Cuticle-degrading enzymes of entomopathogenic fungi.

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Award date:
1985

Awarding institution:
University of Bath

CUTICLE-DEGRADING ENZYMES OF

ENTOMOPATHOGENIC FUNGI

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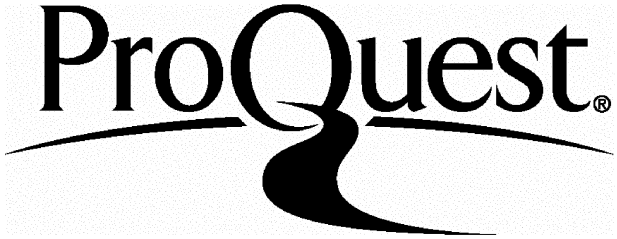
for the degree of Ph.D.

of the University of Bath

1985



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ABSTRACT

A study on cuticle-degrading enzymes (CDE) of three hyphomycete entomopathogens has produced information on enzyme types, levels, characteristics, mode of action, regulation, sequence of production, cellular localisation and production during host penetration. This is the first critical work on CDE of any entomopathogen.

Several pathogenic isolates of *Metarhizium anisopliae, Beauveria bassiana* and *Verticillium lecanii* when grown in buffered liquid cultures containing comminuted locust cuticle as sole carbon source (good growth occurred on most monomeric and polymeric cuticular constituents), produced a variety of extracellular and bound enzymes corresponding to the major components of insect cuticle e.g. 3 endo-proteases, aminopeptidase, carboxypeptidase A, lipase, esterase, chitinase and N-acetylglucosaminidase. Considerable variations occurred in levels of production between spp. and even within a sp., but endo-proteases were exceptional in being produced in large amounts by all the isolates. CDE were produced rapidly and sequentially in culture. The first activities to appear (< 24 h) were those of the proteolytic complex, chitinases were always produced substantially later.

Properties of CDE were investigated in terms of pH and tem-erature optima, substrate specificity, molecular weight, iso-electric point, mechanism of substrate degradation and the effect of specific inhibitors.

Studies with culture filtrates and purified CDE revealed that substrates in intact cuticles are amenable to degradation but the prior action of protease is necessary for significant degradation of the chitin. Staining of chitin by a fluorescent lectin (FITC-WGA) and calcofluor only in cuticles from which protein has been removed (by protease or KOH) also suggests initial masking of chitin. This and determination of amino acid composition of peptides solubilised by endo-protease revealed the potential of CDE in studying the physicochemical structure of insect cuticles.

The apparently localised action of CDE during host penetration may result from molecular sieving, binding to fungal walls or binding to cuticle. The first possibility is lessened by the small size of the endo-enzymes (< 34 K daltons) which could allow diffusion *via* the various canals which traverse cuticle. However, cuticle effectively binds (ionically) CDE, and also activities of several CDE remain partly bound in various ways to hyphae and conidia (by ionic binding to walls, by disulphide bonds, and on or within membrane structures).

The involvement of proteolytic enzymes in infection was suggested by their presence in conidia, penetration structures, and infected cuticle (detected histochemically and following extraction from cuticles). Also the constitutive production of endo- and exo-proteases lends weight to their possible significance in parasitism as synthesis will be subject only to catabolite repression. Chitinase is induced by N-acetylglucosamine and was not detected in infected cuticle.

Possible mechanisms and significance of enzymic degradation of cuticle during infection are discussed, particularly in comparison to host penetration by phytopathogenic fungi.

ABBREVIATIONS

BAPNA	N-benzoyl-L-arginine *p*-nitroanilide
BGGAMN	N-benzoyl-diglycyl-arginine-4-methoxy-β-naphthylamide
BGGAN	N-benzoyl-diglycyl-arginine-β-naphthylamide
BSA	bovine serum albumin
BTEE	N-benzoyl-L-tyrosine ethyl ester
BTPNA	N-benzoyl-L-tyrosine-p-nitroanilide
c.	circa
CDE	cuticle-degrading enzyme(s)
CR	catabolite repression
CTAB	cetytrimethyl ammonium bromide
CWDE	cell wall-degrading enzyme(s)
d	day
diam.	diameter
DNA'ase	deoxyribonuclease
FITC-WGA	Fluorescein isothiocyanate conjugated wheat germ agglutinin
g	gram
h	hour
K	potassium
k_{av}	diffusion coefficient
M	Moles or Molar
M.W.	Molecular weight
min	minute(s)
βNA	β-naphthylamide
NAG	N-acetylglucosamine
NAG'ase	N-acetyl-β-D-glucosaminidase
OD	optical density
pI	iso-electric point
ppm	parts per million

RH	relative humidity
RNA'ase	ribonuclease
R_s	Stokes radius
RT	room temperature
SDA	Sabouraud dextrose agar
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TAME	p-toluene sulfonyl-L-arginine methyl ester
T_{80}	Tween 80
UV	ultraviolet
Ve	protein elution volume
Vo	void volume
Vol	volume
Vt	total volume
Wt	weight

INTRODUCTION

Non-pathogenic fungi and avirulent strains of entomopathogenic fungi are frequently characterised by a reduced ability to penetrate cuticle of insect hosts (e.g. Robinson, 1966; Al-Aidrous and Roberts, 1978). Consequently an understanding of the mechanisms involved could help in the production of strains of entomopathogenic fungi with increased virulence and presumably with an improved potential for pest control. Recently ultrastructural evidence has accumulated that is consistent with an involvement of cuticle-degrading enzymes in the penetration process (e.g. Zacharuk, 1970 b,c). Therefore CDE may be determinants of pathogenicity (e.g. Pavlyushin, 1978). Nevertheless, studies conducted so far have provided only fragmentary information on enzyme types, characteristics, modes of action, regulation, cellular localisation, sequence and levels of production. In addition, little work has been attempted on the production of enzymes *in vivo*, and no information is available on the degradation of isolated cuticle by purified enzymes from entomopathogens.

The main objective of the work reported below was to provide critical information on the characteristics, synthesis and regulation of cuticle-degrading enzymes. This would then facilitate an evaluation of the role of these enzymes in pathogenesis.

The muscardine fungi, *Metarhizium anisopliae*, *Beauveria*

bassiana and *Verticillium lecanii* were investigated in view of their potential as pest control agents, the ultrastructural evidence implicating the enzymes of *M. anisopliae* in penetration, and because the systems seemed amenable to study with techniques already successfully exploited with phytopathogens.

LITERATURE REVIEW

Principal target insect groups

Although it has long been realised that epidemics of fungal diseases occur naturally in insect populations, the exploitation of fungi for pest control has lagged behind that of other disease-causing organisms. However there has been a renewed interest in entomopathogenic fungi in the last 20 years, the result of which is an increasing number of reports of the successful control of insect pests by fungal pathogens (Ferron, 1978). To date the principal target insect groups have been Culicidae (Mosquitoes), Aphidae (aphids), Delphacidae (plant hoppers), Cicadellidae (leaf hoppers), Cercopidae (spittle bugs), Aleyrodidae (whiteflies), Coccoidea (scales), Thysanoptera (thrips), Coleoptera (beetles) and Lepidoptera (caterpillars) (Roberts and Humber, 1984). Unlike those insect-pathogenic micro-organisms which must be ingested to initiate disease (viz. Viruses, bacteria, and Protozoa), entomopathogenic fungi normally invade through the host's external cuticle. Fungi, therefore can infect insect pests which have sucking mouthparts (e.g. Homoptera) even though such insects cannot ingest entompathogens applied to host plants. Fungi are also the pathogens of choice for control of bettle pests as effective viral, bacterial and protozoan alternatives are unknown for most Coleoptera (Roberts and Humber, 1984).

