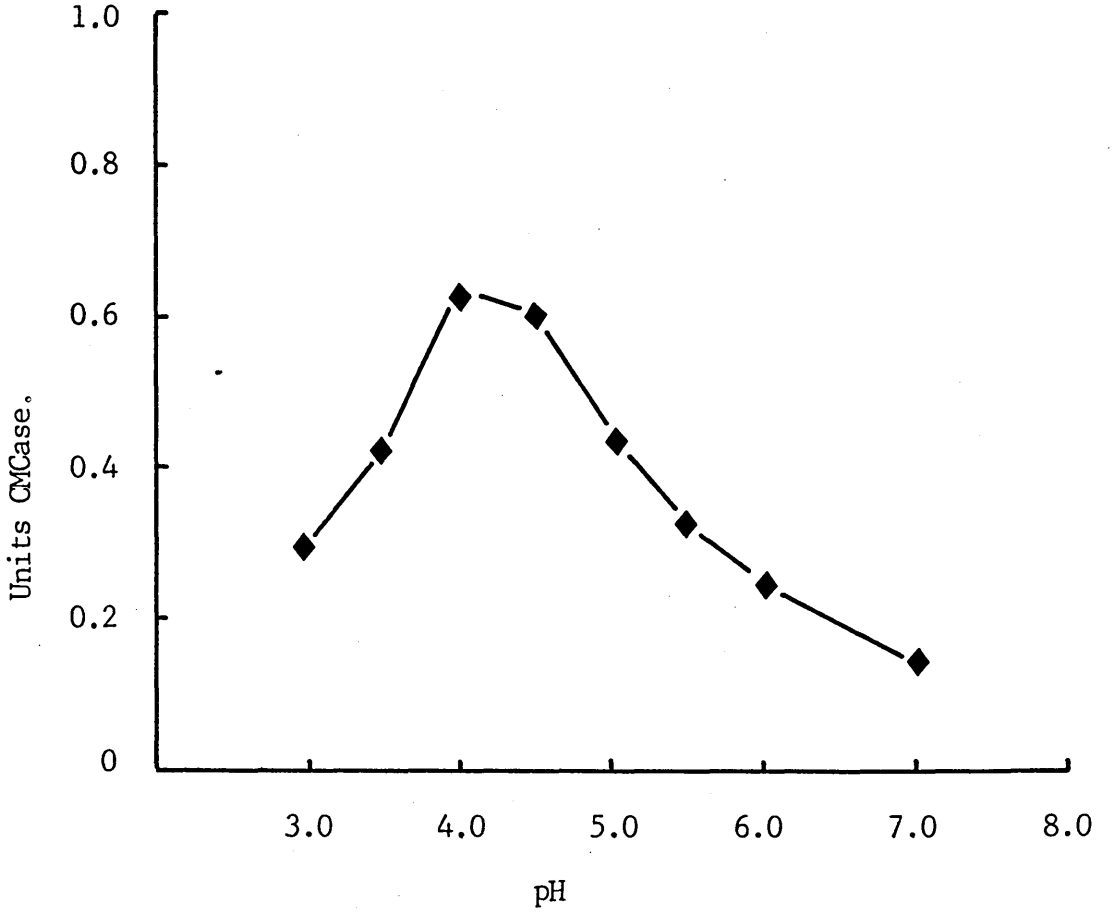


FIGURE 4.1. Effect of pH on activity of CMCase from A. niger. Commercial cellulase, 100 µg in 0.1 ml, 0.1 M sodium acetate buffer, was incubated for 30 min with 5.0 mg of CMC in 0.5 ml of the same buffer at 50°C. reducing sugars were determined using the PAHBAH reagent at 0 and 30 min.

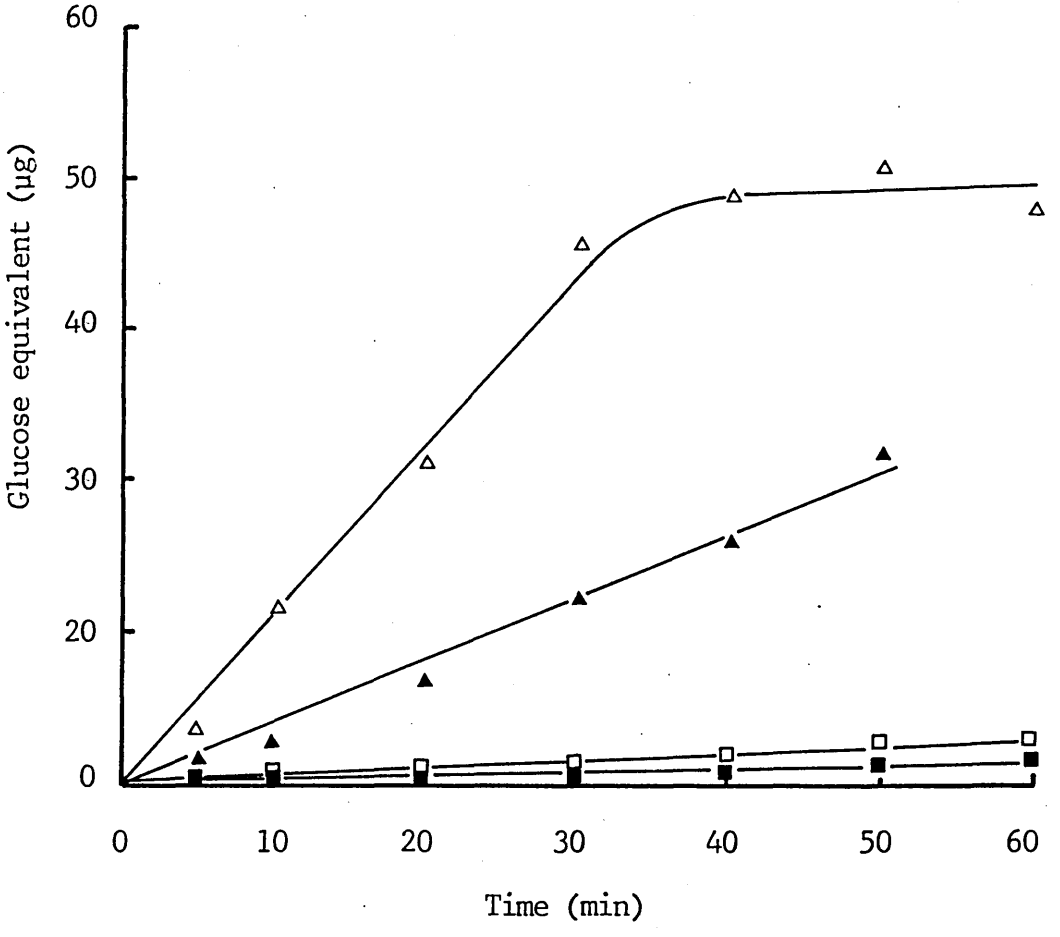


FIGURE 4.2. Effect of time of incubation on reducing sugar production from CMC by CMCase from A. niger. Commercial cellulase, 0.5 to 40 ug, in 0.1 ml, 0.1 M sodium acetate buffer, ph 5.0, at 50°C. Reducing sugars were determined using the PAHBAH reagent. Cellulase concentrations were; (■) 0.5 µg, (□) 1.0 µg, (▲) 10.µg, (△) 40 µg.

developments proportional to enzyme concentrations up to at least 25 µg (Fig. 4.3). From Fig.4.2 it is also apparent that this holds for concentrations over a 10 min incubation.

A similar range of experiments was conducted with culture filtrates of A. fumigatus grown for 7 days on BMC and CMC media. The pH profile of enzyme filtrates from these cultures is plotted in Fig. 4.4 and shows that the CMC filtrate has a slightly lower optimum at pH 4.5 than the BMC filtrate with an optimum at pH 5.0. Time course profiles of the two filtrate activities indicated that these increased linearly with the time of incubation (Fig. 4.5). BMC filtrates consistently showed greater activity per mg of protein than the CMC filtrates although the amount of sugars produced soon leveled off at a maximum due to the upper limits of the assay.

The temperature profile of CMCase activity of a BMC culture filtrate is shown in Fig. 4.6. A broad optimum of activity is evident ranging from 50°C to 70°C.

4.2. GROWTH CHARACTERISTICS OF A.FUMIGATUS ON CELLULOSE MEDIA.

Some initial experiments were conducted using Rautela Cowling tubes to compare the cellulolytic abilities of the A. fumigatus strains with T. viride and A. niger. Boiling tubes were filled with 20 ml of BMC medium and inoculated with equal amounts of mycelia. Readings of the depths of clearing of the media were made after 7 or 10 days incubation at 30 or 50°C respectively. These results indicated that the A. fumigatus strains possessed considerable cellulolytic activity compared to the other species (Table 4.1).

Further initial investigations of the wild type A. fumigatus

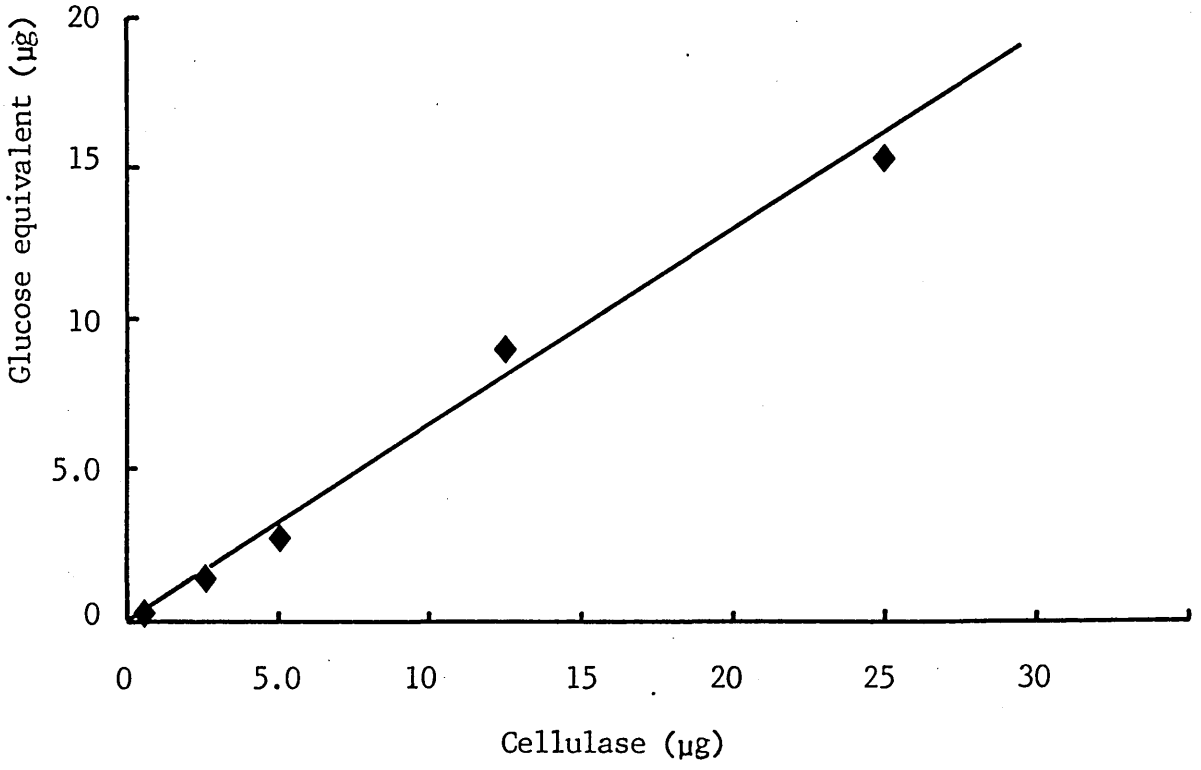


FIGURE 4.3. Effect of enzyme concentration on activity of CMCase from A. niger. Commercial cellulase, 1.0 to 50 µg, in 0.1 ml, 0.1 M sodium acetate buffer, pH 5.0, was incubated for 10 min with 5.0 mg CMC in 0.5 ml of the same buffer at 50°C. Reducing sugars were determined using the PAHBAH reagent.

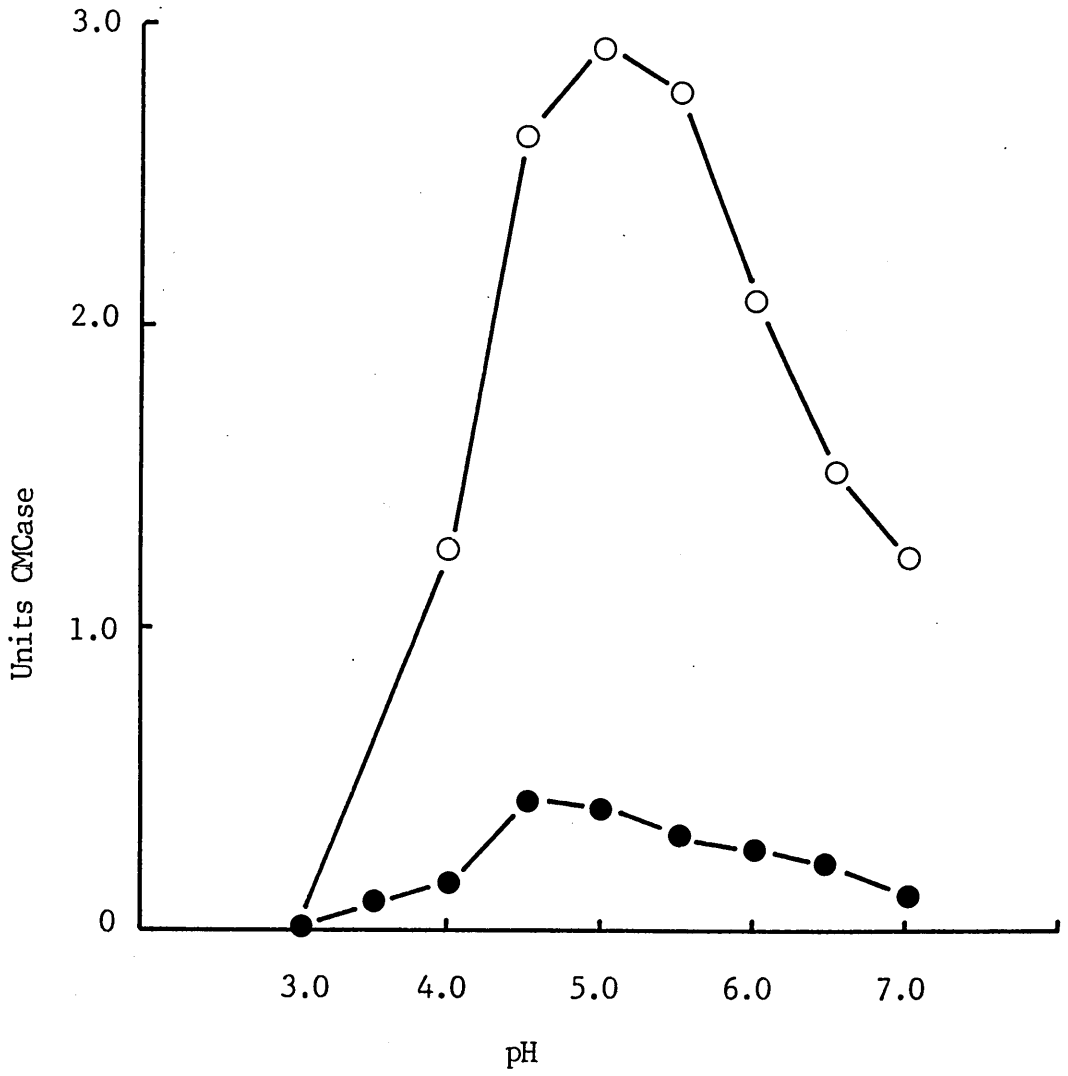


FIGURE 4.4. Effect of pH on activity of CMCase from A. fumigatus culture filtrates. Culture filtrates, 0.1 ml, were incubated for 30 min with 5.0 mg CMC in 0.5 ml, 0.1 M sodium acetate buffer at 50°C. Reducing sugars were determined using the PAHBAH reagent.

(O) BMC medium, (●) CMC medium.

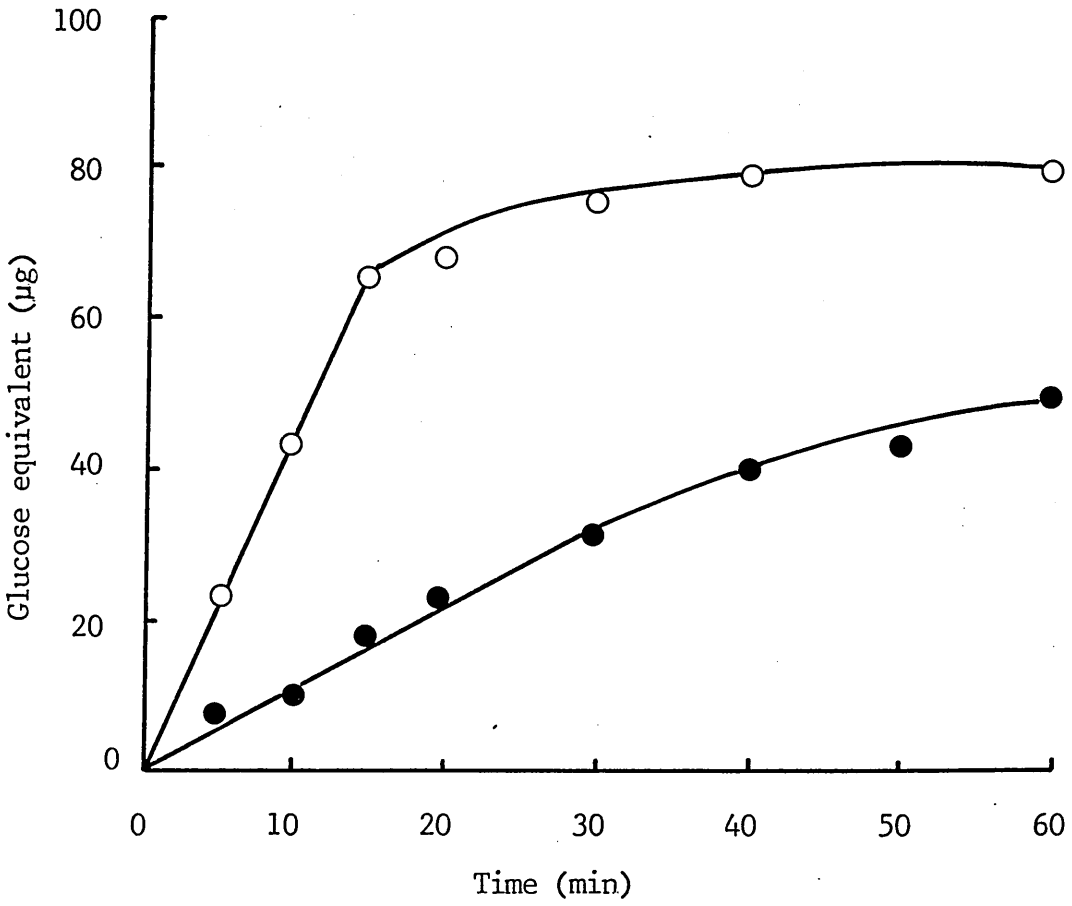


FIGURE 4.5. Effect of time of incubation on reducing sugar production by CMCase from A. fumigatus culture filtrates. Culture filtrates, 0.1 ml, were incubated with 5.0 mg CMC in 0.5 ml, 0.1 M sodium acetate buffer, pH 5.0. Reducing sugars were determined using the PAHBAH reagent. (○) BMC medium, (●) CMC medium.

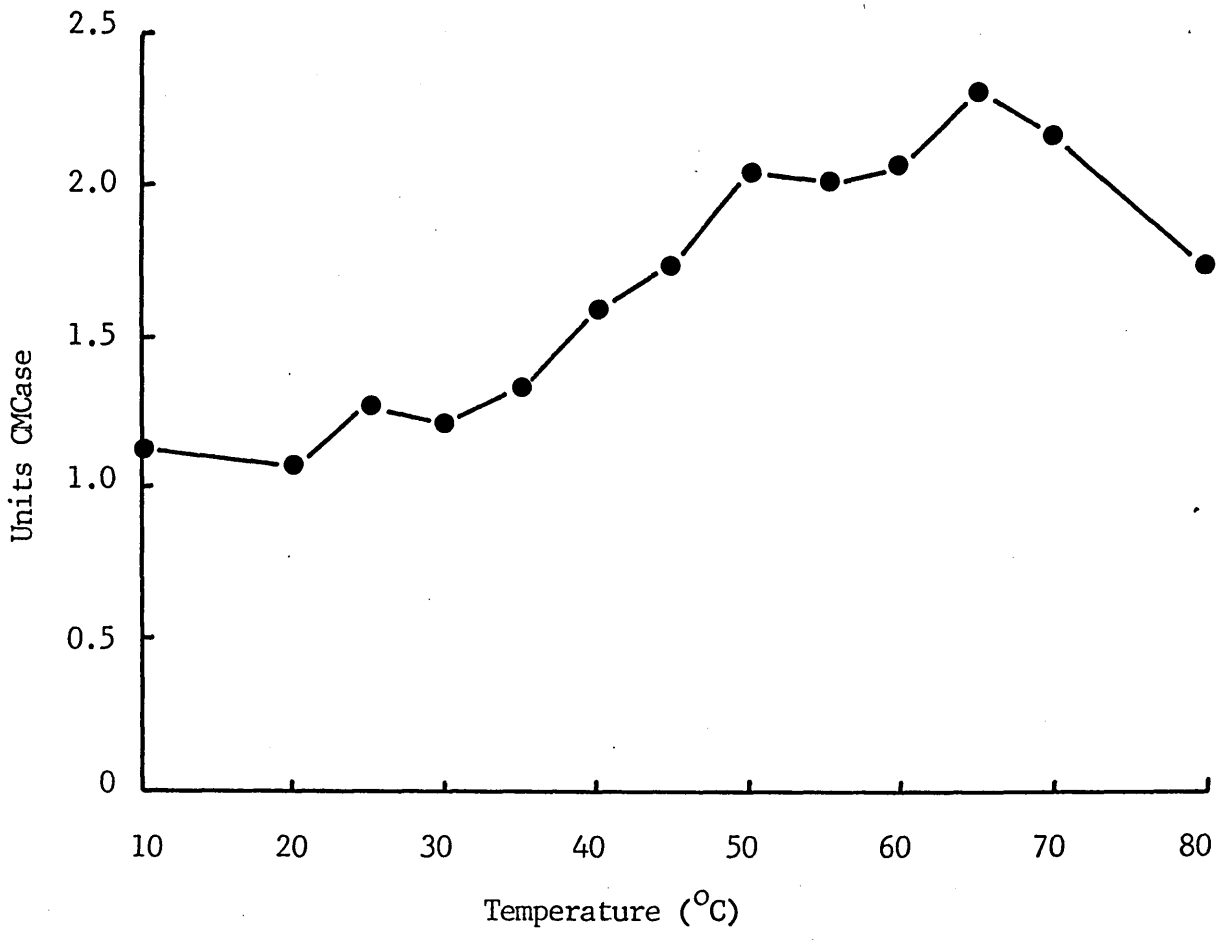


FIGURE 4.6. Effect of temperature of incubation on activity of CMCase from culture filtrates of A. fumigatus. Culture filtrates, 0.1 ml, were incubated for 5.0 min with 5.0 mg CMC in 0.5 ml, 0.1 M sodium acetate buffer, pH 5.0. Reducing sugars were determined using the PAHBAH reagent.

TABLE 4.1.

Organism	Depth of clearing (mm)
30°C	
A. niger	0.0
T. viride	3.8 ± 0.29
A. fumigatus w⁻	5.8 ± 0.29
A. fumigatus +	7.7 ± 0.58
48°C	
A. niger	0.0
T. viride	0.0
A. fumigatus w⁻	11.0 ± 0.55
A. fumigatus +	13.5 ± 0.58

Depth of clearing of BMC medium by test organisms in Rautela Cowling tubes at 7 and 10 days growth. 30 and 48°C respectively.

w⁻ A. fumigatus white mutant strain IMI No 143864.

\+ A. fumigatus wild type strain IMI No 226070.

strain was conducted using Eggins and Pugh medium with impure cellulose residues such as paper and cardboard. Although undigested residues were evident after growth on these a considerable production of CMCase was observed (Table 4.2). Growth on purified cellulose, however, resulted in complete digestion of the substrate and further experiments were conducted with BMC or CMC media to reduce complications due to comtaminates in the substrates.

The basic medium for growth on cellulose substrates was that of Eggins and Pugh (Table 2.1) with BMC, CMC or simple sugars as the carbon source. CMC medium encouraged faster growth initially but BMC plates were more extensively colonised when growth was assessed on solid media. In liquid medium growth was again initially more rapid with CMC as the cellulose form. After 2.0 days the mycelium assumed a compact pellet form (Fig. 4.7), and often grew conidiophores indicating that at least some of the mycelium was not receiving nutrients (Fig. 4.8). Growth in liquid BMC medium was initially slow, taking 5.0 to 7.0 days at 48^{o}C to utilise all the substrate. However, the growth pattern was more diverse than in liquid CMC medium with hyphae growing between and adhering to cellulose particles during their digestion (Fig. 4.9). At later stages of digestion small pieces of cellulose remained attached to the hyphal surface and many hyphae had a roughened surface with indications of a loosely fitting surface wall layer (Fig. 4.10). This appearance was also found on hyphae growing in CMC medium (Fig. 4.11) and it was notable that the surface at the extreme tip had a smoother appearance than the surface more distal to the tip.

The production of CMCase in culture filtrates and in crude mycelial homogenates of A. fumigatus grown on BMC medium is shown in Fig 4.12. After 3.0 to 4.0 days of growth there was a sharpe rise in cellular CMCase

TABLE 4.2.

Substrate	Units CMCase	Reducing sugar mg per ml
Tissue paper	0.97 ± 0.09	0.14 ± 0.04
Newspaper	1.30 ± 0.06	0.11 ± 0.01
Brown paper	1.35 ± 0.03	0.12 ± 0.01
Hand toweling	1.39 ± 0.05	0.14 ± 0.02
Cardboard	1.51 ± 0.02	0.16 ± 0.01

CMCase and reducing sugar production by A. fumigatus cluture filtrates. 7 days growth, 48° C.

FIGURE 4.7. A. fumigatus mycelium. 2.0 days growth in CMC medium. Samples were prepared for SEM as described in Materials and Methods. x 150.

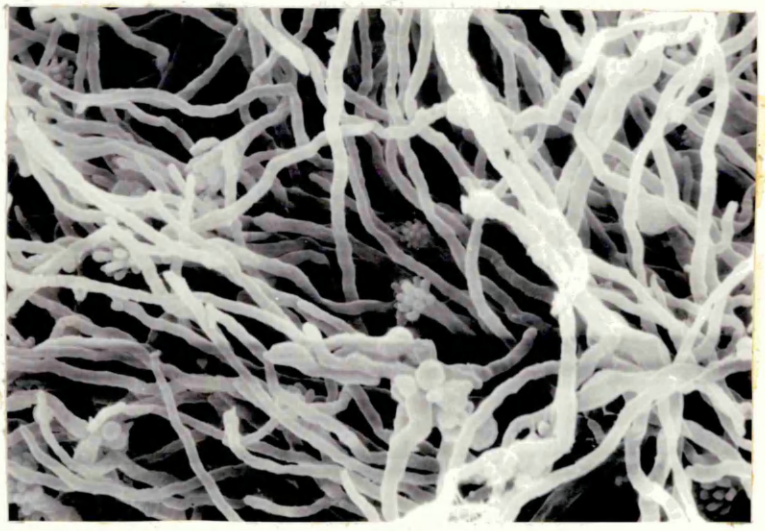


FIGURE 4.8. A. fumigatus mycelium. 2.0 days growth in CMC medium. Samples were prepared for SEM as described in Materials and Methods. x 3,700.

FIGURE 4.9. A. fumigatus mycelium. 4.0 days growth in BMC medium. Samples were prepared for SEM as described in Materials and Methods. x17,000. b = BMC particle, h = hyphae.

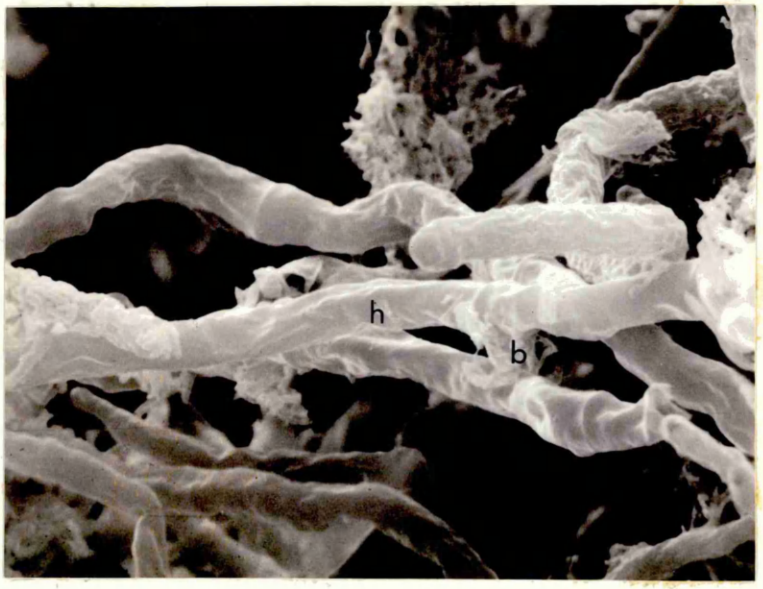


FIGURE 4.10. A. fumigatus mycelium. 4.0 days growth BMC medium. Samples were prepared for SEM as described in Materials and Methods. x 23,000. b = BMC particle, h = hyphae.

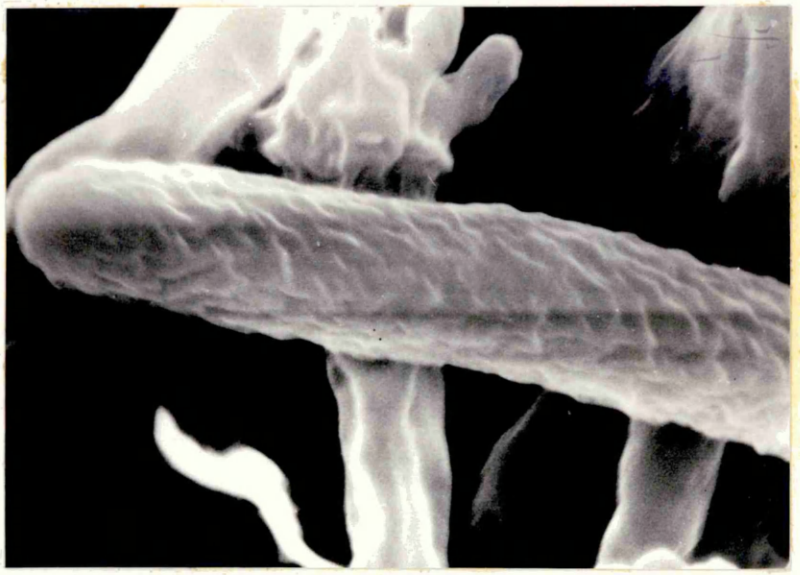


FIGURE 4.11. A. fumigatus mycelium. 2.0 days growth in CMC medium. Samples were prepared for SEM as described in Materials and Methods. x 37,000. Note smooth surface of the hyphal tip and the more roughened surface distal to this.

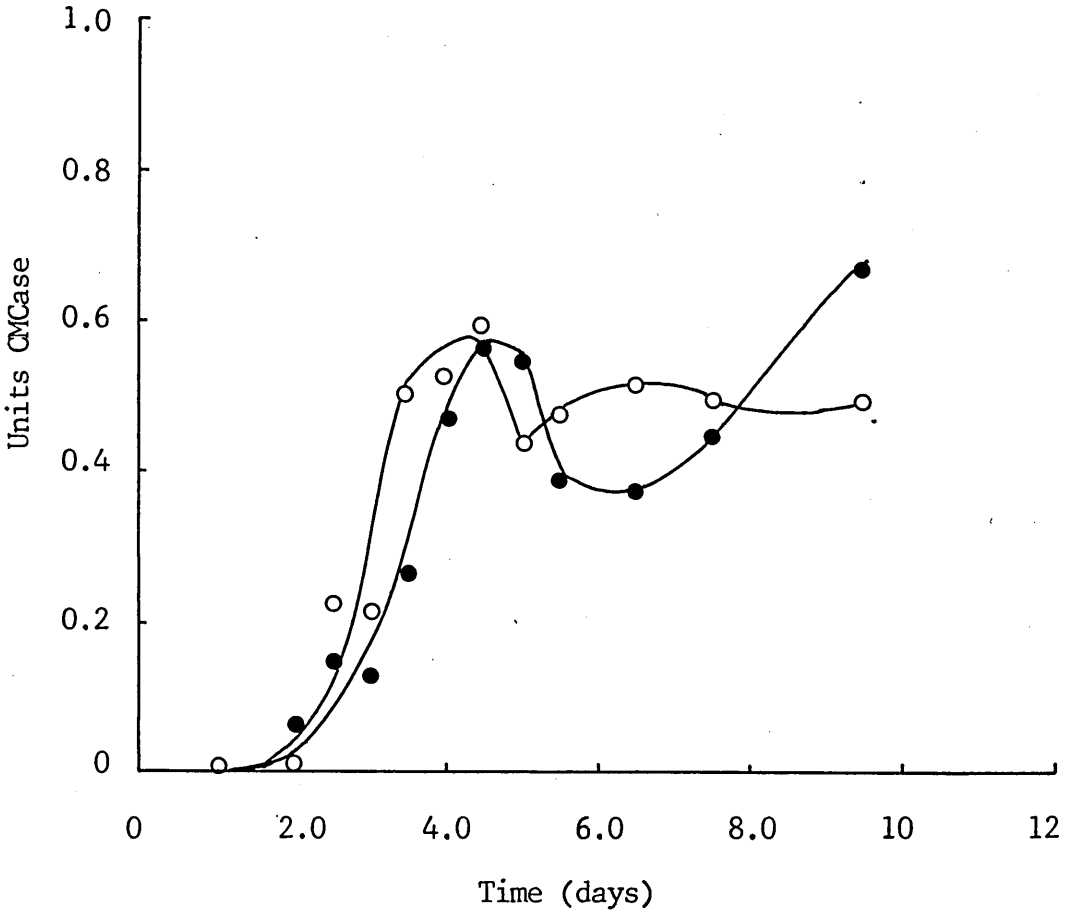


FIGURE 4.12. CMCase activities in culture filtrates (●) and mycelial homogenates (○) of A. fumigatus mycelium growing on BMC medium. Culture filtrates and mycelial homogenates, 0.1 ml, were incubated for 5.0 to 60 min with 5.0 mg CMC in 0.5 ml, 0.1 M sodium acetate buffer, pH 5.0 at 50°C. Reducing sugars were determined using the PAHBAH reagent.

levels which was followed by a slight decline and a levelling off up to 9 days of growth. By this time cultures were noticeably autolysed and contained coloured metabolites in the medium. Extracellular CMCase activity was similar but slightly retarded compared to that of cellular CMCase activity. Again there was an initial rise in activity followed by a slight decline, however, after 6 days, there was a further rise, this time to a level higher than that evident at 4 days (Fig. 4.12).

Since the presence, in BMC cultures, of a solid substrate complicates the use of dry weight determinations of mycelial growth the amount of mycelial and extracellular protein was also measured and the specific activity of the CMCase determined at each time interval (Figs. 4.13 and 4.14). These results indicate that mycelial protein levels followed that expected for the growth curve. The specific activity followed a very similar pattern indicating that the increase in CMCase activity is not necessarily due to an increase in the amount of enzyme produced.

It is also apparent from Figs. 4.12 and 4.13 that the initial lag in enzyme production is probably caused as the organism adapts itself to the substrate. This would result in an initial poor growth of the fungus. In order to reduce this lag and thus obtain maximum enzyme production as soon as possible a number of salts and sugars were added to the growth medium and the rate of growth or CMCase activity in solid or liquid culture determined.

Table 4.3 shows the effect of adding salts, amino acids and trace elements to solid BMC and CMC media. Growth and sporulation were assessed by eye and compared to that on control plates containing glucose as a substrate instead of cellulose. Compared to the glucose control it is evident that only asparagine encouraged appreciable

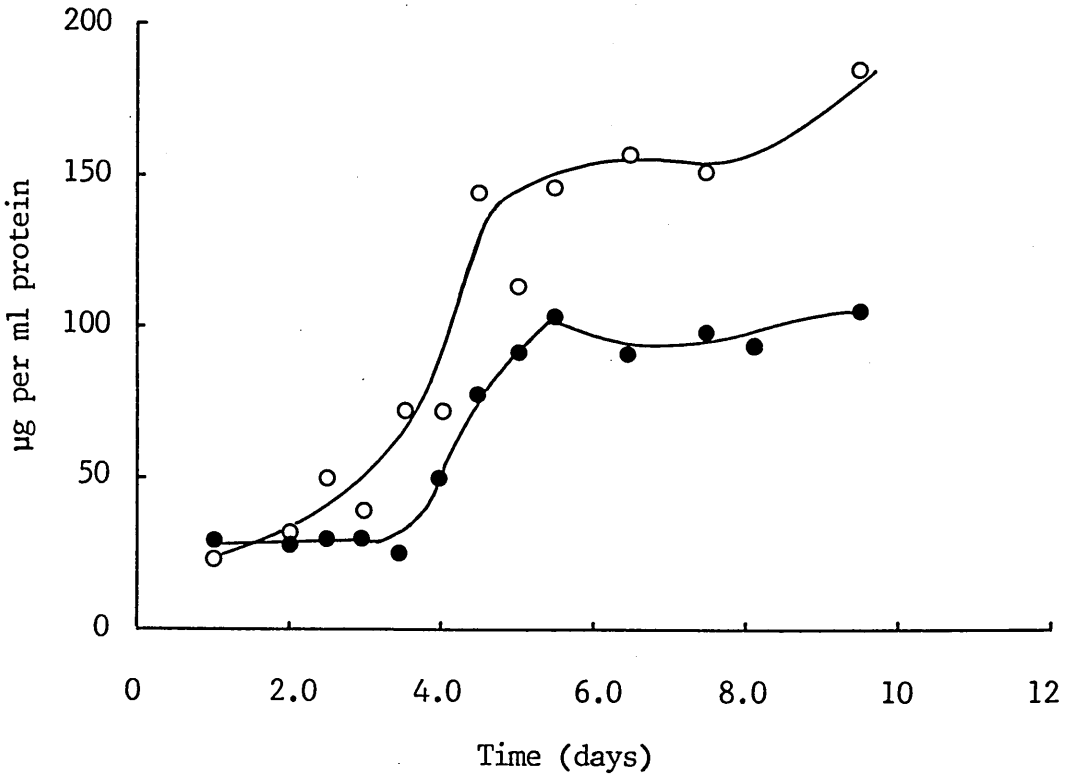


FIGURE. 4.13. Protein content of culture filtrates (●) and mycelial homogenates (○) of A. fumigatus mycelium growing on BMC medium. Culture filtrates and mycelial homogenates, 0.1 ml, were assayed for protein as described in Materials and Methods.