Every major fungal taxonomic groups except the higher basidiomycetes and dematiaceous Hyphomycetes has entomopathogenic

members. Approximately 90 fungal genera and more than 700 species are involved; although to date only 15 are being developed for pest control (Roberts and Humber, 1984). Of these most attention has been focussed on 3 species with broad geographical and host ranges, the hyphomycete fungi, *Beauveria bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii*.

B. bassiana causes white muscardine disease in a wide range of insects including members of the Heteroptera, Homoptera, Lepidoptera (e.g. *Pyrausta nubilalis)*, Coleoptera (e.g. *Scolytus scolytus, S. multistriatus* and *Limonius* spp.). Hymenoptera and Diptera (e.g. *Culex* spp. and *Anopheles* spp.) (Bell, 1974).

M. anisopliae, the cause of green muscardine disease has many hosts in common with *B. bassiana*. It has been reported to infect over 100 different species belonging to a variety of insect orders (Boucias and Pendland, 1984), including Coleoptera (e.g.*Elateridae*; McCauley, Zacharuk and Tinline, 1968), Lepidoptera (e.g. *P. nubilalis* and *Bombyx mori*; Bell, 1974) and Diptera (*Culex* spp. and *Lonchaea palposa;* Leatherdale, 1970). Within the Orthoptera, *M. anisopliae* has been shown to infect *Schistocerca gregaria* (Veen, 1968). Chapman (1976) comments that very occasionally, gregarious locust populations may be almost totally destroyed by fungal epidemics. The development of these epidemics depends on appropriate conditions of moisture and temperature, but once established, they sweep rapidly through the population, which is highly susceptible

because of its gregarious nature.

V. lecanii infects several species within the Homoptera including representatives from the superfamilies Aleurodidae (whitefly), aphidoidia (aphids), and Coccoidea (whitefly). (Hall, 1976). *V. lecanii* has also been found on other orders of insects including Coleoptera (e.g. *S. scolytus;* Barson, 1976).

Route of entry into host

Infection by hyphomycete fungi is effected by conidia and there are four possible routes of entry; directly through the outer integument, via the digestive tract, through other natural openings such as pore canals, or through wounds. Successful artificial infection can be produced by introducing spores into fresh wounds but the natural importance of this route seems to be low(Madelin, 1963).Similarly, except for *Culicinomyces,* infection through the digestive tract does not normally occur (Roberts and Humber, 1984). Although there is little evidence to suggest penetration through pore canals (David, 1967) there are reports of infection via sense organs and tracheae (McCauley *et al.*, 1968; Hedland and Pass, 1968). It is clear however that the major route of entry for most entomopathogenic fungi including *B. bassiana, M. anisopliae* and *V. lecanii* is directly through the external cuticle (Schabel, 1978; McCauley *et al.*, 1968; Veen, 1966; Pekrul and Grula, 1979). The composition and structure of the cuticular barrier is therefore an important consideration for any study on their mode of

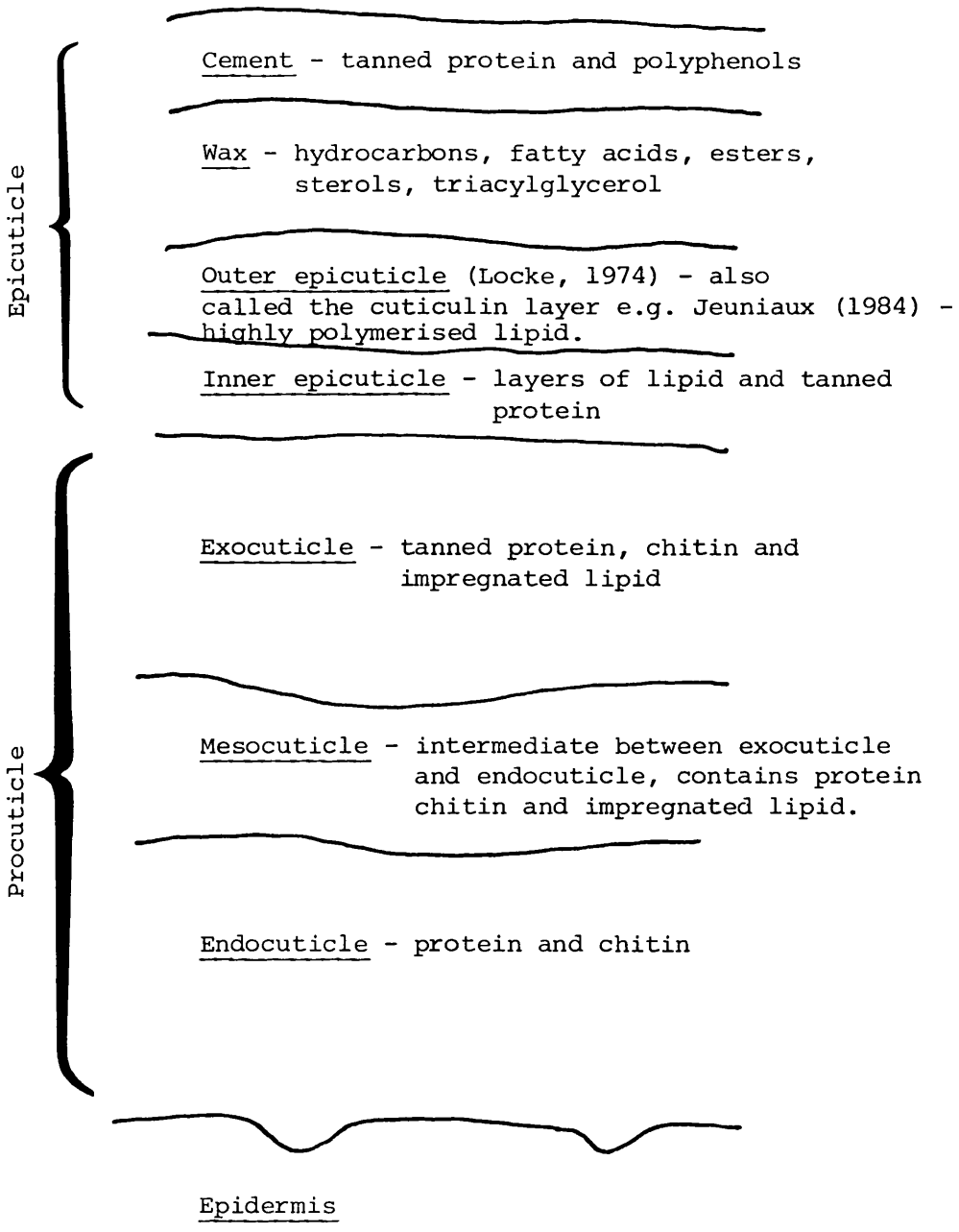
infection by entompathogenic fungi.

Structure and Biochemistry of Insect Cuticle

Most insects possess three main types of cuticle. Hard sclerotized cuticle (Sclerites) which covers most of the body, soft flexible cuticle (arthrodial membrane) which inter-connects the sclerites, and resilin (rubber-like cuticle) which is used for storing kinetic energy elastically (Neville, 1975; Andersen, 1977). The arthodial membrane is often the favoured site of penetration by entomopathogenic fungi (e.g. Schabel, 1978; David, 1967), though invasion can also occur via sclerites (McCauley *et al.*, 1968; Veen, 1966; Takahashi, 1958).

The structure of cuticle is illustrated diagrammatically in Fig. 1. All three types of cuticle are covered externally by the thin but complex epicuticle. This consists of an inner protein epicuticular layer (a cross-linked network of lipids and proteins), a cuticulin envelope (structure unknown) and wax layers, covered by a superficial cement layer (Jeuniaux, 1984; Locke, 1984). The epicuticle determines the size, shape and surface properties of the cuticle (Locke, 1984) and being the interface with the environment is the first barrier that the pathogen must penetrate. It may in fact constitute the most important obstacle to penetration (Jeuniaux, 1984) especially as its non-wetting properties could hinder adhesion by the pathogen (Blomquist, 1984).