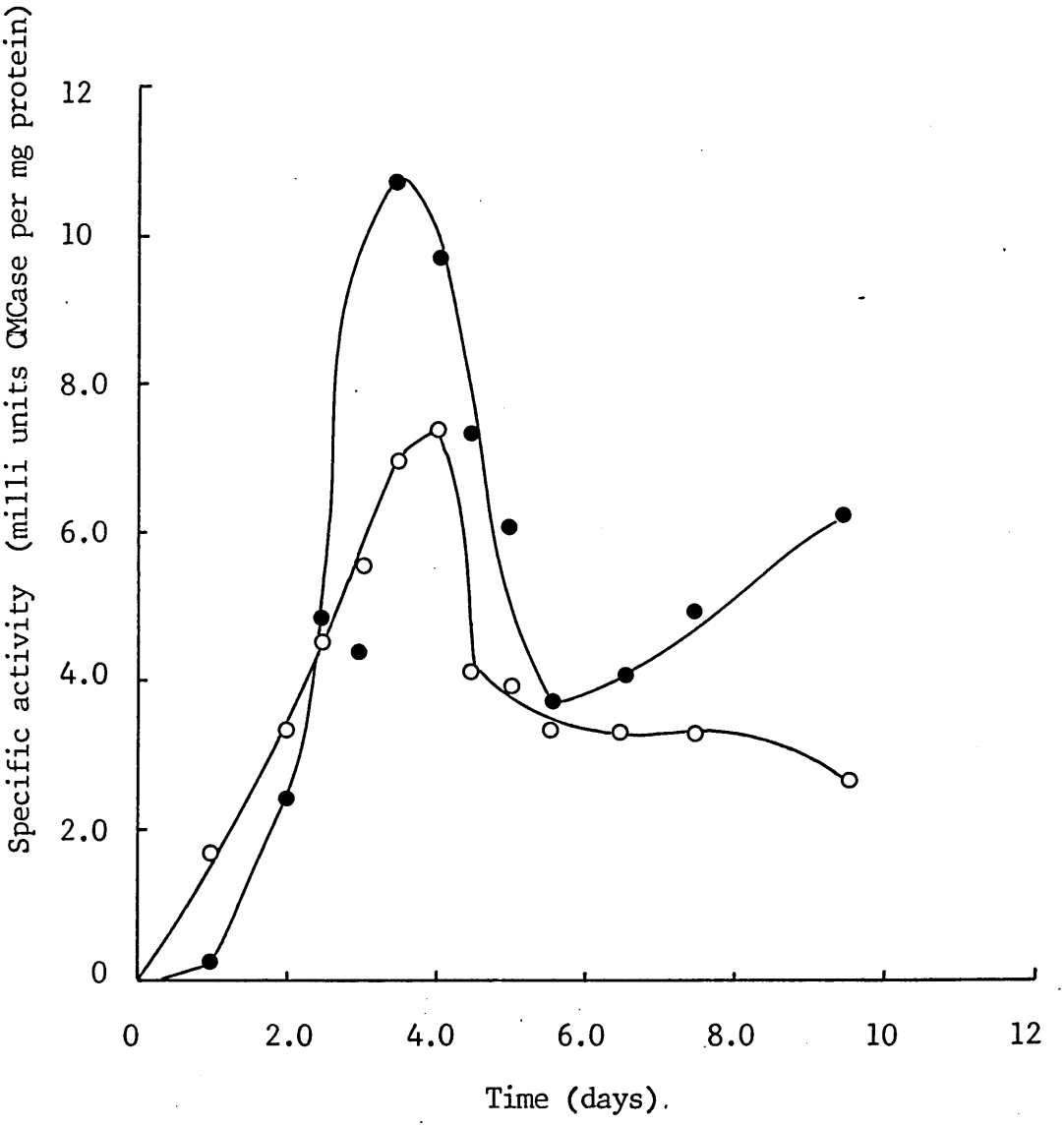


FIGURE 4.14. Specific activities of CMCase of culture filtrates (●) and mycelial homogenates (○) from A. fumigatus cultures growing on BMC medium.

TABLE 4.3.

Medium	Mycelial growth	Sporulation
Glucose	+++++	++++
CMC	++	+
CMC + 0.1% NaCl	+++	++
CMC + Trace elements *	+++	+++
CMC + 0.1% asparagine	++++	+++
BMC		+
BMC + 0.1% NaCl	+	++
BMC + Trace elements *	+	++

Effects of salt, trace elements and asparagine on growth and sporulation of A. fumigatus on solid medium. 24 h growth.

* As detailed in Table 2.2.

\+ Observable growth or sporulation.

+++++ Full plate covered.

growth after 24 h. The combined addition of the trace elements, iron, manganese, copper, zinc and calcium produced some growth with CMC as substrate but little growth with BMC. Sporulation was similarly affected. Dry weight determinations from liquid grown cultures, in CMC medium with the same additions, further indicated that these inclusions did not satisfactorily increase the growth compared to the glucose control (Table 4.4).

In order to investigate the possibility that the growth of the fungus on cellulose would benefit from priming with a small amount of a simpler substrate a number of sugars were added to the basic medium containing CMC and the amount of CMCase in the medium determined after 2 days growth. Table 4.5 shows that cultures with most sugars added at 0.2% (w/v) produced up to 30% more enzyme than an unprimed control. Surprisingly lactose, which possesses a β, 1-4 bond and which has been reported to induce cellulase in T. viride (Mandels, 1975), was the least effective additive. However, A. fumigatus cultures with 0.2% (w/v) glucose in the medium produced over 50% more enzyme (Table 4.5).

While a number of sugars have been reported to induce or repress cellulose synthesis (Mandels, 1975) it is to be expected that most would be readily utilised by the young hyphae and thus increase the initial rate of growth. Once established a greater mass of mycelium will be available to produce enzymes to degrade the more complex substrate.

Since glucose addition produced the greatest increase in enzyme activity this sugar was chosen for further investigation. Varying concentrations of glucose were added to 1.0% (w/v) CMC medium and the inoculated cultures incubated for 2 days at 48^{o}C. CMCase, reducing sugars and protein were determined in the filtrates and the dry weight

TABLE 4.4.

Medium	Dry weight (mg per ml)
Glucose	5.4 ± 0.49
CMC	0.5 ± 0.04
CMC + 0.1% NaCl	0.4 ± 0.08
CMC + Trace elements *	0.6 ± 0.11

Effect of salt, trace elements and asparagine on growth of A. fumigatus in liquid medium. 24 h growth.

* As detailed in Table 2.2.

TABLE 4.5.

Medium	Units CMCase
CMC	1.55 ± 0.06
CMC + 0.2% arabinose	2.27 ± 0.14
CMC + 0.2% fructose	1.68 ± 0.07
CMC + 0.2% glucose	3.17 ± 1.44
CMC + 0.2% lactose	1.44 ± 0.04
CMC + 0.2% maltose	2.35 ± 0.09
CMC + 0.2% mannose	2.20 ± 0.18
CMC + 0.2% starch	2.30 ± 0.07
CMC + 0.2% sucrose	2.27 ± 0.05

Effect of different sugars on CMCase production by A. fumigatus incubated in CMC medium. Units of activity in filtrates of 2.0 day cultures.

of the mycelium measured. Table 4.6 shows that all the factors investigated increased with increasing concentrations of glucose. CMCase activity, however, levelled off at 0.2% (w/v) glucose while dry weight and protein in the filtrate increased continuously up to 1.0% (w/v) glucose. Reducing sugar levels did not increase linearly with the concentration of glucose indicating that the sugar had been consumed by the organism by this time. This latter observation was confirmed by reducing sugar determinations made during the growth period. A comparison of the specific activities of the CMCase produced by the cultures after 2 days of growth indicated that this was greatest in the control cultures with only CMC as the substrate and decreased with increasing amounts of glucose addition (Table 4.6).

Using the supplemented medium the activity of the CMCase, reducing sugar levels and protein in the mycelium and culture filtrates of mycelium growing on BMC and CMC media were determined over 48 h of growth. Figs. 4.15 and 4.16 show that an initial peak of CMCase activity occurred in the mycelium of both BMC and CMC cultures. This peak later subsided and in general the results are similar to those obtained for the organism growing on unsupplemented medium. The peak of CMCase activity in the mycelium grown on supplemented medium, however, occurs at 18 h rather than 3 to 4 days as in the mycelium from unsupplemented medium (Fig.4.12). One noticeable difference between the BMC and CMC media is that the CMCase activity in the filtrates of the latter medium is very low until late stages of growth while BMC medium has a high activity soon after the initial peak in mycelial activity.

The concentration of supplementing sugar in the medium decreased rapidly after 6 h and reached a minimal level at 12 h (Fig.4.17). It is also evident that despite considerable hydrolysis of cellulose to

TABLE 4.6.

Medium	Dry wt mg per ml	Reducing sugar µg per ml	Units CMCase	Protein mg per ml	Specific activity U per mg
CMC	0.23 ± 0.03	22.5 ± 0.70	5.93 ± 0.21	1.95 ± 0.11	3.04
CMC + 0.1% glucose	0.55 ± 0.05	25.0 ± 0.70	6.80 ± 0.45	2.49 ± 0.06	2.73
CMC + 0.2% glucose	0.90 ± 0.09	24.0 ± 0.03	9.08 ± 0.46	3.33 ± 0.11	2.73
CMC + 0.5% glucose	1.28 ± 0.07	24.0 ± 0.05	8.23 ± 0.88	4.06 ± 0.05	2.03
CMC + 1.0% glucose	2.28 ± 0.07	23.0 ± 0.01	9.23 ± 0.77	4.73 ± 0.49	1.95

Effect of glucose concentration on growth and CMCase activity of A. fumigatus incubated in CMC medium. Cultures were filtered and the parameters determined after 2. 0 days of growth .

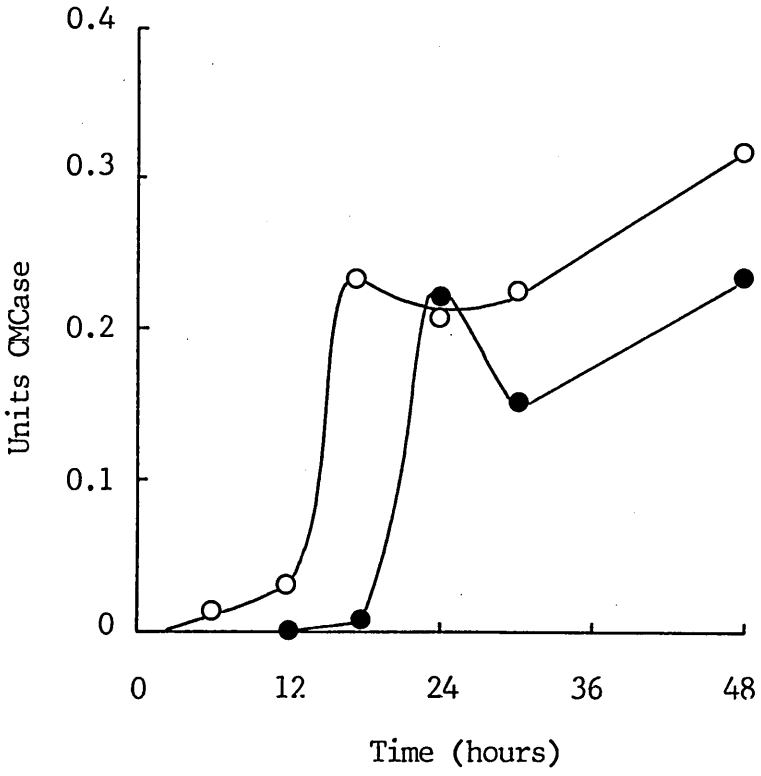


FIGURE 4.15. CMCase activities in culture filtrates (●) and mycelial homogenates (○) of A. fumigatus mycelium grown on BMC medium supplemented with 0.2% (w/v) glucose. Culture filtrates and mycelial homogenates, 0.1 ml, were incubated for 5.0 to 60 min with 5.0 mg CMC in 0.5 ml, 0.1 M sodium acetate buffer, pH 5.0 at 50°C. Reducing sugars were determined using the PAHBAH reagent.

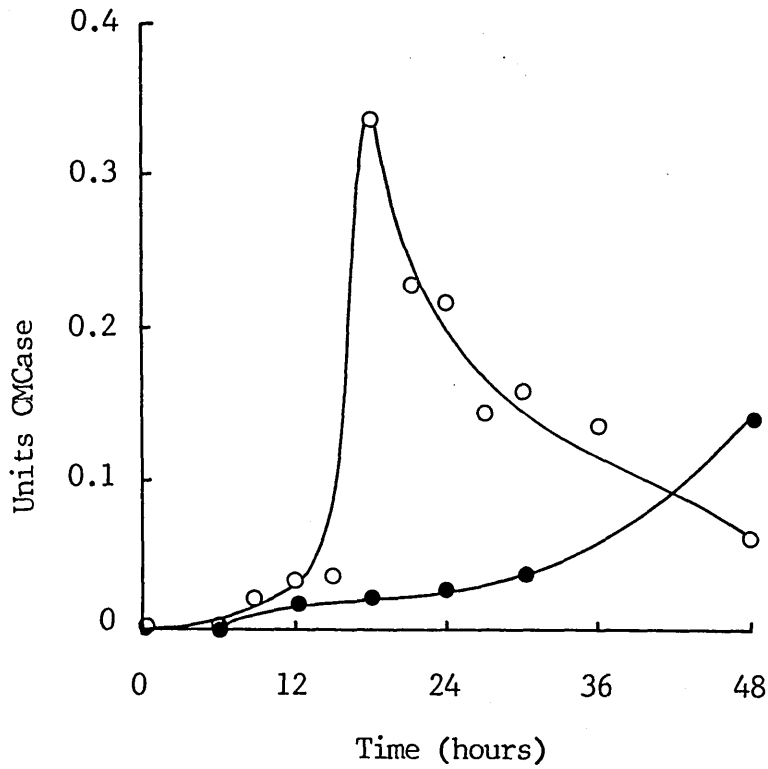


FIGURE 4.16. CMCase activities in culture filtrates (●) and mycelial homogenates (o) of A. fumigatus mycelium grown on CMC medium supplemented with 0.2% (w/v) glucose. Culture filtrates and mycelial homogenates, 0.1 ml, were incubated for 5.0 to 60 min with 5.0 mg CMC in 0.5 ml, 0.1 M sodium acetate buffer, pH 5.0 at 50°C. Reducing sugars were determined using the PAHBAH reagent.

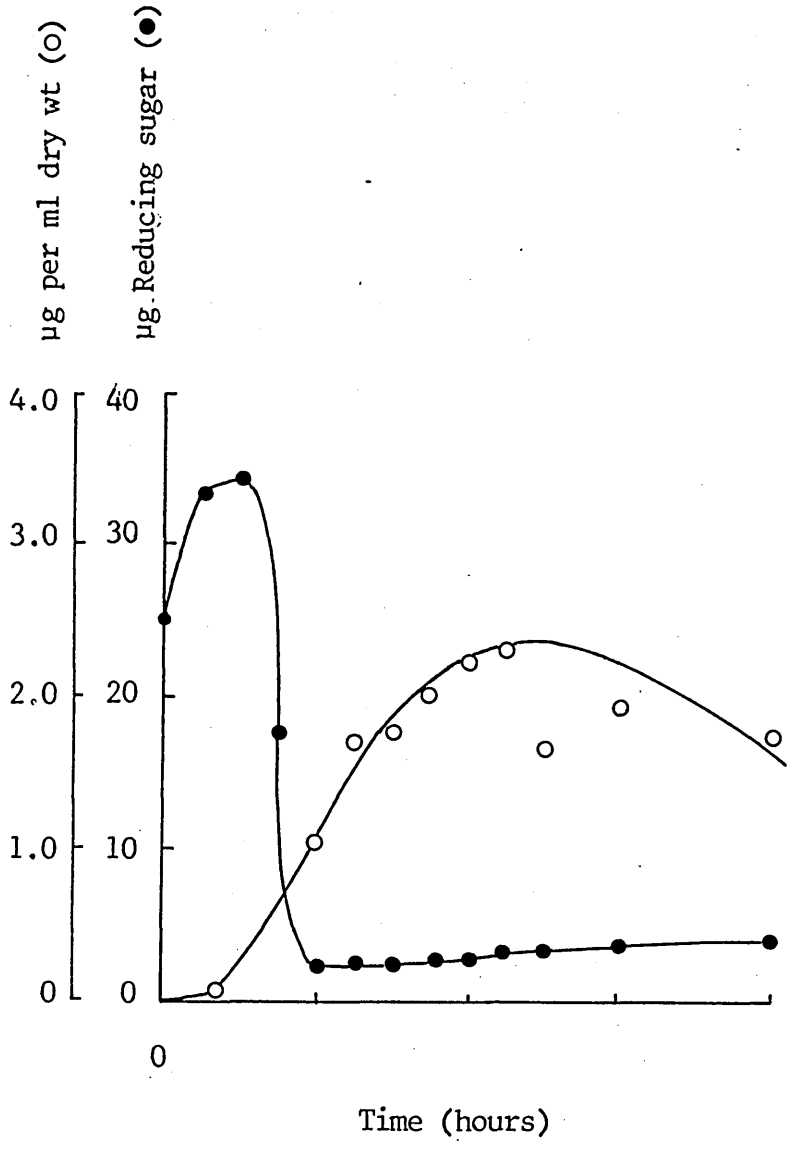


FIGURE 4.17. Reducing sugar (●) and mycelial dry weight (o) of culture filtrates and A. fumigatus mycelium grown on CMC medium supplemented with 0.2% (w/v) glucose. Culture filtrates were assessed for reducing sugar with the PAHBAH reagent. Dry weights were determined on 1.0 ml samples of the culture.

reducing sugars,by the cellulase enzymes produced,no appreciable level of these sugars is maintained in the medium. Mycelial dry weight increased rapidly in CMC cultures once reducing sugars declined and was almost maximal at 18 h (Fig. 4.17). Protein followed a pattern similar to that of dry weight (results not shown).

4.3. CELLULASE LOCALIZATION.

4.3.1. BUFFER EXTRACTIONS.

Two homogenisation techniques were initially conducted to determine the cellular content of CMCase in A. fumigatus ie, grinding with sand and sonication. Both methods released active enzyme from growing mycelium and to further investigate the efficiency of this release different buffer additives were employed, including Triton X-100 and EDTA.

Homogenisation by both methods in the presence of Triton X-100 and EDTA generally increased the yield of both CMCase activity and protein (Table 4.7). Triton X-100 consistently produced a greater release of enzyme than EDTA while a combination of the two was more effective than Triton X-100 alone only when sonication was employed. Triton X-100 is well known to solubilise membranes (Schnaitman, 1971 and Singer, 1974) and its effect in releasing CMCase could indicate that some of the enzyme is membrane bound or enclosed. Control experiments, detailed in the Appendix, showed that neither the detergent nor EDTA had any effect in increasing the specific activity of the enzyme.

TABLE 4.7.

Pestle and mortar		
Buffer	Units CMCase	Protein mg per ml
0.1 M Sodium acetate pH, 5.0.	0.07 ± 0.01	2.52 ± 0.39
+ 0.2% Triton X-100	0.27 ± 0.09	3.27 ± 0.31
+ 0.005 M EDTA	0.19 ± 0.03	2.28 ± 0.17
+ 0.2% Triton X-100 + 0.005 M EDTA	0.21 ± 0.05	2.58 ± 0.18
Sonication		
Buffer		
0.1 M Sodium acetate pH, 5.0	0.19 ± 0.01	1.43 ± 0.04
+ 0.2% Triton X-100	0.23 ± 0.02	1.73 ± 0.05
+ 0.005 M EDTA	0.20 ± 0.01	1.40 ± 0.04
+ 0.2% Triton X-100 + 0.005 M EDTA	0.27 ± 0.02	1.97 ± 0.09

Effect of Triton X-100 and EDTA on release of CMCase activity and protein from A. fumigatus mycelium fractionated by grinding and sonication. 24 h cultures grown in CMC medium at 48°C.