Fig. 1. Structure of insect cuticle



After Locke (1974); Jeuniaux (1984)

The bulk of the cuticle (the procuticle) is composed almost entirely of chitin fibres embedded in a protein matrix (Filshie, 1982). The nature of the proteins associated with chitin and the type of links between proteins gives the cuticle its hardness (hard procuticle = exocuticle) or its flexibility and softness (soft procuticle = endocuticle). Sclerites correspond to cuticular regions where exocuticle makes up a prominent part of the cuticle thickness, while arthrodial membrane is built up almost entirely of endocuticle (Jeuniaux, 1984).

The mechanism by which cuticle is hardened (sclerotization) is still a matter for debate but it clearly involves cross-linking of some sort between protein chains. Among the different theories which have been proposed are:

1. That protein-linked tyrosine residues are oxidised *in situ* by tyrosinase to DOPA residues, which are further oxidised to DOPA-quinone residues able to react with neighbouring protein molecules (Blower, 1950; Brown, 1950).
2. That a tanning agent is formed from the oxidation of the β-carbon atom in the aliphatic side chain of N-acetyl-dopamine. This can then react with the free amino groups of cuticular structural proteins forming a cross-link (Andersen, 1971b; Andersen and Barrett, 1971).

Not all workers accept that the cross links are likely to be covalent. Vincent and Hillerton (1979) believe that chemical evidence for the existence of covalent cross-links is lacking and suggest that their occurrence could not in any event explain

the degree of stiffening of the cuticle at sclerotization. In their opinion and as suggested earlier by Fraenkel and Rudall (1940) and Lipke and Geoghegan (1971a) the strong dehydration which occurs when procuticle turns to exocuticle (from 70% water down to 12% in some cases) would have a marked effect on the stiffness of the cuticle by inducing secondary bonds (hydrogen bonds) between the protein chains. Similar views have also been expressed by Jeuniaux (1984). It is envisaged that the quinones, which appear at the time of sclerotization, control dehydration rather than provide covalent cross-links.

Andersen (1981) considered Vincent and Hillerton's (1979) theory in relation to the process of cuticular tanning in locusts. He concluded that though dehydration may contribute to cuticle stabilization, the observed properties are nevertheless better explained by the existence of covalent cross-links. He concedes however that his own hypothesis (Andersen, 1971b; 1973; 1977) has several inherent difficulties, including steric problems and the nature of the anabolic enzyme(s) involved (Andersen, 1980). Evidently, clarification of the nature of protein cross-linking must await further study. Whatever the mechanism involved it is evident that sclerotization confers exceptional resistance to enzymatic breakdown by hydrolases in moulting fluid or by commercial enzyme preparations (Jeuniaux, 1984; Lipke and Geoghegan, 1971a). In the light of this, pathogen enzymes must have special properties if enzymic degradation plays a part in the penetration of sclerotized cuticle (Charnley, 1984).

The stability of exo- and endo-cuticle could be further enhanced by chemical bonds between chitin and protein. N-acetyl-glucosamine (NAG) as well as its polymer **chitin** can react with α-amino acids (especially tyrosine), peptides and cuticular proteins to give stable complexes (Hackman, 1955). Furthermore, even after careful purification, chitin from various natural sources always contains small amounts of amino acids - mainly aspartic acid and histidine (Hackman, 1960; Strout and Lipke, 1974; Kimura, Strout and Lipke, 1976). This has been taken as evidence that chitin is covalently linked to proteins to form stable glycoprotein complexes (e.g. Jeuniaux, 1984).

Three different types of covalent protein-chitin linkages have been postulated. These are:

1. The formation of N-acyl-glucosamine covalent links from the carboxyl groups of aspartyl- or histidyl-residues in the protein to the free NH_2 group of glucosamine residues in partially de-acetylated chitin molecules (Hackman, 1960; 1976).
2. The formation of N-glycosidic linkages between the terminal NAG of the chitin chain and aspartic acid or aspargine (Brine, 1982).
3. The formation of chitin-protein bonds via neutral sugars in highly sclerotized cuticles such as *Sarcophaga puparium* (Lipke, Grainger and Siakotos, 1965; Lipke and Geoghegan, 1971 a,b).

According to Lipke and Geoghegan (1971a,b) physical interactions may contribute to the stability of the cuticle in addition to covalent bonds. Whatever the chemical nature of the linkages between chitin and protein it is clear that the association protects chitin from hydrolysis by chitinases (Jeuniaux, 1965; Lipke and Geoghegan, 1971a; Samsinakova, Misakova and Leopold, 1971). The experiments of Jeuniaux (1959 a,b) showed that in various cuticles only 4% to 30% of the total chitin is extractable with chitinases. The remaining chitin is presumed to be bound to protein. In insect cuticles, the proportion of free chitin is generally less than 5% of the total chitin, even in soft larval cuticles.

Protein

Although protein is the major component of the cuticle (55 - 80%) there is less known about it than any of the other constituents (Neveille, 1975). It is not even known for certain in what configuration proteins occur in cuticle. Fraenkel and Rudall (1947) claimed from X-ray diffusion studies that cuticular proteins exist in the fully extended β-configuration. More recently Hackman and Goldberg (1979) found that soluble cuticular proteins in blowfly larvae are in a disordered configuration but in the solid state they and other larval cuticular proteins adopt a β-configuration. Many workers have assumed without investigation that proteins exist in the β-configuration (e.g. Andersen, 1973 for *S. gregaria*). However, Arnott, Dover and Neville (unpublished observations, reported in Neville, 1975)

found no evidence from X-ray diffraction work on *Eutropidaaris* tendons for β-extended proteins, and Neville (1975) suggests that the majority of cuticular proteins probably have a globular structure. It is known that the protein chains in resilin are in the random coil configuration (Andersen, 1973).

Ideally single proteins should be used to gather conformational detail but none have yet been purified (Jeuniaux, 1984). However, Fristom, Hill and Watt (1978) and Larson (1975) have reported the partial purification and characterisation of proteins from *Drosophila* larvae and from *Limulus* respectively. So far no primary amino acid sequence or fragments of primary sequence have been ascertained but it is widely recognised that cuticles contain many different proteins. Thus Willis (cited in Weis-fogh, 1970) extracted 10 proteins from locust pre-ecdysial solid cuticle, and 13 from intersegmental membrane while Andersen (1973) found 14 bands in *Schistocera* femoral pre-ecdysial exocuticle.

Descriptions of total amino acid compositions of various hard and soft cuticles abound in the literature but as pointed out by Hackman and Goldberg (1958) the large number of different proteins makes it difficult to interpret the meaning of amino acid contents of total hydrolysates. Nevertheless, despite these difficulties some generalisations have been made. Andersen (1971) noted a correlation between content of high non-polar amino acid residues and cuticle hardness. Thus in *S. gregaria* sclerotized cuticle contains 74 - 81% non-polar

residues (glycine, alanine, valine, leucine, isoleucine); arthrodial membrane 59 - 64%, and resilin 65 - 68%. Similar results have been found for *T. molitor* (Andersen, Chase and Willis, 1973). Early attempts to correlate glycine and alanine contents with cuticle hardness seemed promising but were later shown not to hold for all species subsequently investigated. Thus in *S. gregaria* alanine is the major amino acid in hard cuticle (28 - 40% of the total number of residues) whereas glycine predominates in arthrodial cuticle and resilin (*c.* 20% and 40% respectively) (Andersen, 1971a; 1973). However, high levels of alanine have been detected in soft larval cuticles of *Galleria* (Srivastava, 1971) and *Tenebrio* (Andersen *et al.*, 1973).