4.3.2. COLD OSMOTIC SHOCK.

In order to investigate whether A. fumigatus had any CMCase located in the periplasmic space between the cell membrane and the cell wall, the cold osmotic shock procedure, as described in Materials and Methods, was employed (Heppel, 1967).

The method used is a modification of previous methods in that the mycelium was washed a number of times in buffer at the growth temperature to ensure that any enzyme trapped within pockets between the hyphae did not exaggerate the amount released by the treatments. Control experiments indicated that three washings were required to release this enzyme from CMC grown mycelium but five were required for BMC grown material (Table 4.8). This difference indicated that the solid substrate may have trapped or absorbed some of the enzyme. The possibility that the mycelium was releasing a constant amount of enzyme , as indicated by the acetate buffer washings containing a measurable amount of CMCase activity, was not investigated. The cultures used for cold osmotic shock experiments were 24 h old and in the BMC cultures cellulose particles were still present. The difference between the number of washes required in the initial, acetate buffer indicates the extent of adsorption of CMCase by the particles and by the hyphae.

The results obtained from these experiments indicated that protein and CMCase activity were preferentially released by the hypertonic, mannitol buffer rather that the final hypotonic buffer (Table 4.8). The release observed here for A. fumigatus was consistent and represented 30% of the organism's total cellular activity for BMC grown cultures but only 4% of this activity for CMC grown cultures.

TABLE 4.8.

CMC grown	Units CMCase	Protein mg per ml
Buffer 1. Wash 1.	0.012 ± 0.001	0.35 ± 0.02
Buffer 1. Wash 2.	0.001 ± 0.000	0.48 ± 0.04
Buffer 1. Wash 3.	0.001 ± 0.000	0.37 ± 0.01
Buffer 2.	0.022 ± 0.002	0.77 ± 0.01
Buffer 3.	0.000 ± 0.000	0.38 ± 0.04
BMC grown		
Buffer I. Wash 1.	0.253 ± 0.010	0.35 ± 0.02
Buffer 1. Wash 2.	0.079 ± 0.003	0.32 ± 0.02
Buffer 1. Wash 3.	0.044 ± 0.001	0.31 ± 0.01
Buffer 1. Wash 4.	0.057 ± 0.001	0.32 ± 0.01
Buffer 1. Wash 5.	0.045 ± 0.001	0.31 ± 0.02
Buffer 2.	0.113 ± 0.007	0.90 ± 0.14
Buffer 3.	0.034 ± 0.005	0.38 ± 0.04

Effect of cold osmotic shock on release of CMCase activity and protein from A. fumigatus mycelium. For procedure see Materials and Methods.

Buffer 1 = 0.1 M sodium acetate buffer, pH 5.0, 48°C

Buffer 2 = 0.8 M mannitol + 0.02 M EDTA, 48°C.

Buffer 3 = 0.005 M $MgCl_2$. 0°C.

4.3.3. WALL DIGESTION.

Two approaches were used to assess the possibility of CMCase being specifically bound to or restrained by wall polymers. Firstly, CMC grown hyphae were digested with a combination of enzymes to release protoplasts and the level of CMCase in the medium measured before and after digestion. Secondly, hyphae were treated with chitinase to specifically digest chitin, a wall polymer which has previously been implicated in the binding of enzymes (Yabuki, 1966 and Gascon et al., 1968). Both treatments were conducted in the presence of 0.6 M KCl to prevent rupture of the cell membrane and release of internal CMCase.

Total digestion of the wall was achieved using β-glucuronidase as described in Section 3. Aliquots of the hyphae were incubated with β-glucuronidase at a final concentration of 10% enzyme to mycelium at 30°C for 90 min. Samples of the incubate were removed at 30 min intervals and assessed for CMCase (Table 4.9). CMCase activity was excessively high in the 0 min control sample, however, indicating that the lytic enzyme solution contained a large amount of CMCase or equivalent activity. Activities of subsequent samples were, however, lower than the 0 min control indicating that either protease activity was present in the enzyme solution or that inhibitors of CMCase were being released during the digestion.

Since the presence of such a high enzyme activity in the digestion medium could possibly mask low levels of released CMCase an induced lytic mixture, obtained from culture filtrates of T. viride growing on purified A. fumigatus cell walls, was also used to hydrolyse the hyphal walls. A. fumigatus mycelium, incubated with this induced

TABLE 4.9.

Time (min)	Units CMCase
0	37.9 ± 1.90
30	32.3 ± 0.97
60	27.4 ± 1.37
90	25.3 ± 1.42

CMCase activity of lytic medium during digestion of A. fumigatus mycelium by β-glucuronidase and chitinase as described in Materials and Methods.

TABLE 4.10.

Treatment	Units CMCase
Control (buffer only)	0.78 ± 0.01
Induced enzyme *	−0.41 ± 0.04
Chitinase	2.86 ± 0.05

Release of CMCase activity from A. fumigatus mycelium by digestion with induced enzyme (40 µg per mg mycelium dry wt) and chitinase (10 units per mg mycelium dry wt).

* Prepared as detailed in Materials and Methods.

enzyme at a concentration of 40 µg per mg of mycelial dry weight, produced protoplasts after 30 min. However, despite the enzyme solution having less inherent activity towards cellulose, there was also a decrease in CMCase activity in the medium after 3.0 h incubation (Table 4.10).

Specific digestion of the chitin in the cell wall was, however, more successful in releasing CMCase (Table 4.10). Incubation of A. fumigatus mycelium with chitinase alone at a concentration of 10 units per mg of mycelial dry weight produced a considerable increase in CMCase after 3.0 h.

4.3.4. DIFFERENTIAL CENTRIFUGATION.

Localization of CMCase activity within hyphal homogenates was also conducted using differential centrifugation and comparing the activities of fractions isolated at 2,500 x g, 10,000 x g and 300,000 x g. Initial experiments (Table 4.11) indicated that after homogenisation of 18 h cultures little CMCase activity was obtained in the pellets of 10,000 x g or 100,000 x g fractions and that most activity remained in the 100,000 x g supernatant. Subsequent experiments, however, indicated that centrifugation of this supernatant at 300,000 x g would sediment the activity (Table 4.12). This fractionation of CMCase activity was found to depend quite markedly upon the age of the mycelium used. With homogenates of 18 h mycelium more CMCase activity was found in the pellets than in the supernatants of the 300,000 x g fraction. With homogenates from older cultures the amount of activity remaining in the final supernatant increased until with 48 h cultures only 20% remained in the 300,000 x g pellet. Little activity was

TABLE 4.11.

Fraction	Units CMCase
Homogenate	0.310 ± 0.022
2,500 x g pellet	0.128 ± 0.028
2,500 x g supernatant	0.197 ± 0.018
10,000 x g pellet	0.046 ± 0.004
10,000 x g supernatant	0.139 ± 0.006
100,000 x g supernatant	0.151 ± 0.047

CMCase activity in fractions of mycelial homogenate of A. fumigatus mycelium separated by differential centrifugation as described in Materials and Methods. 18 h cultures grown on CMC medium supplemented with 0.2% (w/v) glucose.

TABLE 4.12.

Fraction	Units CMCase		
	18h	24h	48h
Homegenate	18.07 ± 0.24	37.65 ± 2.48	49.92 ± 2.55
2,500 x g pellet	2.71 ± 0.14	2.45 ± 0.11	10.89 ± 1.14
2,500 x g supernatant	8.21 ± 0.33	25.05 ± 0.43	19.97 ± 1.88
10,000 x g pellet	0.58 ± 0.02	4.44 ± 0.04	0.76 ± 0.01
10,000 x g supernatant	7.32 ± 0.44	14.97 ± 0.35	11.70 ± 0.73
300,000 x g pellet	1.14 ± 0.04	3.58 ± 0.03	2.05 ± 0.06
300,000 x g supernatant	0.72 ± 0.00	10.93 ± 0.20	7.18 ± 0.31

Effect of mycelial age on CMCase activity in fractions of homogenates of A. fumigatus mycelium growing on CMC medium supplemented with 0.2% (w/v) glucose. Differential centrifugation as described in Materials and Methods.

associated with the 10,000 x g fraction but variable amounts remained in the 2,500 x g cell wall fraction.

Fumarase assays were conducted to characterise the location of mitochondria in the fractions and indicated that these mainly sedimented in the 10,000 x g pellet (Table 4.13). A significant amount was also found in the 300,000 x g supernatant indicating some rupture of the organelles. Phosphorylcholine-glyceride transferase activity was assessed as a marker enzyme for endoplasmic reticulum and indicated that most of this activity occurred in the final supernatant fraction after the 300,000 x g centrifugation (Table 4.13). Whether the enzyme was still attached to the endoplasmic reticulum was not determined although observation of this fraction by electron microscopy indicated the absence of membrane structures. The use of this enzyme for localization of endoplasmic reticulum in plants is well characterised (Lord et al., 1972) although little information is available for fungi.

Fractions were also characterised by observing negatively stained samples in the electron microscope. Samples from 10,000 x g fractions were mainly composed of large, irregular bodies showing little detail of internal structures (Fig. 4.18). Samples from 300,000 x g fractions contained small, irregularly sized, electron dense patches, often studded with darkly staining particles (Fig. 4.19). When treated with Triton X-100 neither the particles nor the patches were observable.

The effect of Triton X-100 on release of CMCase activity and protein from samples of the 300,000 x g pellet fraction was also investigated. The results obtained indicated that the detergent released 10 and 20% extra CMCase activity from 18 and 48 h mycelium samples respectively (Table 4.14).

TABLE 4.13.

Fraction	Fumarase Units*	PCGT d.p.m. x 10^6
10,000 x g pellet	0.088 ± 0.013	0.124 ± 0.008
10,000 x g supernatant	0.069 ± 0.008	7.203 ± 0.274
300,000 x g pellet	0.025 ± 0.004	0.145 ± 0.022
300,000 x g supernatant	0.053 ± 0.020	4.794 ± 0.698

Fumarase and Phosphorylcholine-glyceride transferase (PCGT) activity in fractions of mycelial hogomenate of A. fumigatus mycelium separated by differential centrifugation as described in Materials and Methods. 18 h cultures grown on CMC medium.

Fumarase and PCGT were assayed as described in Materials and Methods.

* 1 unit fumarase is that amount of enzyme which causes a decrease in absorbance at 260 nm of 1.0 under the assay conditions employed.

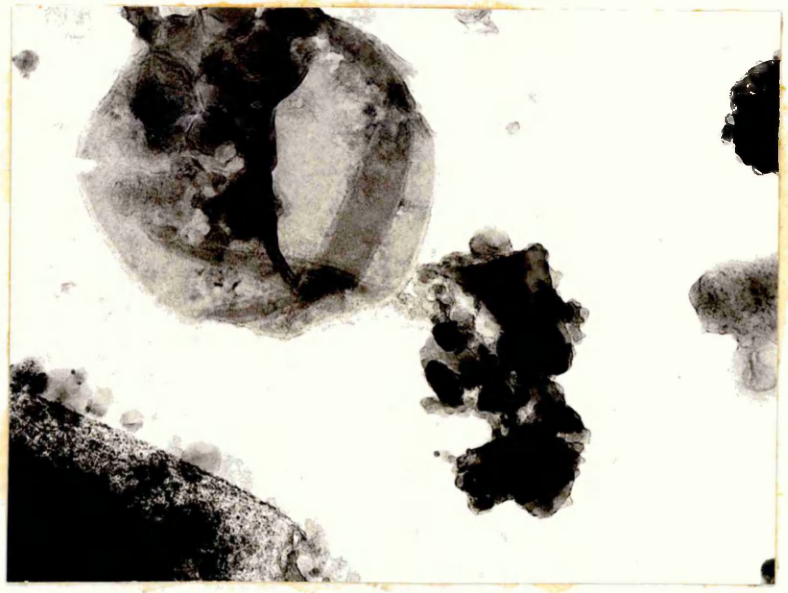


FIGURE 4.18. A. fumigatus mycelial homogenate. Sample from differential centrifugation, 10,000 x g pellet. Samples were prepared for negative staining as described in Materials and Methods. x 100,000

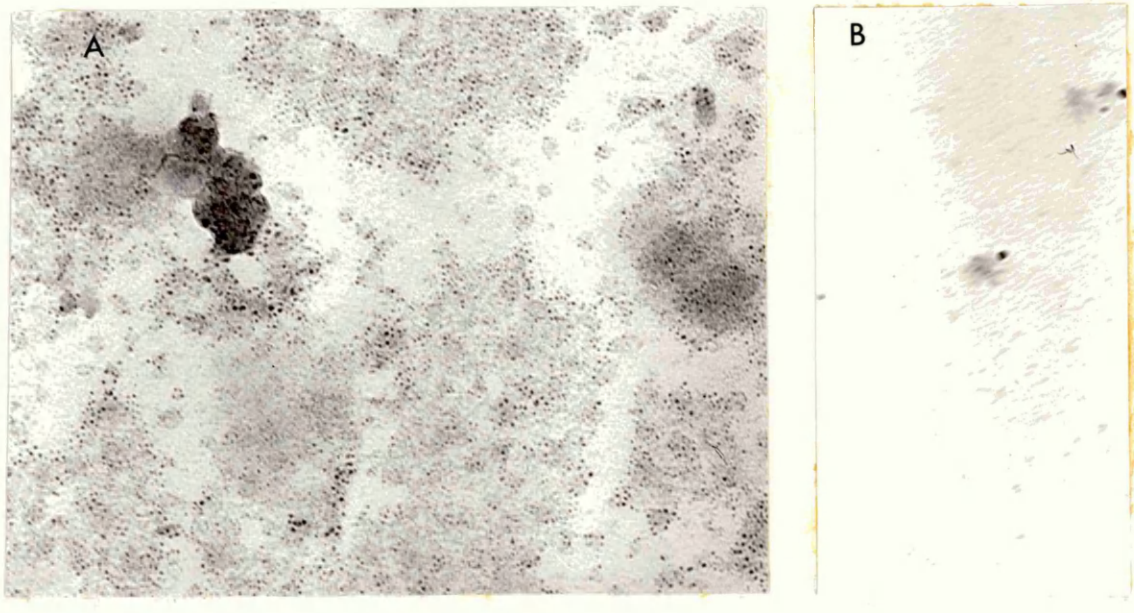


FIGURE 4.19. A. fumigatus mycelial homogenate. Sample from differential centrifugation, 300,000 x g pellet. Samples were prepared as for negative sstaining as described in Materials and Methods. x 200,000 x g.
A, standard sample. B, Triton X-100 treated sample.

TABLE 4.14.

Mycelial age	% Increase	
	Protein	CMCase activity
18 h	13.5 ± 1.46	9.5 ± 1.19
48 h	21.8 ± 2.86	21.5 ± 1.20

Effect of Triton X-100 on release of CMCase and protein from 300,000 x g pellets obtained by differential centrifugation of mycelial homogenates of A. fumigatus.

Samples of pellets were resuspended in 0.1% Triton X-100 in 0.1M sodium acetate buffer pH 5.0 and their CMCase activity compared with similar samples resuspended in the same buffer without Triton X-100.

4.3.5. HISTOCHEMICAL LOCALIZATION OF CELLULASE.

Because refined centrifugation procedures were unsuccessful in further localizing CMCase within hyphal cytoplasm histochemical techniques were employed. These utilize residual enzyme activity within lightly fixed tissue to produce electron opaque deposits which may be taken as indicators of enzyme position (Bal, 1972 and Nolan and Bal, 1974).

Experiments with A. fumigatus indicated a difference in localization of CMCase according to whether the fungus was grown on BMC or CMC medium. Unstained, BMC grown samples, processed to localize CMCase (as described in Materials and Methods) produced dense deposits both within and outside the hyphal cytoplasm (Figs. 4.20 and 4.21). Deposits were particularly extensive along the outer layers of the cell wall and even occurred some distance away from the hyphae (Fig. 4.22). Deposits within the hyphae were consistently located in large vacuoles (Fig 4.21). No evidence was obtained for localization in smaller organelles such as Golgi bodies although the short period of fixation and the processing conditions used may not have stabilized these structures. Control samples showed some non-specific deposition which was generally of a different appearance to that in the test samples. (Fig. 4.23). A further control was conducted using plant tissue to obtain a reference of the expected deposition due to CMCase activity (Fig. 4.24). The deposits produced in the middle lamellae between the growing cells was taken as the reference standard and resembled the appearance shown in previous reports (Bal, 1972).

In contrast to the localization evident in BMC samples, CMC grown tissue produced localization of CMCase only within the cytoplasm and

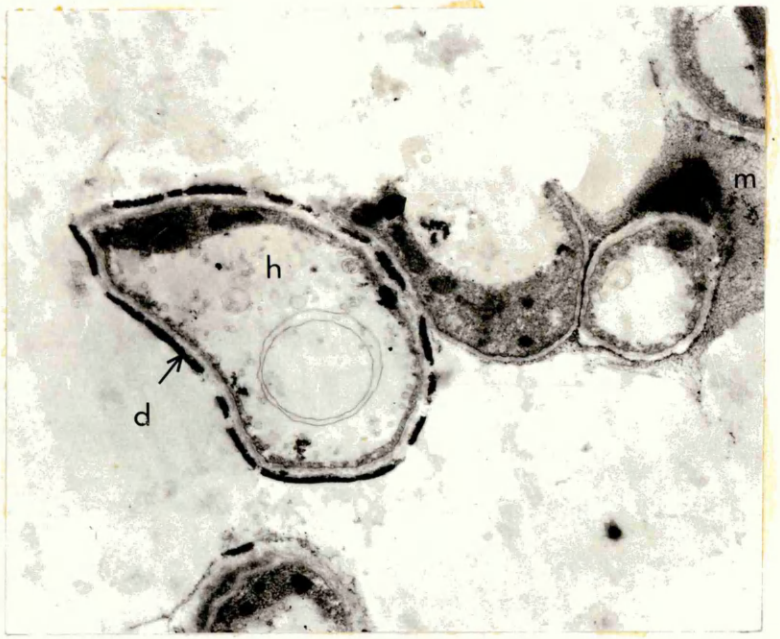


FIGURE 4.20. CMCase localization in A. fumigatus hyphae. Samples from 24 h BMC cultures were prepared for CMCase localization as described in Materials and Methods. x 17,200. d = electron dense deposit, h = hyphae, m = extracellular matrix.

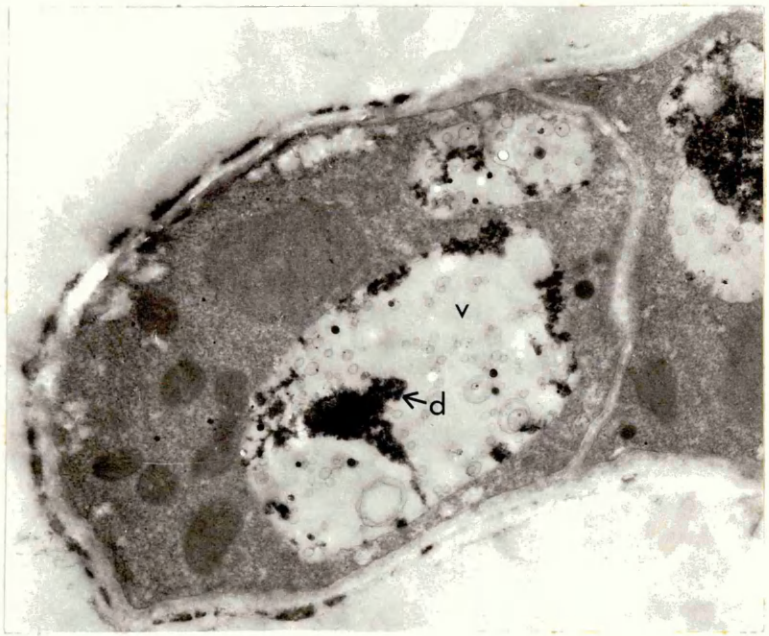


FIGURE 4.21. CMCase localization in A. fumigatus hyphae. Samples from 24 h BMB cultures were prepared for CMCase localization as described in Materials and Methods. x 27,600. d = electron dense deposit, v = vacuole.