Chitin

Apart from protein, the only other major component of procuticle is chitin. There is good evidence that chitin is an unbranched polymer of N-acetylglucosamine (NAG) units linked by β(1-4) glycosidic bonds (Foster and Webber, 1960). The structural repeating unit is chitobiose,the dimer of NAG (Carlström, 1962), a feature that may have significance for the substrate specificity of chitinases (see Stirling *et al.*, 1979). Chitin exists in 3 distinct crystallographic forms (α, β or γ) recognised by X-ray diffraction analysis (Rudall, 1963). Chitin is generally present in the α form in fungi and arthropods viz. antiparallel chains of poly-N-acetylglucosamine stabilised by intra chain and inter-chain H bonds (Rudall, 1963; Rudall and Kenchington, 1973; Carlstrom, 1962). However

insect perithropic membranes (a chitino-proteic muff which is secreted in the midgut) are said to possess γ-chitin (two parallel chains and one antiparallel chain) (Rudall and Kenchington, 1973).

While it is generally assumed that chitin is built up only of NAG, the deacetylated form glucosamine makes up to 10% of the residues released from chitin by enzymic hydrolysis (chitinase and chitobiase) (Waterhouse, Hackman and McKellar, 1961; Hackman and Goldberg, 1965). Indeed Rudall (1963) concluded on the basis of X-ray diffraction analysis, infra-red-absorption spectra and density measurements, that chitin may depart from an idealised poly-N-acetylglucosamine structure in having one out of every 6 or 7 residues de-acetylated, with bound water replacing missing acetyl groups to maintain properties consistent with those of poly-N-acetylglucosamine. As mentioned above, if chitin *in situ* is partially de-acetylated, the free amino groups of glucosamine in the polymer could be involved in covalent linkages with proteins (Hackman, 1960).

Chitin isolated from arthropoid cuticle is highly resistant to many chemical reagents including dilute acids or alkalis and organic solvents. Although isolated chitin retains all the macroscopic morphological features of the cuticle, it exhibits none of its physical properties (e.g. hardness, impermeability, colour, elasticity) (Jeuniaux, 1984); clearly many of the mechanical properties of chitinous structures are not due to chitin itself but to the nature of its complexes with other

components of the cuticle.

Lipid

The third cuticular component with a structural role is lipid. The chemistry of insect cuticular lipids have been reviewed recently by Nelson (1979), Gilbey (1980), Lockey (1980), Blomquist (1984) and Blomquist and Dillwith (1984). There is now a considerable body of data on the composition of cuticular lipids derived from *c.* 100 species of insect. Of the chemical classes of lipids isolated from cuticle, hydrocarbons are the most common and abundant. They are usually mixtures of N-alkanes and methyl-branched alkanes, and sometimes contain mono- or diunsaturated alkenes. In *L. migratoria* and *S. gregaria*, long-chain saturated acyclic hydrocarbons contributed 54% and 76% respectively of the total amount of lipid extracted from the wings (Lockey, 1976). In *S. gregaria* 11 n-alkanes were identified ranging from C_{22} to C_{33} and they accounted for approximately 70% of the alkane fraction. The remainder of the fraction consisted of branched alkane constituents (methyl alkanes) (Lockey, 1976). Like hydrocarbons, fatty acids appear to be ubiquitous in cuticle. In *S. gregaria*, C 18:1 fatty acids (e.g. oleic acid) constituted 44% of the total extracted from abdominal sclerites with palmitic and linoleic acids also identified as major constituents (Thompson and Barlow, 1970). Other oxygenated long chain components, particularly C_{20}-C_{32} primary alcohols

and wax esters can be major components of the cuticular lipids of some species e.g. scale insects (Chibnall, Piper, Pollard, Williams and Sahal, 1934) triacylglycerols, steroids, phospholipids and aldehydes have only been found in small amounts (Gilbey, 1980).

Most studies to date have only characterised those surface lipids readily extracted in organic solvents. Consequently very little is known about the distribution of lipids within cuticle and unfortunately much of the existing information is limited to a very few species of insects ,for example *Calpodes* (Locke, 1974) and *Rhodnius* (Wigglesworth, 1976). The epicuticle appears to be covered by a lipoidal layer but may also contain free lipid in epicuticular canals (Locke, 1974) and bound lipid in the form of 'tanned lipoprotein' and 'cuticulin'. The structure of cuticulin is not known but the fact that it is dispersed in 3% alcoholic potassium hydroxide has led Wigglesworth (1970) to suggest that it is a multiple polyester, consisting of hydroxylated fatty acid chains, cross-linked by ester bonds between carboxyl and hydroxyl groups. This would give it a resemblance to plant cutin (Martin and Juniper, 1970). Lipids may also be involved as a structural component of procuticle (Wigglesworth, 1933, 1970), possibly impregnating it via pore canals (Dennell and Malek, 1955). However the chemical means used to reveal procuticular lipids (chlorated nitric acid and sodium hypochlorite) are so severe in their effects that interpretation of results is difficult (Gilbey, 1980). Evidently further chemical tests are necessary to explore

this possibility.

Cuticular lipids may serve other functions besides helping to maintain structure. They have a well known role in water-proofing cuticle (Hadley, 1981) and may activate the enzyme phenolase (Heyneman and Vercauteren, 1968). Insect cuticular lipids may also provide chemical communication as sex pheromones, species and caste recognition cues,thermoregulatory pheromones and as kairomonal cues for parasites (Howard and Blomquist, 1982). In addition some lipids may have an antifungal action. Several studies have shown that removal of epicuticular lipids enhances pathogenicity (Sussman, 1951; Koidsumi, 1957; Fargues, 1984) oer permits germination or invasion of otherwise harmless fungi (Nyhlen and Unestam, 1975; Smith, Pekrul and Grula, 1981; Fargues, 1984). However, Fargues (1981) demonstrated that although "dewaxed" *Cetonia aurata* larvae become highly susceptible to the species-specific strain *M. anisopliae* C.A. they remained resistant to the non-specific strain *M. anisopliae* O.R. It has been suggested that hydrophobic cuticular lipids protect the insect from micro-organisms by making the insect surface non-wettable preventing adhesion (Blomquist, 1984). However lipids, particularly short chain saturated fatty acids, may also provide a specific chemical barrier to infection (Koidsumi, 1957). Smith and Grula (1982) investigated this hypothesis and found that caprylic acid (C8) which is the major free fatty acid in extracts from the surface of *Heliothis zea* and *Spodoptera frugiperda* larvae inhibited germination of *B. bassiana in vitro* at 0.02% and 0.04%

final concentrations. The significance of these results cannot be determined until the concentrations of free fatty acids on cuticle have been measured, since it may be too low to have any effect (Latge and Vey, 1974). In fact the occurrence of short chain fatty acids on insect cuticle may not be a general phenomenon. They are absent in extracts from larvae of Scarabeids (Fargues, 1981; 1984), *Curculia caryae* (Champlin, Cheung, Smith Burton and Grula, 1981) and *Acyrthosiphon pisum* (Brey, 1982). Also crude chloroform extracts of scarabeid larval cuticles lacked mycostatic activity toward both specific and non-specific strains of *M. anisopliae* (Fargues, 1984).

Growth and Infective behaviour of Entomopathogens

Conidia are the infective propagules of hyphomycetous fungi. Germinating conidia on the surface of the cuticle produce germ tubes which after a variable amount of hyphal growth generally give rise to appressoria-like structures prior to invasion of the cuticle (Pekrul and Grula, 1979; Robinson, 1966; Schabel, 1978; Vey and Fargues, 1977). Appressoria produce one or more penetration pegs which penetrate the epicuticular layer before extending laterally, to form a plate in the outer layers of the exocuticle. Hyphae from the plate complete the passage across the procuticle either by lateral extension within the exocuticle before producing vertical penetration hyphae, or with a minimum of lateral proliferation there is a step wise progression of hyphae towards the endocuticle. In both cases once a pathogen reaches the endocuticle progression to the epidermis is more

or less direct (McCauley *et al.*, 1968; Robinson, 1966; Zacharuk, 1970c).

Penetration of the cuticle,as described above, is considered to be a combination of mechanical pressure and enzymic degradation, but the relative importance of the two mechanisms is not known (Ferron, 1978; Roberts and Humber, 1981). A similar dual mechanism is believed to be employed by phytopathogens (Aist, 1976; McKeen, 1974).

The Production and Activity of CDE *In Vivo*

The first indications of enzymic involvement in penetration are observed prior to germination. Wallengren and Johansson (1929) found that the epicuticle of *Pyrausta nubilalis* larvae become granular and pale around a conidia of *M. anisopliae*. An ultrastructural study of the penetration of larval wireworms by *M. anisopliae* revealed that the wax layer was absent underneath the appressoria (Zacharuk, 1970b). Later, irregular cavities appeared beneath the appressoria but there was no evidence of mechanical displacement of the surrounding epicuticle which suggests their formation is primarily by enzymic action. The outer epicuticle was also found to disappear under encysted zoospores of *Aphanomyces astaci* on crayfish cuticle (Nyhlen and Unestam, 1975). Further evidence for the enzymic degradation of the epicuticle comes from a study on *Heliothis zea* infected with conidia of *B. bassiana*. Scanning electron micrographs revealed neat circular holes around the germ tubes at their point of penetration (Pekrul and Grula, 1979). These observations

are consistent with the secretion of lipases and/or wax degrading enzymes by the pathogens. Histoenzymological tests revealed that pregerminating conidia of *B. bassiana* on the cuticular surface of *Galleria mellonella* produce non-specific esterases and possibly lipase (assay with Tween 80; Michel, cited in Fargues, 1984). Similarly, Soderhall and Unestam (1975) found non-specific esterolytic activity in zoospores of *A. astaci* germinating on crayfish cuticle.

Evidence for enzymic involvement in penetration of the procuticle by pathogens varies considerably from study to study. This may be a reflection of variability in both insect cuticular structures and fungal synthetic capabilities (Charnley, 1984). Several workers have observed clearing zones which presumably reflect hydrolysis of the exocuticle immediately surrounding the penetration pegs and plates of *M. anisopliae*. McCauley *et al.* (1968) using elaterid larval cuticle found that the semicircular clearing zones were associated with appressoria which suggested diffusion of the enzymes from these structures. Zacharuk (1970c) concluded from his study that enzymes secreted by penetration pegs and appressoria diffused primarily along pore canals, resulting in a localised digestion of the cuticle. In contrast other workers have found clearing zones of uniform diameter along the length of infection threads and have consequently suggested that enzymes are secreted solely from their tips (Notini and Mathlein, 1944; Robinson, 1966; Wallengren and Johansson, 1929). The clearest histochemical evidence for production of fungal enzymes in insect cuticle was provided by Ratault and Vey (1977) using *M. anisopliae* and the beetle

Orycetes rhinoceros. They localised a non-specific esterase in clearing zones and an N-acetylglucosaminidase apparently bound to the fungal cell wall. An ever present problem in interpretation of studies such as these is that many enzymes probably have a role in fungal germination or metabolism and may not be involved in penetration.

Evidence for the degradative action of enzymes *in vivo* is controversial and is largely based on conflicting interpretations of results obtained using the periodic acid-schiff (PAS) reaction. Several groups have found that insect cuticle infected with fungi changes from PAS negative to PAS positive. Sanassi (1969) noted this phenomenon in the endocuticle of queen *Odontermes obesus* infected with *Aspergillus flavus*, Benz (1963) in the mite *Malacosoma alpicola* infected by *Spicaria* sp., and Sanassi and Oliver (1971) in the mite *Dinothrombium giganteum* attacked by *A. flavus*. Benz (1963) suggested this change in PAS reflected the action of both protease and chitinase. Delachambre (1969), however concluded that protease alone could be responsible; removal of protein would expose free amino groups in the chitin which as a result should give a positive PAS reaction. A further interpretation is that of Gabriel (1968a) who found in his study of the penetration of *Galleria mellonella* larvae by *E. coronata* that the cuticle was weakly positive except in areas immediately around the penetrating fungus, which were negative. He suggested that removal of chtin by the pathogen would produce a PAS negative reaction. The results of a mercury/bromophenol test

forprotease also indicated restricted hydrolysis of protein around the invading hyphae. Takahashi (1958) found that the endocuticle of *Bombyx mori* infected with *B. bassiana* stained with fuchsin in contrast to the aniline blue reaction of intact endocuticle; it was suggested that this reflected a change in its nature from basophilic to acidophilic. Similar results were obtained with the mite *Dinothrombium giganteum* when infected with *A. flavus* (Sanassi and Oliver, 1971). Both authors conclude that this change is the result of degradation by fungal enzymes. However the situation is complicated by the fact that the fuchsin dye molecule is smaller than that of the aniline dye and its staining must reflect a decrease in the magnitude of the inter-molecular spaces (Neville, 1975); this is opposite to that expected if enzymic degradation had occurred (Charnley, 1984).

Most ultrastructural and histological studies suggest that if enzymic degradation of the cuticle does occur it is limited to the immediate vicinity of the fungus. The possibility that cuticle-degrading enzymes (CDE) are primarily wall bound has not apparently been considered in entomopathogens. In phytopathogens, such binding is a better documented phenomenon although most attention has focussed on the more amenable extracellular forms (Lisker, Katan, Chet and Henis (1975), Eberhart *et al.*, 1970; Polacheck and Rosenburger, 1978). Lisker *et al.* (1975) studied the cellular location of cellulase and polygalacturonase, two enzymes which might play a role in the early stages of *Rhizoctonia* damping off diseases. Location of activity was established by assay of culture fluids and

chemical extractions from hyphae. Such techniques showed that each enzyme was produced in both extracellular and wall bound forms. The cell bound enzymes were released in varying degrees by H_2O, salt solutions and detergents, but not by the sulfhydryl reagent 2-mercaptoethanol. Lisker *et al.* (1975) suggested this release is probably enhanced by plant exudates so that bound enzymes may become extracellular during the initial infection stages.

In the case of phytopathogens Cooper, Wardman and Skelton (1981) has suggested that enzyme activity in host cell walls could be further reduced by binding to wall components and by the effects of inhibitors within walls. Covalent binding (reversible by high ionic concentrations) of both host and pathogen enzymes to plant cell walls is a well documented phenomenon (Jansen, Jang and Bonner, 196o: Parr and Edelman, 1975; Cooper *et al.*, 1981) while low molecular weight phenolic precursors and free radicals produced during lignin polymerization may be toxic to the fungus or inactivate its enzymes (Ride, 1983). Also movement of enzymes in host walls could be restricted by molecular sieving of larger enzymes by the molecular matrix of the wall polymer (Cooper *et al.*, 1981). Comparable mechanisms *re* cuticle binding, inhibition or molecular sieving may operate in insect cuticles but have not been generally investigated. However some components of insect cuticle such as quinones are known to inhibit many enzymes (Hoffman-Ostenhof, 1963), and melanin, the resulting polymer, can also inhibit microbial proteases and chitinases (Kuo and Alexander, 1967; Bull, 197O).

These results conflict with those of Soderhall and Ajaxon (1982) who failed to detect inhibition of *A. astaci* protease by pre-formed melanin, quinones or L-dopa *in vitro*.