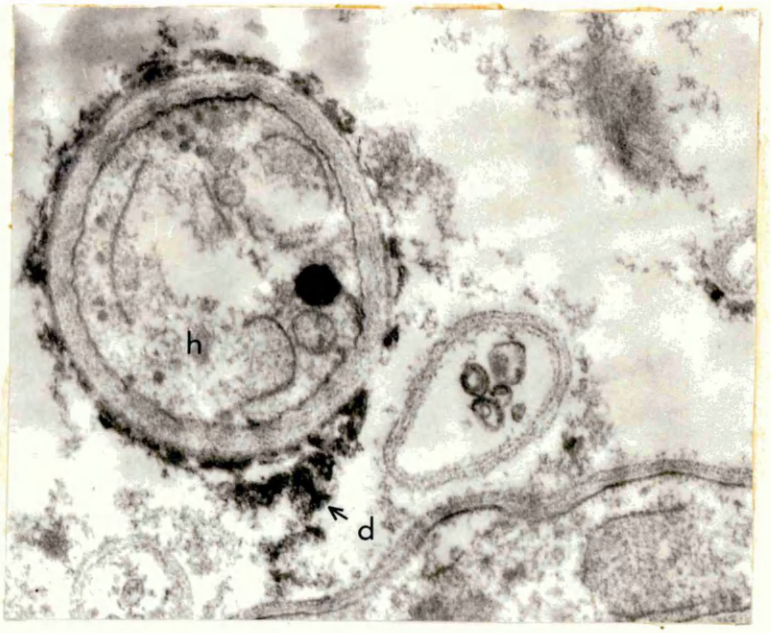


FIGURE 4.22. CMCase localization in A. fumigatus hyphae. Samples from 24 h BMC cultures were prepared for CMCase localization as described in Materials and Methods. x 28,900. d = electron dense deposit. h = hyphae.

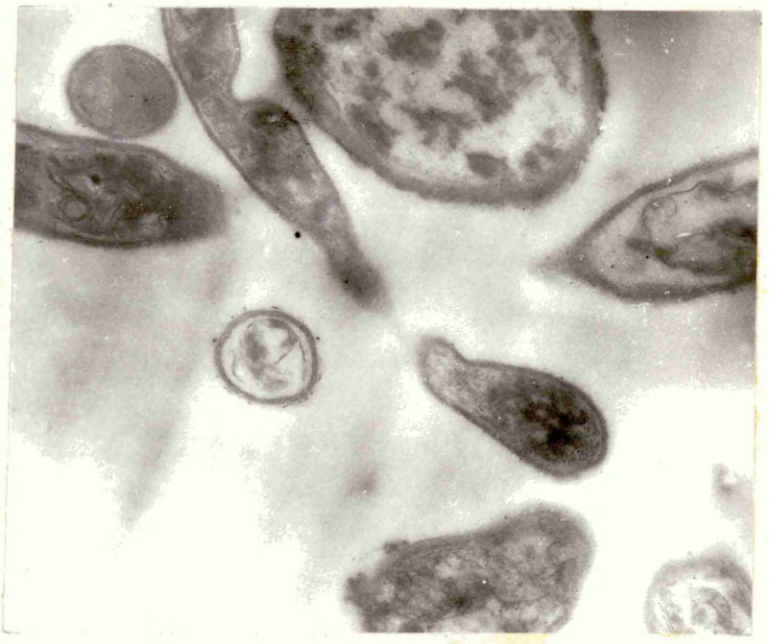


FIGURE 4.23. CMCase localization in A. fumigatus hyphae. Control samples from 24 h BMC cultures were prepared as described in Materials and Methods. x 25,000.

FIGURE 4.24. CMCase localization in Brassica pekinensis Rupr. Samples from young, actively growing leaves were prepared for CMCase localization as described in Materials and Methods. x 25,000. c = chloroplast, d = electron dense deposit.

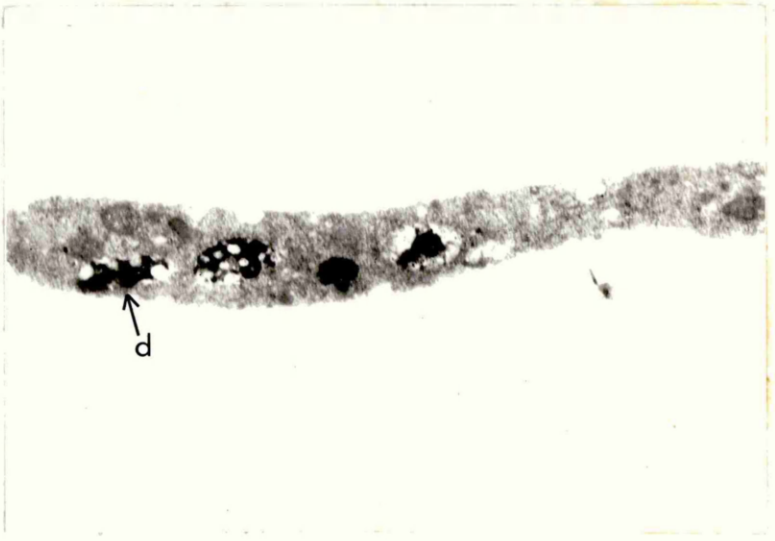


FIGURE 4.25. CMCase localization in A. fumigatus hyphae. Samples from 24 h CMC cultures were prepared for CMCase localization as described in Materials and Methods. x 18,500. d = electron dense deposit.

no deposits were evident in the cell walls or in the medium beyond these (Fig. 4.25). Within the cytoplasm large vacuoles were found to stain heavily but were different in appearance to those observed in BMC grown hyphae (Fig. 4.26). Control samples showed no deposition within these vacuoles (Fig. 4.27).

A further observation made during these investigations was that of an extensive coating of amorphous material surrounding some of the hyphae growing in BMC cultures (Figs. 4.20 and 4.28). This matrix was often considerably thicker than the cell wall and frequently extended around groups of hyphae. No CMCase activity was localized within this matrix and similar structures were not observed on CMC grown hyphae.

4.3.6. CMCase RELEASE FROM A. FUMIGATUS PROTOPLASTS.

Experiments were conducted to assess CMCase activity and synthesis in protoplasts isolated from A. fumigatus mycelia grown on CMC medium. Further experiments were performed to investigate the synthesis of CMCase by regenerating protoplasts.

Initially protoplasts were obtained from CMC grown mycelia and their internal content of CMCase assessed to determine whether protoplasts could be used to provide a more precise cellular localization of the enzyme than that allowed by standard fractionation procedures. This approach, however, carries the complication that the digestive enzymes used to release the protoplasts possess a high lytic activity towards cellulose and because of this there is the strong possibility that any activity found in protoplast homogenates may have been carried over from the protoplast incubation medium.

To determine whether this occurred CMCase activity was measured

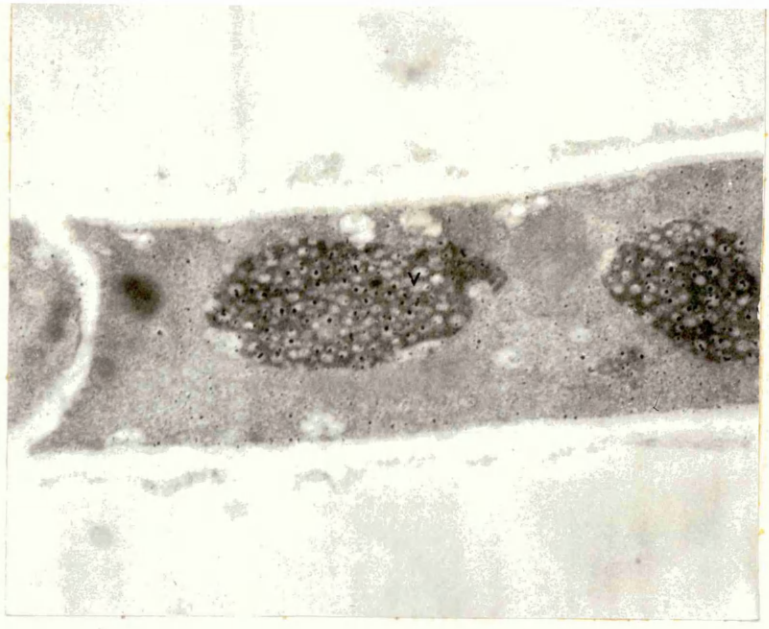


FIGURE 4.26. CMCase localization in A. fumigatus hyphae. Samples from 24 h CMC cultures were prepared for CMCase localization as described in Materials and Methods. x 34,000. v = vacuole.

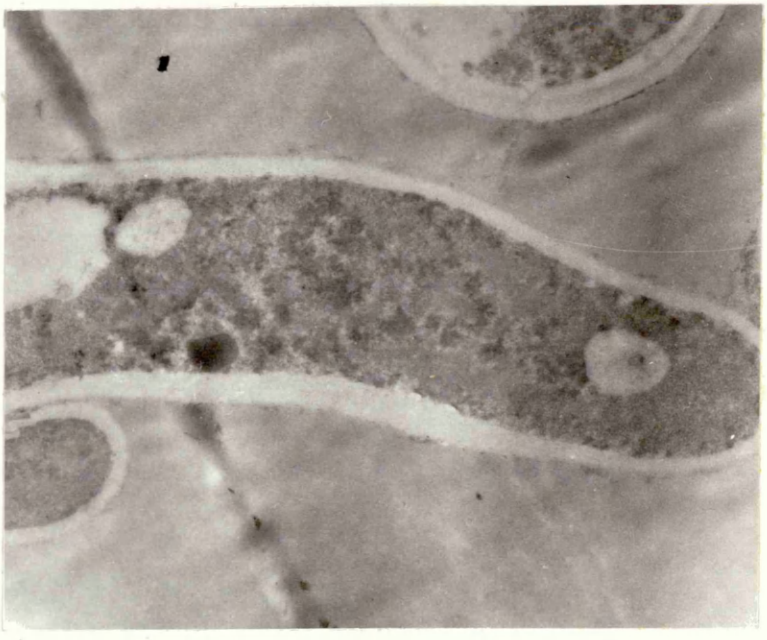


FIGURE 4.27. CMCase localization in A. fumigatus hyphae. Control samples from 24 h CMC cultures were prepared as described in Materials and Methods. x 30,000.

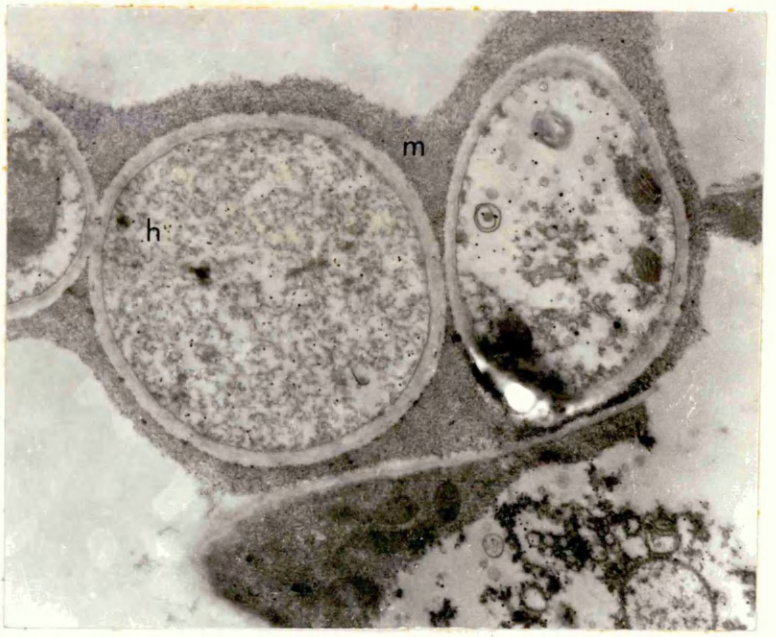


FIGURE 4.28. Extracellular matrix material surrounding A. fumigatus hyphae growing in BMB medium. x 23,400. h = hyphae, m = extracellular matrix.

in samples of buffer washes from protoplasts obtained using the standard procedures outlined previously with β-glucuronidase and chitinase. Samples of protoplast contents and the remains obtained after bursting in water were also assayed (Table 4.15). A considerable activity was initially associated with the protoplasts but this was soon removed by washing in buffer indicating that the enzyme was not adsorbed strongly onto the cell membrane. CMCase activity in the protoplast fractions was, however, very low indicating that the high activity previously noted in the intact mycelium (Fig. 4.13 and Table 4.7) was lost during the production of protoplasts.

Experiments were subsequently conducted to determine whether CMCase could be induced in protoplasts regenerating in osmotically buffered CMC medium. After 5.0 h incubation in this medium the CMCase activity of the medium external to the protoplasts had increased six-fold (Table 4.16). Cellular CMCase, however, either in the soluble fraction or in that sedimenting at 2,500 x g, showed no increase indicating that the enzyme was not accumulated within the protoplast cytoplasm to a significant degree. Control incubations were also conducted to ensure that the increase of CMCase in the medium was due to a specific, induced release from the protoplasts. For this protoplasts were incubated in an equivalent sample of medium without CMC. No increase in activity was observed in this incubation. A further control consisting of protoplasts which had been burst in water before incubating with CMC was also conducted to ensure that the synthesis and release of the enzyme required active cytoplasm and was not due to contaminating wall fragments. Again this control incubation failed to show an increase (Table 4.16).

Intact and burst protoplasts were incubated in CMC medium to

TABLE 4.15.

Treatment	Units CMCase
Lytic medium	3.32 ± 0.04
1st buffer wash	0.35 ± 0.03
2nd buffer wash	0.17 ± 0.02
3rd buffer wash	0.01 ± 0.00
Protoplast contents *	0.01 ± 0.00
Protoplast residue #	0.03 ± 0.00

CMCase content of protoplasts and buffer washes after isolation. Protoplasts were obtained by digestion of 18 h A. fumigatus mycelium with β-glucuronidase and chitinase as described in Materials and Methods. Mycelium was grown on CMC medium supplemented with 0.2% glucose, 30°C.

Wash buffer = 0.05 M HEPES, pH 5.5 + 0.6 M KCl + 0.2 M $CaCl_2$.

* Obtained by bursting protoplasts in 0.1 M sodium acetate buffer, pH 5.0 and removing debris by centrifugation at 2,500 x g.

\# Debris from above procedure resuspended in an equal volume 0.1 M sodium acetate buffer, pH 5.0.

TABLE 4.16.

Treatment		Milliunits CMCase
Control	Contents	2.0 ± 0.25
	Residue	5.5 ± 0.32
Burst protoplasts 5 h incubation CMC medium + 0.6 M KCl	Supernatant	3.0 ± 0.12
	Contents	1.0 ± 0.00
	Residue	3.0 ± 0.10
Intact protoplasts 5 h incubation 0.6 M KCl	Supernatant	3.0 ± 0.10
	Contents	1.0 ± 0.00
	Residue	4.0 ± 0.19
Intact protoplasts 5 h incubation CMC medium + 0.6 M KCl	Supernatant	13.0 ± 0.45
	Contents	2.0 ± 0.02
	Residue	4.0 ± 0.03

CMCase activity of protoplast fractions after 5 h regeneration in CMC or incubation in control buffers. Protoplasts obtained as described in Materials and Methods, incubation procedure as described in text.

determine the extent and time scale of regeneration. Complete regeneration of protoplasts to pelleted mycelium was observed to occur within 18 h (Figs. 4.29 to 4.33). The initial morphological developments were an aggregation of the intact protoplasts into clumps and a deformation of the spherical shape to produce a round body with a slight protuberance (Figs. 4.29 and 4.30). These features were observed between 3.0 h and 6.0 h although a wide variety of synchrony was evident and some protoplasts did not regenerate at all. Further swelling of this protuberance resulted in one or more elongated, swollen bodies growing out of the original protoplast (Figs. 4.31 and 4.32). Narrower hyphae subsequently grew from these bodies after 9.0 to 12 h of incubation (Fig. 4.33). Control samples of protoplasts which were burst before incubation did not show any signs of mycelial growth indicating that contaminating spores and mycelial fragments were few or inactive (Fig. 4.34). Experiments on CMCase synthesis in regenerating protoplasts were conducted within the first 9.0 h of regeneration since after this time the protoplasts were no longer osmotically fragile. Production of CMCase activity was, nevertheless, shown to increase with the time of incubation at least up to six hours (Table 4.17).

Regenerating protoplasts were used to determine two factors of CMCase synthesis and release. Firstly, whether they produced a greater activity of extracellular CMCase than an equivalent amount of intact hyphae and secondly, whether the synthesis of the enzyme depended upon the previous cultural conditions of the hyphae used to obtain the protoplasts. This latter investigation was further extended to obtain some indication of the cellular controls regulating the synthesis of CMCase.

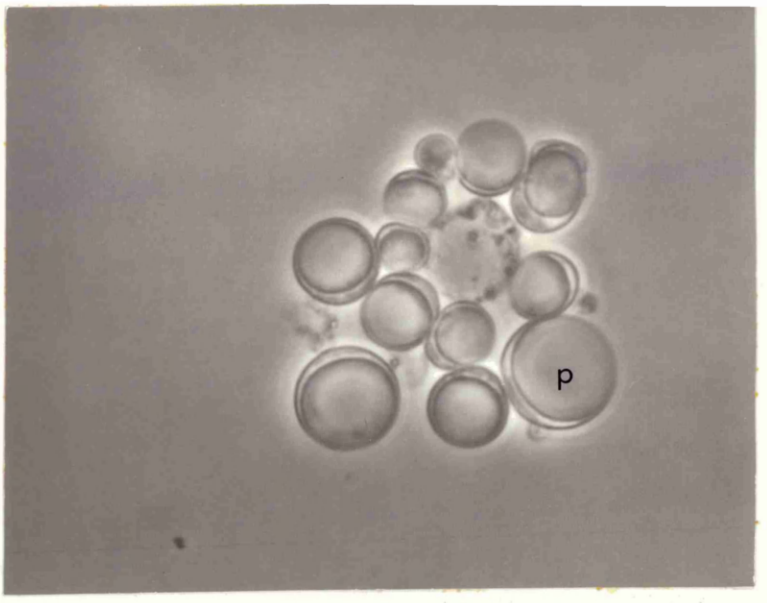


FIGURE 4.29. A. fumigatus protoplasts incubated in CMC regeneration medium for 3.0 h. x 1,600. p = protoplast.

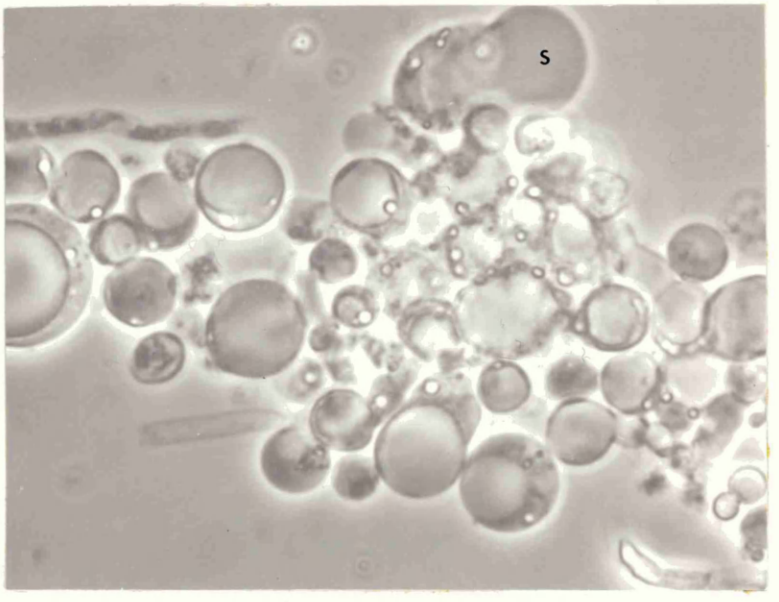


FIGURE 4.30. A. fumigatus protoplasts incubated in CMC regeneration medium for 6.0.h. x 1,600. s = protoplast swelling.