The histochemical and cytochemical evidence for the *in vivo* production of cuticle degrading enzymes is better for phyto-pathogens than for entomopathogens, albeit sparce when compared to the wealth of biochemical information. Dudderidge and Sargent (1978) studying *Bremia lactucae* infecting lettuce cotyledon cytochemically localised "lipase" (using Tween 80) in that part of the appressorial wall in contact with the host cuticle. Convincing evidence for involvement of a cutinase during infection by *Fusarium solani* was provided by detection with ferritin-conjugated antibody raised against the pure enzyme (Purdy and Kolattukudy, 1975a; Shaykh *et al.*, 1977). It was also found that unless the stem surface was wounded, infection was greatly reduced when conidia were mixed with anticutinase prior to inoculation (Maiti and Kolattukudy, 1979; Köller *et al.*, 1982). Cutinase production would therefore appear to be a pre-requisite for infection. Dickerson and Pollard (1982) used immuno-fluorescent techniques for the detection of 2 enzymes of *Claviceps purpurea;* a β-glucanase and a β-glucosidase which *in vitro* are known to occur both extracellularly and bound to cell walls. They found fluorescence was largely absent in the early stages of infection of its cereal host but, after 10 days both enzymes were identified to varying degrees, in areas of active growth, particularly at hyphal tips and at the host parasite interface. Dickerson and Pollard (1982) postulated that these enzymes may be involved

in fungal wall growth and hydrolysis of host cell wall polysaccharides, particularly callose, a β-1,3 glucan produced in response to wounding. These enzymes do not appear however to have the same primary role in infection as the cutinase produced by *Fusarium solani* (Shaykh *et al.*, 1977).

The Role of Mechanical Pressure in Cuticle Penetration

There is considerable evidence from ultrastructural studies in entomopathogens, of a mechanical component to the penetration of both epicuticle and procuticle. Zacharuk (1970b,c) reported that before histolysis, penetration pegs which form from appressoria act as pressure points indenting the epicuticle. Several groups of workers report that lamellae in the exo- and endocuticles are physically displaced during the penetration process (Lefebvre, 1934; Nyhlen and Unestam, 1975; Sanassi, 1969; Wallengren and Johansson, 1929; Zacharuk, 1970). McCauley *et al.* (1968) suggested enzymatic activity ceases with the commencement of lateral development from the penetration peg and Zacharuk (1970c) concluded that apart from the initial stages, penetration of the procuticle is largely mechanical. Vey and Fargues (1977) found no evidence for enzyme activity even during the penetration of the outer lamellae of the procuticle of larval *Leptinotarsa decemlineata* by *B. bassiana* and suggested that penetration of the inner lamellae could be facilitated at the appropriate time by the lytic action of the insect's moulting fluid. The importance of physical pressure in the penetration of *Trichophisia ni*

by *Entomorphthora apiculata* was demonstrated by the way the growing hyphae wedge procuticular layers apart (Lambiase and Yendol, 1977). However they also found substantial epidermal lysis in the area of initial penetration which may be the result of fungal enzymes.

Cuticle-Degrading Enzymes

Many studies have shown that entomopathogenic fungi can *in vitro* produce enzymes capable of digesting the major constituents of insect cuticle, *viz* protein, chitin and lipid (Neville, 1975), e.g. *B. bassiana* and *M. anisopliae* (Leopold, *et al.*, 1970), *Cordyceps militaris* and *A. flavus* (Huber, 1958), several *Entomorphora* sp. (Gabriel, 1968b), *Nomuraea rileyi* (Mohamed *et al.*, 1978) and *Cephalosporium* spp. (= *Verticillium*) (Pisano, *et al*, 1963).

Extracellular depolymerase enzymes have been extensively studied in many fungi and bacteria, but relatively few critical studies have been performed on entomopathogens.

Proteolytic Enzymes

Amongst fungi as a whole two different types of extracellular proteolytic enzymes have been recognised (Cohen, 1977). These are peptide exohydrolases such as aminopeptidases or carboxypeptidases, which remove the terminal amino acids of proteins and peptide endohydrolases which have varying specificities for internal peptide bonds. The exohydrolases

participate in but cannot by themselves rapidly complete the digestion of proteins. They have not apparently been studied in entomopathogens or indeed many other fungi except for yeasts. The aminopeptidases (α-aminoacylpeptide hydrolases, EC 3.4.11-) are still under intensive investigation. Although sub-divided into several groups they show considerable overlapping of specificities. Leucine aminopeptidase is the term for an aminopeptidase which has a preference for leucine. The activity of this enzyme however is not limited to N-terminal leucine and also does not always show a preferential hydrolysis of L-leucylpeptides, e.g. L-alanylpeptides; amino acid amides and arylamides may also be hydrolysed (Appel , 1974). Catheptic aminopeptidases hydrolyse various peptides often with broad non-specificity. This group includes the amino acid aryl-amidases which preferentially or exclusively hydrolyse "chromogenic" substrates like naphthylamides or anilides of amino acids (Appel, 1974).

Carboxypeptidases (EC 3.4.12-) hydrolyse peptides with a free COOH group on the adjacent peptide or ester bond. The most studied are the pancreatic carboxypeptidases A and B (Neurath, 1960). These enzymes hydrolyse amino-substituted peptides with different specificities and are inhibited by strong chelating agents. Carboxypeptidase in plants,bacteria and fungi are generally termed cutheptic carboxypeptidases (Appel, 1974). They are not usually affected by EDTA but are inhibitied by sulfhydryl reagents (Fruton, Irving and Bergmann, 1941; Greenbaum and Sherman, 1962). Several new

classes of carboxypeptidase are still under investigation (Appel, 1974).

The endopeptidases, commonly called proteases, can cleave peptide bond distant from the termini of polypeptide chains and thus solubilise insoluble proteins. These enzymes are further characterised by reactivity of inhibitors with particular residues in the active site region. In general the serine enzymes (EC 3.4.21) are most active under alkaline conditions with an optimum around pH 8.0. The thiol (EC 3.4.22) and metallo-enzymes (EC 3.4.24) are generally most active from pH 4.0 *c* 8.0 and are often known as 'Neutral proteases'. The acid proteases (EC 3.4.23) show optimal activity below pH 4.0. Kucera (1980) using inhibitors classified the two proteases produced by a strain of *M. anisopliae* as a thiol protease ($pH_{opt.}$ 6.5) and a serine protease ($pH_{opt.}$ 9.0). To the author's knowledge this is the only such study involving enzymes from an entomopathogen.

Chitinolytic Enzymes

Chitinolytic enzymes have not been isolated or characterised from any entomopathogen. In other systems however, it has been established that degradation of chitin generally involves the sequential action of chitinase, an endo-N-acetyl-β-glucosaminidase (poly-β1-4-(2-acetamido-2-deoxy)-D-glucosideglucanohydrolase, EC 3.2.1.14), and chitin oligosaccharase - an exo-N-acetyl-β-glucosaminidase (EC 3.2.1.29) (Stirling, Cook and Pope, 1979). Chitinases are sometimes compared with

cellulases since they both act on β(1-4) linked glucose polymers, which share the common physio-chemical properties of microfibril formation resulting from inter and intra molecular hydrogen bonding. Two types of cellulases C_1 and C_x are distinguished by their activities towards highly ordered cellulose and its soluble derivative, carboxymethylcellulose. Cellulase C_1, a β(1-4)-glucan cellobiosidase degrades microcrystalline cellulose while C_x, an endo β(1-4) glucanase is active towards CM-cellulose and also participates in the hydrolysis of microcrystalline cellulose along with cellobiase (Halliwell and Griffin, 1973; Berghem, Petterson and Axio-Fredriksson, 1975; Wood and McCrae, 1979). There are some indications that chitinases may also exist in two or more forms distinguished by their activities towards crystalline and swollen chitin. Monreal and Reese (1969) reported that the chitinolytic system of *Serratia marcescens* is composed of an endochitinase, a chitobiase and a factor Chi required for the hydrolysis of crystalline chitin,although these components were not purified. Tiunova, Pirieva and Feniksova (1976a); Tiunova, Pirieva, Feniksova and Kuznetsov (1976b) found that the ratio of chitinase activity on crystalline chitin and colloidal chitin varied by a factor of 2 in culture filtrates from 9 strains of Actinomycetes. They interpreted this as evidence of Ch_1 and Ch_x chitinases analogous to the C_1 and C_x enzymes in the cellulase system, but again the components were not isolated. Better evidence came from fractionation of the enzyme system of *Actinomyces kurssanovii*, which suggested the presence of Chi and Chx chitinases plus an acetylglucosaminidase(Bezborodov,

Rodionova and Tuinova, 1982). To the author's knowlege there has been no categorical demonstration to date that the Ch_1 chitinase component is an exochitobiosidase.

There have been few systematic studies of the substrate specificities of highly purified chitinases but it seems that most chitinases are inactive on cellulose or the deacetylated portions of chitosan though they are active towards a variety of other chitin derivatives (Stirling *et al.*, 1979). An enzyme distinct from chitinase that depolymerises chitosan has been detected in the culture fluids of several organisms including *B. bassiana* (Monaghan, Eveleigh, Tewari and Reese, 1973).

The mode of chitinase action and the products of chitin hydrolysis are currently in dispute. Stirling *et al.* (1979) has suggested that the end product of chitinase attack is chitobiose, and the appearance of mono and trisaccharides is due to contaminating chitobiase activity. However pure preparations of chitinase from *V. albo-atrium* or tomato plants (Pegg and Young, 1982) and a spider (Mommsen, 1980) have shown that NAG can either be the sole reaction product or a major end product along with di- and trisaccharides. It seems most likely that both the nature of the chitin substrate and the bond preferences of individual chitinases determine how much NAG is produced (Jeuniaux, 1963; Powning and Irzykiewcz, 1965). The pH optima for chitinases is usually in the range pH 4.5 to 6.5 (Jeuniaux, 1966), but an exception

to this is the enzyme from *Serratia marcescens* which has optimal activity between 8.5 and 9.0 (Lysenko, 1976). Chitinases resemble most other glycanases and glycosidases in not requiring co-factors (Stirling *et al.*, 1979). Several microbial chitinases are inhibited by Na^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+} and Hg^{2+} (Jeuniaux, 1966; Stirling *et al.*, 1979) and melanins have been reported to inhibit the action of chitinases on fungal cell walls (Bull, 1970).

Lipolytic Enzymes

Lipolytic enzymes may be defined as long chain fatty acid ester hydrolases with "long chain fatty acids" meaning aliphatic acids, saturated or unsaturated, with 12 or more carbon atoms (Nagata, 1975; Brokerhoff and Jenson, 1974). The alcohol moiety of the ester may be glycerol (Lipase EC. 3.1.1.3) or a glycerol derivative (e.g. phospholipases, galactolipase) or a sterol (cholesterol esterase). The site of action of lipase is the interface between the oil drops and the aqueous phase, so that the degree of emulsification plays an important part in establishing the effective concentration of substrate (Fielding, 1970). This distinguishes lipases from esterases which can react with water soluble substrates.

The pH optima of most microbial lipases lies between 8.0 and 9.0 (Lawrence, 1967), although there are exceptions, for instance the lipase in *Mucor pusillus* is most active *c.*

pH 5-6 (Somkuti, Babel and Somkuti, 1969). Although many microorganisms produce lipases, few have been closely studied. Most recent attention to the enzymes has arisen because the production of lipases may assist in classification of micro-organisms and detection of those that are pathogenic to vertebrates (Lawrence, 1967; Brokerhoff and Jenson, 1974). Although lipolytic activity has been considered of taxonomic importance most investigations have been based within rather than across genera eg. both lipolytic and non-lipolytic strains were found in *Staphylococcus aureus* and *S. albus* (Davies, 1954). Alford, Pearse and Suggs (1964) in a cross-generic study observed hydrolysis of mainly the primary ester of triglycerides by several species of *Pseudomonas* and a variety of fungi. *S. aureus* and *Aspergillus clavus* attacked all three ester bonds, while *Geotrichum candidum* released oleic acid regardless of position. Zvyagintseva and Melikhovskii (1982) isolated 57 organisms from waxy plant surfaces which comprised 20 bacteria,5 yeasts and 32 fungi. All bacteria, yeasts and 16 of the fungal cultures exhibited low lipase activity. The other fungal cultures produced moderate or high activities.

The interpretation of results obtained in the few studies on lipases of entomopathogens is handicapped by fundamental deficiencies in enzyme assay. Thus in every case lipase has been assayed with either tweens or tributyrin which are not specific lipase substrates but may be degraded by carboxyesterases or enzymes of the proteolytic complex (Brokerhoff and Jensen, 1974; Desnuelle and Savory, 1963;

Sierra, 1964; Benoehr, Franz and Krisch, 1966).

Regulation of Enzyme Synthesis

In contrast to phytopathogens (see Cooper, 1977; 1983; 1984 for reviews) relatively little is known about the regulation of enzyme synthesis in fungal pathogens of insects. Leopold and Samsinakova (1970) studied production of enzymes by *B. bassiana* in stationary and submerged (shaken) cultures. In both cases several days incubation was necessary for maximal protease, chitinase and "lipase" (assayed with tributyrin) activities. The media they generally used contained several carbon sources (e.g. glucose 2.5%, starch, 2.5% and corn steep 2%) besides chitin such that production of CDE may reflect induction or release from catabolic repression (CR), alternatively enzymes may derive from autolysing hyphae.

In contrast to the above situation where chitinase was only produced in well established cultures, Huber (1958) found chitinase in hyphal tips and germ tubes but presumably its role here could be concerned with alterations to the cell wall during growth. Soderhall *et al.* (1978) found protease in ungerminated and germinated spores of *Aphanomyces astaci* whereas no chitinase was demonstrable until the germ tube-hyphal complex began to branch *in vitro* after 12-18 h. They suggested that the delay before chitinase production corresponded to the time for a germ tube to penetrate the epicuticle and reach the chitinous procuticle. However this

highly speculative claim and the remarkably long assay period required for detecting the enzymes (protease, 2 wks, chitinase, 12 wks) casts some doubt on the validity of their results (see Charnley, 1984).

Leopold and Samsinakova (1970) claimed that in an isolate of *B. bassiana* chitinase was constitutive as production was not enhanced by the presence of chitin. It is not clear however whether they ever used chitin as sole source of C and N in these experiments and assay periods were extended to 7 d to detect significant activity. Gabriel (1968b) found that chitinase production by four species of *Entomorphora* and various isolates of *M. anisopliae* and *B. bassiana* was prevented in solid (agar) medium containing other energy sources besides chitin, presumably as a result of CR. If chitinase is adaptive, it is unlikely that chitin *per se* is the inducer as its insolubility and large size would preclude entry into the fungal cells. Mono or oligosaccharides which would result from chitin breakdown are more likely effectors. It is interesting therefore that growth for 4 d on amino sugars (1% w/v) allowed production of small quantities of chitinase, whereas no chitinase was detected in alanine or glucose cultures (Smith and Grula, 1983). It is possible therefore that even under conditions where CR may be operating there is still some specificity of induction.

In recent studies on protease production by *B. bassiana* (Kucera,1971) and *M. anisopliae* (Kucera, 1980) it was found

that the "proteolytic complex" consists of two components with acid and alkaline pH optima. The *Metarhizium* proteases were much less adaptive than those of *Beauveria* as indicated by the relatively high production by the former on organic non-protein nitrogen sources. In *Metarhizium* several nitrogen sources increased production of both proteases. The type of nitrogen source also influenced the proportions of the two proteases. Regrettably no account was taken in Kucera's work of the effect on enzyme production of CR, pH of the medium or autolysis.