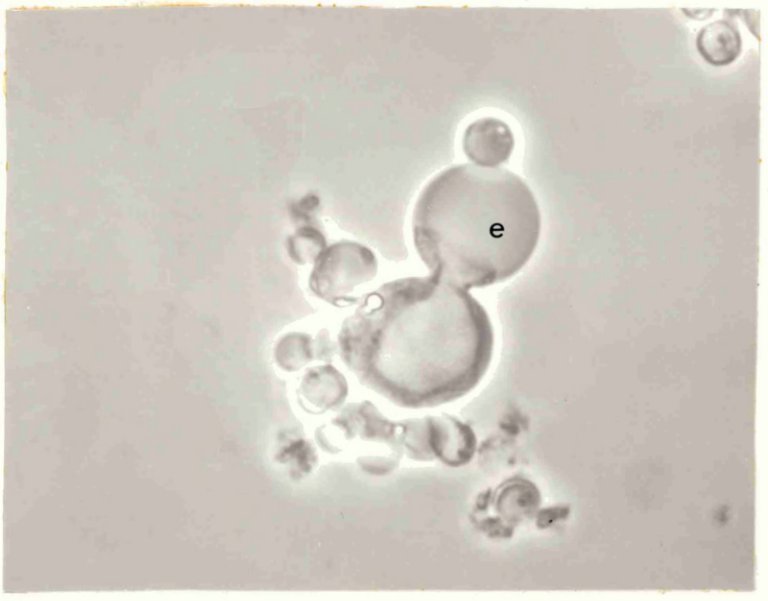


FIGURE 4.31. A. fumigatus protoplasts incubated in CMC regeneration medium for 9.0 h. x 1,600. e = enlarged swelling.

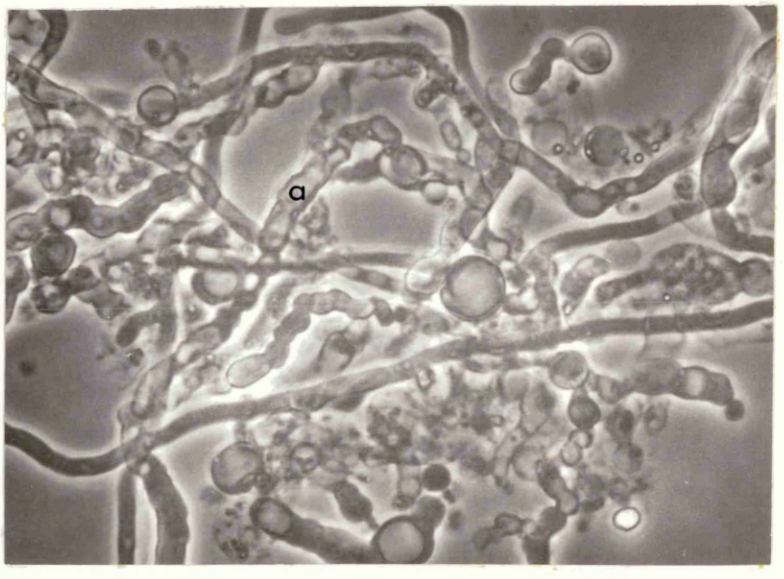


FIGURE 4.32. A. fumigatus protoplasts incubated in CMC regeneration medium for 12 h. x 800. a = abnormal hyphae.

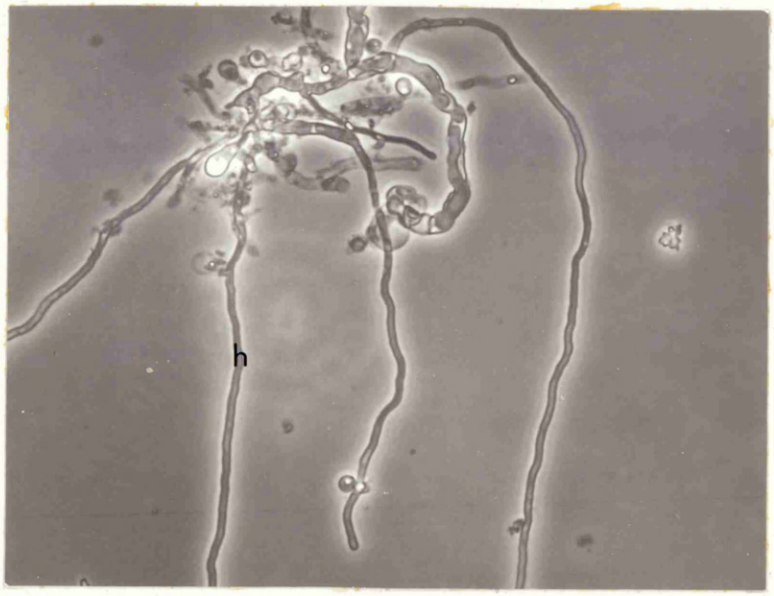


FIGURE 4.33. A. fumigatus protoplasts incubated in CMC regenaration medium for 18 h. x 400. h = hyphae.

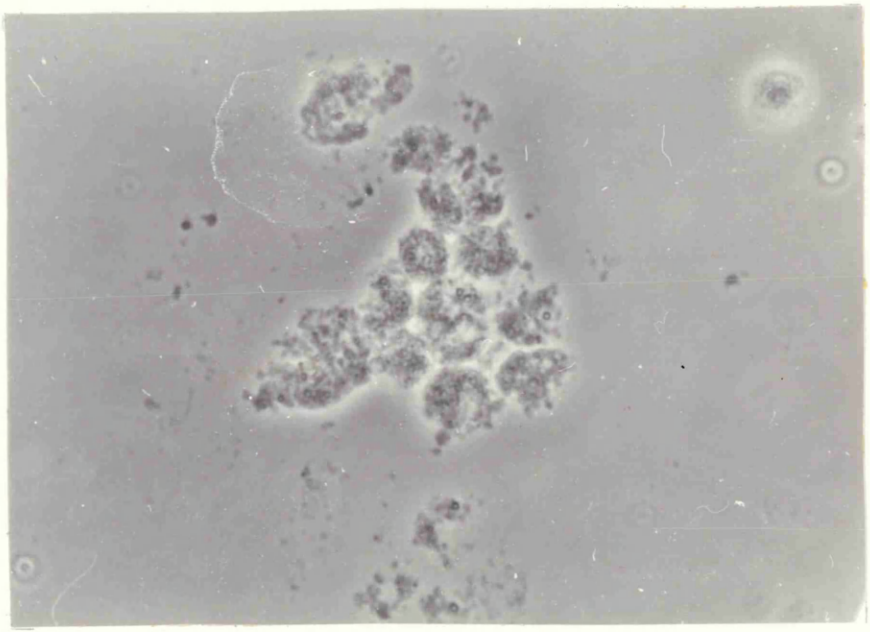


FIGURE 4.34. A. fumigatus protoplasts incubated in CMC regeneration medium after bursting in water. 12 h. x 1,600.

The results of experiments comparing CMCase synthesis in protoplasts from CMC grown and glucose grown mycelia are shown in Table 4.18. No significant difference is evident, however, between the extracellular levels produced by protoplasts or the equivalent amount of hyphae, regardless of the protoplast or hyphal origin. In these comparisons protoplasts were consistently found to release greater amounts of the enzyme than the mycelium, sometimes up to 25 times more (Table 4.18). Unfortunately, the CMCase content of the hyphal samples was not determined since the amount of hyphae used was too small to ensure an adequate recovery after homogenisation.

Protoplasts from mycelium actively synthesising CMCase were found to contain very little CMCase activity (Table 4.15). Alternatively, protoplasts from both induced and non-induced mycelium produced equal activities of CMCase on incubation in CMC medium (Tables 4.18 and 4.19). This result raises the possibility that CMCase synthesis depends upon newly formed mRNA. To investigate this, the activity of protoplasts and extracellular medium fractions induced in the presence and absence of cycloheximide (CH), was compared. The results from these experiments indicated that CH, 10 mg per L, severely depressed CMCase synthesis from both CMC and glucose grown mycelia (Table 4.19).

TABLE 4.17.

Fraction	Milliunits CMCase		
	0 h	3.0 h	6.0 h
Supernatant	9.7 ± 0.5	28.3 ± 0.7	66.7 ± 3.4
Contents	2.3 ± 0.2	4.0 ± 0.1	7.2 ± 0.6
Residue	2.2 ± 0,3	3.5 ± 0.2	7.8 ± 0.5

CMCase activity in fractions of regenerating protoplasts of A. fumigatus on CMC medium. Fractionation procedures as described in Materials and Methods.

TABLE 4.18.

Fraction	Milliunits CMCase			
	Glucose grown		Cellulose grown	
	Protoplasts	Mycelia	Protoplasts	Mycelia
Supernatant	127.0 ± 15.5	4.0 ± 0.2	166.0 ± 7.1	4.0±0.2
Contents	53.0 ± 6.9		56.0 ± 3.7	
Residue	35.0 ± 11.0		1.0 ± 0.1	

CMCase activity in fractions of regenerating protoplasts of A. fumigatus grown on glucose and CMC medium compared to CMCase activity produced by intact mycelium. Protoplasts fractionated at 4 h as described previously.

TABLE 4.19.

Fraction	Milliunits CMCase			
	Glucose grown		Cellulose grown	
	-CH	+CH	+CH	+CH
Supernatant	22.83 ± 5.90	4.17 ± 0.21	20.78 ± 0.69	4.16 ± 0.07
Contents	1.83 ± 0.01	1.67 ± 0.00	3.30 ± 0.04	1.00 ± 0.06
Residue	3.00 ± 0.47	2.67 ± 0.94	2.87 ± 0.56	0.29 ± 0.01

Effect of cycloheximide on CMCase activity in regenerating protoplasts of A. fumigatus. Protoplasts were fractionated as described previously. +CH = Cycloheximide, 10 mg per L. -CH = control.

The determination of cellulase activity involves a number of problems which can influence the range and type of information obtained. Primarily this is a result of the natural substrate for the enzyme being an insoluble polymer and generally possessing a high degree of microcrystalline organisation (Cowling, 1975). Owing to this at least three,interrelating enzymes are found to be necessary for the full hydrolysis of the naturally occurring substrate. Moreover, the use of an insoluble substrate in an assay imposes dispensing difficulties as well as the problem of enzyme adsorption onto the surface of the substrate. This latter ability has been found to occur with great tenacity (Halliwell and Griffin, 1978), and can pose a particularly influential variable when working with low concentrations of enzyme. Further it has been noted that the different cellulase enzymes are adsorbed differently according to their type (Bisaria and Ghose, 1977) and their activity (Halliwell and Griffin, 1978).

The three enzymes recognised as major components of the cellulase complex are endo-1,4-β-glucanase which preferentially hydrolyses internal glucose - glucose bonds within the cellulose chains, exo-cellobiohydrolases which preferentially liberate cellobiose units from the non-reducing ends of the chains and cellobiase which hydrolyses cellobiose to glucose (Fig. 4.35). To totally convert cellulose to glucose at least two of these enzymes must be present and it is well noted that a combination of glucanases act synergistically to produce a rapid hydrolysis (Li et al., 1965; Wood, 1975 and Ghose and Bisaria, 1979). It has also been shown that these two enzymes are essential for the hydrolysis of highly crystalline regions (Wood, 1975) and that adsorption of

the enzyme is a prerequisite for hydrolysis (Peitersen et al., 1977 and Ghose and Bisaria, 1979). For the investigation of cellulase synthesis in A. fumigatus discrimination was not made between the types of enzymes present although it was noted that three types could be separated by polyacrylamide gel electrophoresis (Appendix, Fig. A1). Full details of the assay method chosen and its relevance to the types of cellulase noted by other workers are given in the Appendix.

Application of the CMCase assay procedure to cultures of A. fumigatus growing on cellulose produced four groups of results. Firstly, some indication of the range of cellulose breakdown by the organism was provided by growing mycelium on a variety of substrates and under different conditions. Secondly, the time course of cellulase production by cultures growing on more purified cellulose substrates was determined and compared. Thirdly, the localization of cellulase within the hyphae was investigated by a number of procedures and finally, some aspects of cellulase synthesis during protoplast regeneration on cellulose media were determined.

Initial experiments using Rautela Cowling tubes as described in Materials and Methods provided evidence that the A. fumigatus strain investigated was considerably more cellulolytic compared to strains of A. niger or T. viride. A range of culture conditions or temperatures were not investigated for these species, however, and the Trichoderma strain used was not necessarily one of the most cellulolytic available. The observation that A. fumigatus produces a greater clearing of the cellulose substrate at 48°C than at 30°C agrees with the thermotolerant characteristics of this fungus and this is further illustrated by the elevated temperature profile for the cellulase enzymes isolated from culture filtrates (Fig. 4.6).

Since the production of reducing sugar from cellulose as well as degradation of the substrate is also a desired end product of cellulase applications more detailed studies on the synthesis of cellulase enzymes in A. fumigatus were undertaken.

Initial experiments, to ensure that A. fumigatus could hydrolyse naturally occurring cellulose substrates, were conducted using a range of paper and cardboard wastes incubated in standard medium (Table 2.1) but without purified cellulose. The CMCase activity of these culture filtrates were determined after 7.0 days (Table 4.2). As may be seen considerable amounts of reducing sugars were released during the assay indicating that the filtrate contained an active cellulase. Levels of reducing sugar in the growing medium were however low, (Table 4.2) indicating that any sugar produced from the natural substrates was rapidly taken up by the organism. Considerable residues were evident at the end of these experiments indicating that either the enzyme became inactive during the course of growth or that the organism was unable to hydrolyse some part of the substrate. Since it is well known that many organisms have difficulty hydrolysing the additional polymers often found associated with cellulose, such as lignin, and to prevent the presence of any enzymes produced against these polymers complicating the assays, further experiments were conducted with purified BMC or soluble CMC media.

The time course of mycelial and extracellular CMCase activity was followed in BMC cultures and indicated that a lag phase of 2.0 days occurs before enzyme activity and protein synthesis become measurable (Fig. 4.12). At this point there is a large increase in both mycelial and extracellular CMCase activity although this soon subsided. Full degradation of the substrate did not occur until 4.0 to

5.0 days after which there was a second increase in extracellular levels. Mycelial CMCase activity, however, levelled off or declined slightly in this period. One possible explanation for the increase in extracellular CMCase activity after the final degradation of the substrate is release of enzyme bound to the cellulose particles. Protein levels were also measured during the growth of the organism and were found to follow a similar pattern but without a peak after 2.0 or 3.0 days (Fig 4.13). Cultures after 5.0 days growth were noticeably aged and autolytic and it is also possible that the rise in extracellular CMCase activity could have resulted from non-specific release from the mycelium. However, the lack of an equivalent decline in activity in the filtered residues and the lack of a protein increase in the medium would tend to discount this. The observation that the specific activity of the protein in the mycelium and the medium increases at 3.0 to 4.0 days adds a further complication but no information was obtained on the presence of a co-factor.

In order to shorten the growth period of the fungus and to prevent the possibility of autolysis affecting the investigations into specific release of the enzyme the basic CMC and BMC media were supplemented with a range of salts and sugars. Peptone has previously been included in cellulose medium for T. viride to achieve this end (Sternberg, 1976) and a number of studies have investigated the effects of low levels of sugar addition to cellulose fungi (Peitersen, 1975 and Mandels and Andreotti, 1978). The benefits of obtaining an earlier, maximal production of the enzyme are obvious, particularly in economic terms. Whether this results from a specific induction or simply from a greater body of mycelium being present at an earlier time will depend upon a number of factors. Because of this the amount

of supplement added and the time of addition may need to be carefully determined.

From the results obtained with A. fumigatus it is evident that sugar supplementation is most profitable, although the specific activity of the enzyme produced in supplemented cultures is decreased according to the amount of sugar added. Nevertheless the time scale of enzyme production is reduced dramatically from about 60 h to 18 h for maximum yields. Attempts to enhance growth by the addition of various salts were largely unsuccessful and a similar lack of effect has also been found in other studies with A. fumigatus (Malik et al., 1980). The observation that asparagine did produce some increase in CMCase activity in A. fumigatus is most likely to be due to the organism utilizing the compound as an additional carbon source.

Further investigations on a number of sugars indicated that most supplements, added at 0.2% (w/v), increased CMCase activity in filtrates compared to filtrates from cultures with CMC as the sole substrate (Table 4.5). Cultures with lactose, however, produced negligible CMCase activity. Dry weight determinations indicated that this was due to a lack of growth and this was confirmed by microscopic observations. Such a result is unexpected since lactose does contain a β-1,4, polymer bond and has been reported to act as an inducer in a number of cellulase systems (Mandels, 1975 and Ryu et al., 1979). One explanation for this is that for a sugar to act as an inducer a critical concentration is necessary (Lobanok and Pavlovskaya, 1977). Further, concentrations greater than this may be inhibitory and thus act to reduce growth. Other sugars which are not inducers will,however,support growth at a greater range of concentrations.

The activity of CMCase from mycelial extracts growing on

supplemented CMC and BMC media is very similar to that obtained for unsupplemented BMC medium and both are characterised by a peak of activity at an early stage after a lag period. In this respect it is relevant that a number of reports on cellulase synthesis show patterns with various initial peaks of cellulase activity (Berg et al., 1972a; Oso, 1978 and Ramasamy and Verachtert, 1980). Correlation of the enzyme activity with protein in A. fumigatus show that this initial peak in activity is not necessarily due to an increase in the amount of enzyme produced but possibly to a more active enzyme and raises the possibility of inducer or inhibitor involvement. Alternatively it is worth noting that the pH optima of extracellular enzymes has been noted to vary with the age of the culture (Nagasaki, 1968) and that a number of studies have shown pH to be of critical importance in cellulase synthesis (Sternberg, 1976 and Mandels and Andreotti, 1978). The levels of pH were not measured throughout the growth of A. fumigatus cultures but it was noted to fall from 6.2 at the start of growth to 3.8 at the end and this agrees with similar values from other reports on batch cultures (Sternberg, 1976 and Mandels and Andreotti, 1978).

Determinations of reducing sugar in the medium of supplemented cultures indicated that little CMCase was produced until the reducing sugar had been utilized. From this and the observation that the specific activity of the enzyme is not increased by supplementation with glucose it may be concluded that the sugar is acting to enable a large bulk of mycelium to develop rapidly rather than inducing the enzyme. Since no CMCase was observed until the supplementing glucose had been consumed it would appear that the sugar may repress CMCase synthesis as reported in other studies (Mandels, 1975; Singh and Kunene, 1980 and Trivedi and Rao 1980) This is also supported by studies where glucose is added to

cultures already synthesising cellulase (Mandels and Andreotti, 1978). In this study 0.4% (w/v) glucose was added to T. viride cultures growing on 2.0% (w/v) cellulose. As a result growth and cellulase synthesis were reduced considerably.

One interesting difference observed between the enzyme activities of A. fumigatus cultures is that between BMC and CMC media (Figs. 4.15 and 4.16). BMC and CMC grown mycelia both show the initial peak in CMCase activity but only BMC cultures show this in the culture medium indicating that there may be a difference between the response of the mycelium towards the secretion of the enzyme. One possible explanation of this would be that the enzyme would only need to be secreted from the hyphae if the cellulose substrate was insoluble. Soluble CMC substrates would be able to penetrate the cell wall or approach it closely enough to be hydrolysed by wall bound enzymes. A similar distribution of cell bound and cell free cellulase enzymes in similar growth media has been noted to occur for the cellulolytic bacteria Cellvibrio fulvus (Berg, 1975) and for Pseudomonas fluorescens (Suzuki et al., 1969) although not for T. viride (Berg and Pettersson, 1977). In the latter organism the presence of cell free cellulase activity was explained by a closer penetration of the soluble CMC to the cell wall resulting in the adsorption and subsequent removal of enzyme from the cell. If this mechanism can operate then it would be expected that the type and substitution of the substrate would be important variables in the secretion of cellulase enzymes. Similarly the structure and porocity of the cell wall will influence the process and would be worth investigation.

Since one of the most potentially valuable uses of cellulolytic organisms is in the production of readily extractable cellulase enzymes

the problem of how the enzymes are synthesised and secreted warrants careful investigation. In order to investigate these aspects in A. fumigatus, experiments were conducted on cell fractions of homogenised mycelium. CMC grown cultures were mainly used in these investigations since the presence of residual BMC particles from BMC grown cultures may tend to adsorb the cellulase enzymes and some cell organelles and thus confuse the assay.

From the results obtained it can be concluded that at 18 h at least some of the CMCase is contained within or bound to membranes. Specific evidence for this is mainly that homogenisation with 0.2% (w/v) Triton X-100 increased the yield and specific activity of CMCase by about four-fold (Table 4.7). When hyphae were sonicated in the presence of Triton X-100 a similar increase was not evident. This difference was possibly due to the greater degree of membrane rupture afforded by the sonication itself, as also indicated by the greater activity released by sonication compared to grinding in the absence of Triton X-100. The addition of Triton X-100 to pellets obtained by differential centrifugation of homogenates also released CMCase activity (Table 4.12). The absence of membrane like structures from these treated fractions, when compared in the electron microscope with fractions not treated with Triton X-100, further implicates the involvement of membranes (Fig. 4.19). Results from similar experiments with Triton X-100 by Hill and Mullins (1979) using Achlya ambisexualis, have also implicated the enzyme as a membrane bound protein.

Finally the histochemical localization procedures employed consistently produced deposits in membrane bound vacuoles within the cytoplasm (Figs. 4.21 and 4.26). Application of this technique to

hyphae of Achlya ambisexualis resulted in localization within vesicles as well as in the cell wall and dictyosomes (Nolan and Bal, 1974). Similar work with Aspergillus terreus, however, only found localization at the surface of the cell wall (Ismailova et al., 1976) although it is possible that this may have been a result of insufficient penetration. Unfortunately the fixative procedure used in this investigation with A. fumigatus produced a poorly resolved cytoplasm with little detail of individual organelles. Further studies using protoplasts to avoid incomplete fixation may be valuable in this respect.

Little CMCase activity was associated with the pellet sedimenting from cell homogenates at 10,000 x g, even after treatment with Triton X-100, and indicates that large organelles contain little of the enzyme or that it is transported through them very rapidly. Alternatively the presence of the enzyme in very delicate organelles such as Golgi may have been masked by their rupture during the homogenisation procedure. Attempts to use protoplasts to circumvent this possibility and isolate these organelles by density gradient centrifugation were, however, unsuccessful due to the low yield from protoplast homogenisations and interference with the CMCase assay by the gradient materials. A considerable amount of activity was, however, found associated with the fraction sedimenting at 300,000 x g from 18 h samples and observation of this fraction in the electron microscope indicated that membrane bound particles were present (Fig. 4.19). Similar investigations by Ismailova et al . (1976) using 24 h A. terreus cultures indicated cellulase activity remained in the supernatant after centrifugation at 180,000 x g. A more extensive investigation of cellulase on bacterial ribosomes has been provided by Carpenter and Barnett (1967) and indicated that membrane bound ribosomes contained

more cellulase activity than free ribosomes. This would imply that cellulase is processed as for many secreted enzymes although more fractionation studies are needed to fully elucidate the pathway.

The amount of CMCase activity in the 300,000 x g pellet from A. fumigatus fractionations compared to that remaining in the supernatant varied markedly with age and growth, a much greater proportion remaining in the supernatant at 24 and 48 h (Table 4.12). This result could imply that an early synthesis occurs rapidly and is followed by subsequent distribution throughout the cytoplasm and cell wall.

Although more detailed experiments such as density gradient separations were not successful with the mycelium homogenates, experiments were conducted to determine the extent of CMCase localization within the cell wall and the periplasmic space. To demonstrate this latter localization the cold osmotic shock procedure of Heppel (1967) was employed as modified for yeast by Patching and Rose (1971). Results obtained on organisms investigated by these authors and from similar experiments conducted on Neurospora (Wiley, 1970) show that greatest release of enzyme occurred in the third buffer wash, ie the cold, hypotonic buffer. With A. fumigatus, however, greatest release was obtained in the preceding, hypertonic buffer (Table 4.8). Recent work by Hill and Mullins (1979) using similar procedures with Achlya ambisexualis, also found that this buffer released cellulase, although of a different type than that released in the final buffer.

It has long been noted that many secreted proteins contain bound carbohydrate moieties (Eylar, 1966 and Bull, 1972) and that these may be important for the process of secretion. Invertase molecules, for example, when secreted from yeast cells contain approximately 30, variously sized chains of mannan which are linked to the protein by

glucosamine residues (Gascon et al., 1968). Similarly chitin has been implicated in the binding of this enzyme in walls of Aspergillus amstelodami (Yabuki, 1966) and the enzymes amylase (Yabuki and Fukui, 1970) and penicillinase (Coles and Gross, 1967) have been found to have specific binding sites in the cell wall. In order to determine whether glucosamine residues are involved in binding CMCase in walls of A. fumigatus the release of CMCase was determined after incubating hyphae with chitinase. Compared to a control incubated in buffer a considerable release of enzyme was apparent (Table 4.10). No increase in CMCase activity was, however, observed during wall digestion with β-glucuronidase, although this may well be due to masking by high inherent levels of activity in the β-glucuronidase itself. CMCase activity decreased continuously throughout the incubation with this enzyme and indicated that inhibition of CMCase may have been occurring. This result would agree with similar experiments conducted during the protoplast investigations (Fig. 3.8) and with the observations of Bull (1970b).

Further evidence that the cell wall is active in restricting release of CMCase was provided by experiments on protoplasts regenerating in CMC medium. Initial experiments indicated that CMCase was produced by protoplasts incubated in this medium but that little remained within the cytoplasm of protoplasts isolated directly from hyphae growing on CMC. Crude fractionation of the protoplasts regenerating on CMC medium indicated that little CMCase was retained by the cytoplasm, a situation quite different to that found in the intact mycelium (Fig. 4.17). More pertinently perhaps is the comparison between the amount of CMCase activity released by regenerating protoplasts and an equivalent amount of intact hyphae (Table 4.18).

Considerably more CMCase activity was found in the medium of protoplast incubations than in the medium of hyphal incubations. This further emphasises the involvement of the cell wall in retaining the enzyme.

Further experiments with regenerating protoplasts produced evidence that the enzyme is synthesised de novo upon contact with the substrate. Firstly, a comparison between the amounts of enzyme produced by protoplasts derived from glucose grown and cellulose grown mycelium indicated that the latter medium did not predispose the protoplasts to a greater enzyme synthesis. Secondly, the addition of 10 mg per litre cycloheximide decreased the synthesis of CMCase in both glucose and CMC derived protoplasts by up to 80% (Table 4.18). Although experiments with cycloheximide are subject to considerable reservations (McMahon, 1975) the use of this inhibitor has been proposed as a tool for the identification of possible repression controls acting on cellulase synthesis at the gene level (Nisizawa et al., 1972). Cycloheximide and other inhibitors have been used to provide similar information on the control of synthesis of chitin synthetase (Ryder and Peberdy,1979). The effect of cycloheximide on A. fumigatus protoplasts and the similarity in CMCase synthesising ability between protoplasts from induced and non-induced mycelium indicates that de novo synthesis is an important aspect of cellulase induction. The identification of the peak time of CMCase synthesis in intact hyphae and the possible involvement of membranes in this indicates this period to be an important time in the production of the enzyme and one at which further investigation may well prove profitable.

Despite many extensive investigations into the factors controlling protoplast release from fungal species, a great deal of individual variation is evident with regard to the efficiency of release and overall yield (Rodriguez Aguirre et al., 1964; Musilkova and Fencl, 1968; Brown, 1971 and Peberdy et al., 1976). The reasons for this variation are as yet undetermined but since many factors affect protoplast release there are a number of possibilities. Owing to this variation, a number of species of fungi routinely used in protoplast studies have been investigated in detail for these factors in order to maximise protoplast yields (de Vries and Wessels, 1972 and Peberdy et al., 1976). These studies have shown that if protoplasts are to be used as the basis for detailed cellular studies it is desirable that the main factors affecting release in the species in question, are determined and that the types and variation of protoplasts produced are investigated.

Two problems currently under investigation in the production and utilization of protoplasts are how to increase the yield of released and purified protoplasts and how to determine and regulate the type and quality of those released. The factors determining the first consideration, that of quantitative yield, are the most commonly investigated in protoplast studies, for example, pH, temperature, enzyme activity and osmotica type and concentration, and much information is now available regarding these parameters in some species (Strunk, 1970 and Peberdy, 1979). However, a full elucidation of the causes for each factor's effect would go a long way towards a more controlled and efficient release of protoplasts and perhaps enable cellular studies to relate more directly to the intact hyphal system in question.

Moreover, the results from studies comparing protoplast release from different species of fungi (Aguirre and Villanueva, 1962; de Vries and Wessels, 1973b and Peberdy et al., 1976) indicate that it would be unwise to extrapolate from one species to another. The parameters for optimal release from A. niger and A. fumigatus were thus investigated for a number of factors. The results obtained from these experiments indicated that digestion of chitin is one of the most important requirements to ensure adequate release. This is supported by a range of biochemical evidence involving the use of chitinase enzymes (Figs. 3.3a, 3.3b, 3.4, 3.5, 3.6 and Tables 3.1 and 3.2). Morphological observations were also made and found to be relevant to this conclusion (Figs. 3.23, 3.24 and 3.25). Further, evidence from samples observed by SEM indicated that the surface wall layers are very readily digested but protoplasts are only released when the inner layers are ruptured (Fig. 3.34). A similar conclusion has also been made for giant cells of A. niger (Dr B Davis, personal communication) and the presence of chitin as an inner wall layer in many fungi (Bartnicki-Garcia, 1968; Hunsley and Burnett, 1970 and Burnett, 1979) implicates this polymer as an important factor in controlling protoplast release.

A further consequence of this is that chitin may be responsible for restraining the cytoplasm and providing the hyphal shape (Katz and Rosenburger, 1970 and Trinci, 1978). Observations on the chitin distribution between yeast and mycelial forms of the dimorphic fungus Paracoccidioides brasiliensis have provided support for this theory (Kanetsuna et al., 1969). Regeneration studies on T. viride protoplasts have further provided evidence that a lack of chitin in fungal cell walls may be responsible for the aberrant hyphae initially

formed from these protoplasts (Benitez et al., 1975). Normal hyphae which later develop do, however, contain chitin. To more fully substantiate the chitin microfibrillar layer as being the main restraint on protoplast release in Aspergilli it would be desirable to investigate the same features of protoplast release on a species with microfibrils of a different polymer, for example, cellulose, and determine whether this polymer was instrumental in controlling release. Similarly, it would be interesting to compare the ease of protoplast release from a chitin - deficient mutant of an Aspergillus species.

This possible role for chitin in Aspergillus cell walls has a number of practical implications with regard to protoplast release, both useful and problematical. Firstly, in order to release as many protoplasts as soon as possible, those parameters relating to the digestion of the restraining wall layer(s), in this case chitin, should be optimised and appropriate measures taken to regulate pH, temperature and other factors such as osmoticum. The observation that individual enzymes are ineffective in ensuring release, (Table 3.3), indicates that combinations are necessary, if only to allow one key enzyme access to its wall polymer substrate. While producing the most efficient release of protoplasts, however, this approach does result in the less desirable presence of undigested fragments of cell wall. These may not only cause complications in purification procedures through clogging filters, but also carry the potential for causing physical damage to protoplasts during manipulations. Similarly, a rapid release may also involve a greater danger of damage to the protoplasts from the wall layers through which the protoplast extrudes. SEM observations on protoplast integrity during release (Figs. 3.33 and 3.34) did not indicate, however, that this occurred

for A. niger and A. fumigatus, although this method of assessment is by no means the most comprehensive.

The alternative procedure to a rapid and incomplete wall digestion is to allow the incubation to proceed until all the wall layers are fully digested leaving only protoplasts. This has been previously reported for protoplasts of Fusarium culmorum (Villanueva and Garcia Acha, 1972), giant cells of A. niger (Davis et al., 1977) and also with Schizophyllum commune (de Vries and Wessels, 1972). However, with this mode of digestion residual wall layers may still remain undetected except by electron microscopy (Darling et al, 1969). Resistant wall residues have also been noted to remain on protoplasts of Rhizopus nigricans after release (Gabriel, 1968). A further factor relevant to this is the observation that some lytic enzymes are rapidly inactivated during the digestive process (Fig. 3.8) and Bull (1970b) has noted that melanin is often influential in inhibiting lytic enzymes. The results presented in Table 3.2 agree with this and it is possible that in some species or under some conditions of growth a full digestion of the wall would be extremely difficult.

Although total wall digestion yields a greater number of protoplasts, it is not commonly regarded as the preferred technique because prolonged incubation in the lytic medium may have detrimental effects on the protoplasts obtained (de Vries and Wessels, 1973b). Further, some protoplasts begin to regenerate in the lytic medium (Gabriel, 1968) and others, such as A. niger giant cell protoplasts, become metabolically inert or are killed (Dr B Davis, personal communication).

Owing to these considerations it was decided to maximise the speed of digestion to release A. niger and A. fumigatus protoplasts

in the shortest possible time. Electron microscope observations of the protoplasts were conducted to determine whether any physical damage occurred or wall layers adhered. The results obtained indicated that most protoplasts were morphologically intact and free from cell wall debris (Fig. 3.30). Whether the lack of debris was due to the rapid release or to some other factor such as electrokinetic repulsion was not determined.

Although an assessment of wall polymers is important in investigations of protoplast release, other factors need to be considered, for example procedures for determining the quantitative amount of the protoplast cytoplasm released. The maximisation of protoplast yield has been studied in a number of systems mainly by haemocytometer counts of the protoplasts released in response to the treatment provided (Musilkova and Fencl, 1968; de Vries and Wessels, 1972 and Peberdy et al., 1976). While protoplast numbers are in part related to cytoplasmic biomass there are further aspects which may modify this relationship. Firstly,the size and cytoplasmic density of the protoplasts are of obvious importance since a large number of very vacuolated protoplasts may contain in total relatively little active cytoplasm compared to a smaller number of dense protoplasts having a high cytoplasmic content. Therefore,sheer numbers do not necessarily provide an accurate indication of cytoplasmic yield. Moreover,since protoplasts vary in size according to the concentration of osmoticum employed (Table 3.15) it is difficult to obtain an accurate assessment of percentage conversion of hyphal cytoplasm to protoplast by simple counting procedures. Little work has been done on other assessments of yield, but investigations on biochemical parameters may provide more valid results in this respect. For

example DNA determinations have indicated that yields of protoplasts may represent only 18% of the cytoplasm of Penicillium and Cephalosporium species (Fawcett et al., 1973) and Isaac et al. (1978) have shown that protein measurements indicate only a 16% yield from A. nidulans.

A consideration of protoplast quality is also relevant since a dichotomy in protoplast type has frequently been noted in protoplast systems (de Vries and Wessels, 1972 and Peberdy, 1979). In particular, populations of small dense protoplasts and large, vacuolated protoplasts have been observed and separated on a density basis (de Vries and Wessels, 1972) and these have been noted to possess different biochemical properties (Isaac et al., 1978). Attempts were madeto isolate populations of protoplasts from A. fumigatus by layering protoplast suspensions on density gradients. However, these were unsuccessful due to aggregations of wall fragments disrupting any protoplast banding that occurred. Morphological differences were nevertheless noted in the protoplasts released (Figs. 3.19 and 3.20) and some evidence is presented for a difference in the release mechanism of tip and distal protoplasts. Other workers have suggested that different protoplast populations may correlate to different hyphal origins (Buckley, 1973 and Peberdy, 1979) and further investigation of this problem is desirable.

The use of protoplasts in the study of cellulase synthesis in A. fumigatus was initially disappointing since insignificant levels of activitywere found within protoplasts after release and isolation. This consequently prevented protoplasts providing a valuable comparison to cruder mycelial homogenisation procedures but does illustrate the potential difficulty afforded by any attempt to use

protoplasts for immediate fractionation studies. Such a result may be due to cellular shock and changes during the lytic incubation or simply leakage and loss of cytoplasm. A similar paucity of cellulase in Pseudomonas protoplasts has also been noted by Yamane et al. (1971).

This problem has been overcome in this study on A. fumigatus by investigating CMCase activities during the regeneration of protoplasts on CMC medium. Perhaps the most pertinent observation from these studies is that considerably more CMCase activity was evident in the medium of the regenerating protoplasts than in the medium of an equivalent amount of mycelium (Table 4.17). This result complements others obtained with enzyme digestion and cold osmotic shock procedures on intact hyphae from cellulose grown cultures, which implied some retention of CMCase by the cell wall. Results of similar osmotic shock experiments on Pseudomonas species (Yamane et al., 1971 and Ramasamy and Verachtert, 1980) and on Cellvibrio fulvus (Berg, 1975) have also implicated the periplasmic space in the localization of cellulase. However, information on fungal species is not available at present.

The localization of cellulase within the cell wall of microorganisms is supported by a number of studies on sphaeroplast formation with 80% of the enzyme being released by this procedure in Pseudomonas species (Suzuki et al., 1969). Periplasmic localization in this study only accounted for 20% of the enzyme and the possibility of specific adsorption or bonding to wall polymers should also be considered. Evidence that enzymes do attach to the cell wall has been provided for a number of enzymes, for example glucoamylase in Neurospora crassa (Gratzner, 1972), and a number of general models have been proposed to explain the involvement of the cell wall in the secretion of enzymes

have been implicated in the wall binding of some enzymes, for example chitin and glucosamine residues have been shown to be involved in the wall binding of invertases (Yabuki, 1966 and Gascon et al., 1968). Furthermore, glucosamine residues have been found attached to bacterial cellulase (Yamane, 1970b).

The results of chitinase digestion of A. fumigatus cell walls indicates that in this fungus CMCase is released by the action of chitinase enzymes (Table 4.10) although whether this is due to digestion of the chitin polymer itself or removal of subsidiary residues from the CMCase enzyme was not determined. Studies on some extracellular enzymes, for example, invertase (Islam and Lampen, 1962; Trevitheck and Metzenberg, 1964 and Andres and Peberdy, 1974) and cellulase in bacteria (Suzuki et al., 1969 and Berg, 1975) have benefited from digesting the cell walls so as to release protoplasts and any enzyme held within these and in the periplasmic space. Unfortunately, this procedure was inapplicable to similar study of A. fumigatus since the lytic enzyme used possessed too high an inherent activity towards the cellulose used as substrate for the assay and thus masked any slight release during digestion. Also the possibility of inhibition of CMCase by factors released during digestion was indicated (Table 4.9). These results are probably analogous to those noted in Table 3.2 concerning the activity of the lytic enzymes after a period of incubation.

Additional evidence for the localization of CMCase activity in the cell walls was provided by histochemical staining of the tissue specifically for the enzyme and subsequent observation in the electron microscope. By this method activity was observed within and at the surface of the cell wall (Figs 4.20 and 4.26). Similar localizations

of cellulase by this technique have also been reported for Achlya ambisexualis (Nolan and Bal, 1974) and for Aspergillus terreus (Ismailova et al., 1976). The localization of CMCase within the cytoplasm of A. fumigatus was not as detailed as that for Achlya ambisexualis (Nolan and Bal, 1974) but did indicate presence of the enzyme within large vacuoles (Figs. 4.21 and 4.25).

The presence of a surface wall sheath (Fig. 4.10) and an external amorphous layer to the wall when growing on BMC (Fig. 4.28) provides some indication of the organism's physiological response to the presence of a solid substrate. Similar surface structures have also been observed on Colletotrichum (Akai et al., 1967), Pyrenochaeta (Hess, 1969) and Helminthosporium (Wheeler and Gantz, 1979), and in bacteria the ability to degrade cellulose fibres has been correlated with the presence of an extracellular slime layer (Berg et al., 1972b). Experiments were not conducted to determine whether the outer layer noted in A. fumigatus contained CMCase or whether it was particularly adhesive to cellulose particles. However, the low level of staining observed in this layer in histochemical preparations (Fig. 4.28) and the close attachment of cellulose particles to the hyphae (Fig 4.11) would argue for the latter possibility.

The investigation of cellulase localization within fungal cell walls is pertinent to the industrial consideration of the enzyme since retention within the wall could decrease the enzyme yield from fungal cultures. In this respect it is relevant to note that considerable differences have been found between the levels of extracellular and cellular cellulase depending on the substrate employed. The presence of solid cellulose has been noted to strikingly enhance levels of extracellular cellulase (Yamane et al., 1970a; Suzuki et al., 1969 and

Berg, 1975) and this may result from the solid substrate being unable to penetrate the cell wall and thus requiring the enzyme to be secreted. Alternatively, it was found in these studies that soluble cellulose substrates such as cellobiose induced high cellular levels of enzyme but low extracellular levels. Presumably these substrates could be taken into the cell or cell wall prior to digestion. In A. fumigatus a similar difference was noted with solid BMC inducing a rapid rise in extracellular CMCase activity (Fig. 4.15), while with CMC extracellular activity was only significant at later stages of growth and probably was a result of autolysis rather than specific secretion (Fig. 4.16). The further observation that the increase in enzyme activity with BMC may be due to an increase in specific activity of the enzyme rather than a greater synthesis of the enzyme does, however, indicate the possible release of a specific co-factor, for example, a protease as suggested by Nakayama (1975).

The implications of these observations are that in order to attain the greatest culture filtrate yields of cellulase the organism(s) should be grown on an insoluble substrate. However, the use of insoluble substrates does introduce the possibility of adsorption of the enzyme onto the substrate and to avoid this the use of inducers such as lactose has proved successful (Ryu et al., 1979). In order to further increase yields it would be relevant to isolate the mycelium grown for cellulase recovery under conditions which impose an osmotic shock on the organism and thus induce further release from the cells.

Another treatment which may be relevant in this context is that of detergents such as Triton X-100. This has been shown to enhance release of β-1,3 glucanase from cell walls of Achlya ambisexualis (Hill and Mullins, 1979) and this implies localization of the enzyme in the

wall in an active form. A positive effect on cellulase release has also been noted from the inclusions of detergents in the culture medium (Reese and Maguire, 1969 and Sternberg, 1976) and experiments conducted with A. fumigatus (Table 4.7) indicated that release from this organism was similarly affected by Triton X-100. Finally, the presence of low Mg^{2+} levels in cellulose media has also induced release of periplasmic enzymes (Nev and Chou, 1967) and the relevance of ionic effects on cell wall permeability and the secretion of extracellular proteins may prove a valuable topic for further investigation as noted previously with the effects of Ca^{2+} on protoplast release.

Cellulase loss due to the retention properties of the cell may also be reduced by using protoplasts directly upon the substrate. In this case very little enzyme would remain in the cell (Tables 4.15 and 4.16). Unfortunately, the tendency for protoplasts to regenerate under such conditions may mitigate against such a system being used until the control of regeneration processes are better understood.

Other information obtained, which may concern the production of cellulase, was that of shortening the lag time before cellulase synthesis was maximal. The addition of sugars to cellulose cultures produced a considerable decrease in this lag time and agrees with other results obtained on supplementation with various nutrients (Lee and Blackburn, 1975; Sternberg, 1976 and Trivedi and Rao, 1980). Since commercial cellulase production is critically dependent upon the time period of incubation before the enzyme becomes recoverable any means of decreasing this lag period has obvious advantages. With T. viride growth on unsupplemented BMC medium, 80% of the substrate was hydrolysed by 45 h growth although only 10% of the cellulase enzymes had been secreted (Mandels and Andreotti, 1978). Moreover, maximum production

of cellulase by T. viride in shake flask culture has been reported to take 13 days (Mandels 1975).

The value of glucose addition to cellulose cultures has however been questioned in view of the strong repressive effect of the sugar on cellulase production (Sternberg, 1976). A number of studies have shown that glucose addition supresses cellulase synthesis (Lobanok and Pavlovskaya,1977; Mandels and Andreotti, 1978 and Trivedi and Rao, 1980) and it has been proposed that this is due to catabolite repression (Nisizawa et al., 1971). Other reports have,however,indicated that while this may be so at high concentrations,low concentrations of less than 5.7 mM actually stimulates cellulase production Peitersen,(1975) and Chahal et al. (1977) have noted that concentrations as high as 8 mM do not affect cellulase levels. BeMiller et al. (1969) further noted that although cellulases may be produced in the presence of glucose, they are not released into the medium, an observation which would agree with the results relating to substrate influence on cellulase secretion (Yamane et al., 1970a; Suzuki et al., 1969 and Berg, 1975). In this respect it is relevant that most investigations on cellulase synthesis only measure activity in culture filtrates and not in the mycelium.

Little CMCase activity was detectable in A. fumigatus cultures supplemented with glucose until the sugar had been utilised. By this time, 12 h, a sizeable growth of mycelium was evident and available for enzyme production. Some comparison experiments with different sugars indicated that glucose actually increased the specific activity of A. fumigatus cellulase (Table 4.5). One possible explanation for this is that the sugar may have been contaminated with the inducer sorphorose as noted by Mandels et al., (1962). Other supplementation

experiments, however, (Table 4.6) did not show this effect. In a commercial situation the addition of glucose, as here, may not be acceptable due to high costs and it would be pertinent to investigate whether there are any alternative waste products which contain suitable sugars for this purpose.

Despite numerous investigations into the synthesis and properties of secreted cellulase enzymes little work has been conducted on the details of synthesis or the intracellular localization of the enzyme, particularly in fungi. However, since extracellular yields ultimately depend upon cellular synthesis a full understanding of these may well be vital to increase a final yield. Studies on A. terreus have shown that cellulase is preferentially localized in the supernatant fraction of cell homogenates after differential centrifugation (Ismailova et al., 1976). More detailed fractionations by Carpenter and Barnett (1967) have found that membrane bound ribosomes contain 7% of cellular cellulase but only 0.02% of the cellular β-glucosidase.

Similar results have also been found for plant cellulase (Koehler et al., 1976) and this would relate well to the observation that cellulase is generally a secreted enzyme while β-glucosidase remains intracellular. The involvement of cellulase synthesis with rough endoplasmic reticulum agrees also with current views on the role of this organelle in preparing enzymes for secretion (Shore and Tata, 1977).

In fractionation studies of A. fumigatus, some CMCase activity was sedimentable at 300,000 x g and electron microscopical evidence was obtained that this was membrane bound (Fig. 4.19). However this sedimentation of CMCase activity was only found at early stages of growth on cellulase while later samples showed much reduced activity

but with a concomitant increase in supernatant activity. Attempts to localize the enzyme in other organelles were unsuccessful although histochemical observations did indicate some activity in membrane bodies in the cytoplasm (Figs. 4.21 and 4.26). Application of this technique to Achlya ambisexualis has localized the enzyme in dictyosomes and cell vesicles (Nolan and Bal, 1974) and this has also been indicated by freeze etching studies (Mullins and Ellis, 1974). Further observations by Hill and Mullins (1979) on the effects of Triton X-100 in Achlya ambisexualis have indicated that the enzyme is an integral membrane protein.

A final observation on the synthesis of CMCase in A. fumigatus is that, at least under conditions of protoplast regeneration, the enzyme requires newly synthesised protein, and most probably is newly synthesised itself. This conclusion is based on both inhibitor effects and a comparison between the synthetic abilities of protoplasts from glucose and cellulose grown mycelia. If the enzymes were coded for by long lasting mRNA then cycloheximide should have produced no inhibition in synthesis. Although an inhibition was noted with cycloheximide, it has been pointed out that due to possible subsidiary effects of such inhibitors the only acceptable result from such studies is the lack of effect of the inhibitor (McMahon, 1975). Supporting evidence for the reality of the observed inhibition was, however, provided by the observation that protoplasts from both glucose and cellulose grown mycelia produced equal activities of CMCase upon incubation with CMC (Tables 4.18 and 4.19). This indicated that the latter mycelium was not predisposed towards CMCase synthesis. The observations by Shockman and Lampen (1962) that protoplasts are 4 times more sensitive to cycloheximide further emphasises the potential of protoplasts in

cellular studies.

Referring back to the introductory discussion that cellulase synthesis is one of the most cost-limiting aspects of cellulose digestion and conversion,it is evident that these latter observations on the localization of the enzyme are particularly pertinent for determining the basis for maximising yields. Since the initial synthesis is the ultimate determining factor for subsequent extractions,information on this process is of prime importance. Further information which is required to this end is of a molecular genetic nature, for example,to determine what components can activate the cellulase genes and how inducers of cellulase relate to these. More practical considerations would particularly be related to manipulative possibilities such as whether a cell-free-synthesising-system could be constructed for cellulase. With regard to current practices it will also be important to determine whether the appropriate genes may be cloned and combined so as to allow a much greater productivity. The use of protoplasts could considerably simplify such procedures. In this context it would also be relevant to determine whether thermophilic organisms possess any particular genetic modifications which enhance cellulase synthesis or affect their rate of growth on cellulose substrates. The inclusion of these in a genetic engineering programme could be of considerable value.

The present lack of information on the cellular synthesis of the enzyme would in these cases need to be advanced for such possibilities to be pursued and the use of protoplasts in these studies would appear to be justified by the results obtained with A. fumigatus.

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Natural cellulose is an insoluble polymer with regularly repeating glucose subunits (Cowling, 1975). The ability of these subunits to form hydrogen bonds between adjacent chains results in the polymer being highly microcrystalline and consequently with a great resistance to enzymatic hydrolysis. In order to overcome this resistance micro-organisms produce a range of cellulase enzymes which both digest different parts of the cellulose polymer and act synergistically to ensure digestion of the most crystalline regions. The full details of how these enzymes interact is still under debate at present (Wood and McCrae, 1977 and Ghose and Bisaria, 1979).

Initially theories proposed that a 'C_1' enzyme is responsible for opening the crystalline regions of cellulose by a specific hydrolysis or by inducing fibre swelling (Reese et al., 1950). Thereafter more general acting 'C_x' cellulase enzymes would be able to hydrolyse the exposed chain ends. More recently the identification of C_1 enzymes with exo β-1,4-glucanases, preferentially able to remove cellobiose units from the non - reducing ends of cellulose chains, has been confirmed (Wood, 1975; Berghem and Pettersson, 1973 and Eriksen and Goksøyr, 1977). From this information the synergistic requirement for both types of enzyme to hydrolyse crystalline areas has become more understandable, since, in the absence of exoglucanases to extend the initial hydrolysis created by the endoglucanases the break would rapidly close through the attraction of the chains reforming hydrogen bonds.

This multiplicity of cellulase enzymes is further extended by the presence of subsidiary structural and protective polymers such as xylose and lignin against which hydrolytic enzymes may also be produced (Kirk, 1975, and Ghose and Bisaria, 1979). It is thus not surprising that culture filtrates may contain a wide range of enzymes with

varying degrees of activity towards cellulose and that procedures to assay these activities may suffer considerable problems due to lack of specificity. Similarly the range of cellulose substrates available will affect which aspect of cellulose hydrolysis is being determined.

These problems may be overcome by fully purifying the enzyme using gel filtration procedures as detailed in numerous reports (Eriksen and Goksøyr, 1977; Wood and McCrae, 1977 and Ghose and Bisaria, 1979). However, while providing valuable information on the range and properties of the cellulase enzymes present this approach is obviously time consuming and inapplicable to small volumes of material such as cell fractions. The selective use of different substrates may, however, be sufficiently discriminating, particularly for the major classes of cellulase enzymes. Thus the release of reducing groups from insoluble cellulose has been employed to assay for the complete range of cellulase enzymes since these will all be needed to digest the insoluble substrate. Alternatively o-nitrophenyl-β-D-glucopyranoside is a specific substrate used to assay β-glucosidase.

As well as identification of hydrolysis products the physical condition of the cellulose substrate may provide a good assessment of activity. Assays using physical parameters of cellulose range from measurement of the tensile strength of cotton or filter paper (Mills, 1973, Latter and Howson, 1977) to measurement of the viscosity of a soluble cellulose solution (Joos et al., 1969). Recent assays have been developed as modifications to these basic procedures and include the use of 3,4-dinitrophenyl glycosides as specific substrates for some cellulase reactions (Capon and Thomson, 1979), the coupling of glucose liberation by cellulases to oxygen consumption to allow a

polarographic assay (Green et al., 1977) and the binding of a dye to the substrate to permit a simple colorimetric assay upon hydrolysis (Poincelot and Day, 1972 and Leisola and Linko, 1977).

The clearance of opaque cellulose agar has also been used as an indication of cellulase activity (Walsh and Stewart, 1969) and has been further developed towards providing a quantitative estimation (Stranks and Bieniada, 1971). However, although these methods are a more direct assessment of the cellulolytic ability of an organism the degradation of the substrate does not necessarily relate to the synthesis of cellulase enzymes (Rautela and Cowling, 1966 and Wabnegg et al., 1978).

Of the methods currently used to assay cellulase activity the release of reducing sugars from filter paper strips is the most informative for estimating total cellulase activity (Mandels et al., 1976) while the reduction in viscosity of a CMC solution is the most sensitive to detect endoglucanase activity (Whitaker, 1971). A combination of reducing sugar release and viscosity reduction has also been used successfully to separate the activities of endo- and exoglucanases (Eriksen and Goksøyr, 1977). However in using these methods the applicability of the samples must be considered, for example, the use of solid substrates will involve the possibility of enzyme and even organelle adsorption and this may be a particularly important restriction with low concentrations of enzymes. Moreover, solid substrate reactions generally require relatively large volumes of enzyme. Alternatively the use of viscometry, while sensitive to low concentrations of enzyme, is a more selective procedure for exoglucanases and would tend to exclude the influence of other cellulases. Also the exact hydrolysis measured by this method is still under discussion (Whitaker, 1971). From the practical point of view this assay is also time

consuming since each assay must be performed individually and to be accurate requires a relatively long procedure. This and the effect of viscous materials such as density gradient media makes it unsuitable for many cellular and kinetic studies, particularly ones of a routine nature with many samples. Even an automated procedure recently devised for cellulase determinations by this technique is only able to conduct 20 determinations per hour (Bartels et al., 1979).

The release of reducing sugars from CMC solutions, however, is rapidly assessed and carries no danger of sequestration of the enzyme by adsorption. Further, both endo- and exoglucanases will be represented in the determinations. Consequently this procedure was used to assay cellulase activity from A. fumigatus culture filtrates and cell fractions according to the details outlined in Materials and Methods.

Ideally a more expansive investigation into the types of cellulase present in different cell fractions would be desirable and other types of cellulase assay would be relevant to this. Detailed examination of A. fumigatus cellulases were not conducted in these investigations although for some experiments culture filtrates were subjected to ammonium sulphate precipitation to partially purify the cellulase enzymes. An attempt was, however, made to determine the number of cellulase enzymes present by the polyacrylamide gel electrophoresis technique of Goren and Huberman (1976). Results from these experiments indicated that purified cellulase from A. fumigatus contained at least three separate enzymes (Fig. A.1).

Since a number of different buffers were used in CMCase determinations the effect of a number of these on CMCase activity was assessed by incubating equal amounts of the purified enzyme in each buffer with CMC and comparing the reducing sugar levels released. The results

indicated that little difference in activity was evident between the buffers although EDTA did seem to have a slight lowering effect. (Table A.1). The effect of a number of density gradient media was also investigated and none were found which did not seriously affect the assay method used. Sucrose for instance produced an excessively high background absorbance while Percoll and Metrizamide interfered with the development procedure to produce precipitates or high backgrounds, the latter possibly due to Metrizamide being a derivative of glucose. These results seriously limited the application of density gradient procedures to the localization of A. fumigatus CMCase.

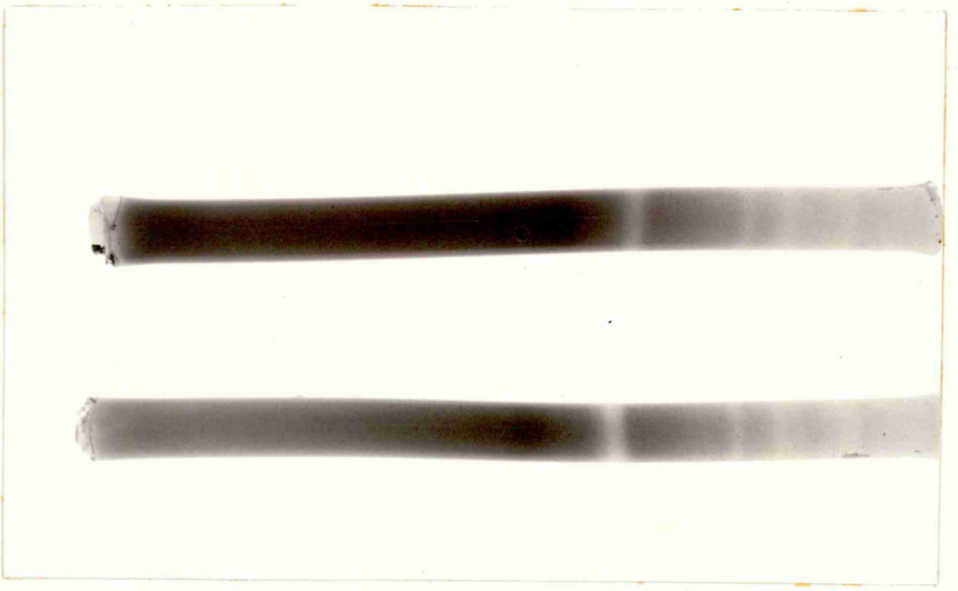

FIGURE A.1. Polyacrylamide gelelectrophoresis of cellulase enzymes. Gels were prepared and run as described in Materials and Methods. Cellulase enzymes were purified from A. fumigatus culture filtrates as described in Materials and Methods.

TABLE. A.1.

Buffer Additive	Units CMCase
Control	1.42 ± 0.07
0.2% Triton X-100	1.30 ± 0.05
0.005 M EDTA	1.28 ± 0.11
0.2% Triton X-100 + 0.005 M EDTA	1.50 ± 0.08
0.8 M Mannitol + 0.02 M EDTA	1.38 ± 0.05

Effect of buffer additives on CMCase activity. Control buffer; 0.1 M sodium acetate, pH 5.0. Purified CMCase, from A. fumigatus culture filtrates, was used in all incubations as described in Appendix.