In spite of numerous studies on *in vitro* production of enzymes by entomopathogens, it has on the whole contributed little to our understanding of the mechanisms regulating the synthesis of these enzymes or in evaluating their contribution to penetration. The biochemical studies to date have generally involved long term trials in batch cultures under conditions where the enzyme potential will not be realised. As described above workers in this field have ignored or barely considered such fundamental considerations as the effects of CR, physiological age of the pathogen, autolysis, pH and medium composition. Thus fungi have often been grown on high levels of energy sources (e.g. Leopold and Samsinakova, 1970; see above) far in excess of that required to effect CR of synthesis of typical catabolic enzymes (ca. 0.01%, Cooper, 1977). In some cases inducing substrates have been omitted in cultures designed to produce enzymes and under these conditions attempts have been made to correlate the minute amounts of enzymes obtained with virulence (Samsinakova and Misakova, 1973). Interpretation

of results obtained in these studies is also handicapped in many cases by fundamental deficiences in enzyme assay e.g. lipases (see above).

More rigorous microbiological and enzymological techniques have been used with fungal pathogens of plants and higher animals, and with fungi having potential industrial use. These have contributed considerably to our understanding of enzyme regulation.

Cooper and Wood (1973, 1975) in a study on the production of cell wall degrading polysaccharidases produced by the plant pathogens *Verticillium albo-atrum* and *Fusarium oxysporum* reduced the possibility of CR by supplying potential inducers from diffusion capsules in restricted cultures. None of the various cell wall degrading enzymes were produced above a low basal level on restricted supplies of glucose as would be expected if synthesis was constitutive with CR the main or only form of control mechanism. Synthesis of each polysaccharidase was induced specifically by the monomer or dimer predominant in the enzymes specific polymeric substrate. Cooper and Wood (1975) suggested that as wall polymers are insoluble slight degradation by low levels of basal enzymes may be necessary to produce sufficient inducers to initiate enzyme synthesis.

Chitinase synthesis can be induced in many microorganisms by growing them in a medium containing chitin (Stirling *et al.*, 1979). However as described above, chitin itself is unlikely to be the actual inducer. In studying the regulation of chitinase

production by *Serratia marcescens* in batch liquid culture, Monreal and Reese (1969) demonstrated that on NAG in relatively low concentrations (0.2%) the organism produced *c.* threefold higher levels of chitinase than at a higher concentration of NAG (1%). More recently Young and Carroad(1981) studied chitinase production by *S. marcescens* in continuous culture with NAG as limiting substrate. Enzyme activity in the culture filtrate increased in relation to dilution rate, a phenomenon which was explained as a balance between induction and CR. However as the effect of restricting supplies of other carbon sources was not determined, their results could be explained solely in terms of CR.

The mechanism of protease regulation has been studied in several species of fungi. The dermatophytes *Microsporum canis* (O'Sullivan and Mathison, 1971) and *Trichophyton rubrum* (Meevootisum and Niederpruem, 1979) produced extracellular protease with protein as sole source of C and N; repression occurred upon addition of more readily available sources of C and N. In *Neurospora crassa* protease production is induced by protein but is also subject to CR (Cohen and Drucker, 1977), while extracellular proteolytic enzymes of *Aspergillus nidulans* are regulated by de-repression, release was found only when the medium was deficient in C, N or S (Cohen, 1973).

To the author's knowledge regulation of lipase synthesis has only been studied in microbes producing hydrolytic rancidity of food products. Extracellular lipase production by *Penicillium chrysogenum* was reported to be constitutive as

synthesis was not enhanced by triglycerides (Chander, Singh and Khanna, 1980). In contrast *Penicillium roqueforti* (Eitenmiller, Vakil and Shaham, 1970) *Geotrichum candidum* (Tsujisaka, Iwai, Fukumoto and Okamoto, 1973) and *Mucor hiemalis* (Akhtar, Mirza and Chughtai, 1980) produced increased amounts (cf. with glucose cultures) of extracellular lipase in response to triglycerides and other lipids. This was interpreted as induced synthesis. However as glucose can repress lipase production e.g. *Syncephalastrum racemosum* (Chopra and Chander, 1983) these results can be explained in terms of CR alone. There would appear to be no critical evidence to date of an inducible microbial lipase.

Mechanism of Cuticle Degradation

The relevance of enzyme production to penetration of cuticle by entomopathogenic fungi is far from clear. The nature and inter-relationships between the main polymers as they exist in insect cuticles must be taken into account in appraising involvement of enzymes active *vs*. model substrates. Cuticular proteins vary qualitatively in various regions of the cuticle of an individual insect (Neville, 1975). In addition although some of the proteins are free, much of the protein is covalently linked to chitin (Hackerman, 1960). The complete digestion of cuticular protein would require the action of many different types of proteases but it is possible that an invading fungus may only need to digest certain kinds or regions within protein in order to expose chitin fibrils to the action of chitinase (Gabriel, 1968a); it should be recalled

at this stage that at least part of penetration may be effected by mechanical means (Zacharuk, 1970c).

Circumstantial evidence that chitin fibrils are exposed by proteases to the action of chitinase has been provided by Samsinakova *et al.* (1971). They looked at the effect of semi-pure commercial preparations of the enzymes lipase, chitinase and protease on isolated pieces of cuticle from larvae of the wax moth *Galleria mellonella*. Sequential application of the enzymes in the order lipase, protease and chitinase produced the most efficient degradation of cuticle. Chitinase was only effective if applied after protease. Similarly Smith, Pekrul and Grula (1981) also using commercial preparations showed that complete degradation of *Heliothis zea* larval cuticle was achieved with a sequence of protease followed by chitinase. This sequential action of enzymes suggests that the chitin framework is masked from enzymic attack by protein whose decomposition opens the way for chitinase attack. This situation appears analogous to that of some plant cell wall polysaccharides which were found not to be amenable to the action of hemicellulases and cellulases of *Colletotrichum indemuthianum* and *Trichoderma viride* until polygalacturonide of the cell wall had been degraded by endo-pectic enzymes (Karr and Albersheim, 1970).

As protein is the major constituent of the matrix in insect cuticle and apparently screens the chitin component, *primae facia* one would expect pathogen-produced proteases to

have a primary role in cuticle degradation *in vivo*. However chitin may also be important in conferring integrity upon the cuticle as evidenced by a recent ultrastructural study by Hassan (1983) with dimilin, an insecticide which blocks chitin-formation in insects. *M. anisopliae* caused substantially greater areas of degradation in *Manduca sexta* cuticle which lacked chitin than in untreated insects where there appeared to be a substantial mechanical component to penetration.

The majority of histochemical and ultrastructural studies including those of Samsinakova *et al*. (1971) and Smith *et al*. (1981) have been performed using non sclerotized cuticle. It may be inferred from the work of Lipke and Geoghegan (1971**a**) that the resulting data are not applicable to tanned cuticle. These workers found that sclerotized pronotal cuticle of larval *Periplaneta americana* was highly resistant to enzymolysis such that trypsin, pepsin, collagenase, papain, panprotease and pronase hydrolysed less than 3% of the available linkages in the cuticular protein, while a variety of carbohydrases had little or no effect. However as described above there are reports of fungal penetration of highly tanned cuticle (e.g. Takahashi, 1958). In addition it is reported that chitin in the exuviae of the crayfish *Orconectes sanborni* is readily hydrolysed by chitinase (Stevenson, 1969), so not all sclerotized cuticle may be as resistant to digestion as that of *P. americana*.

The Importance of CDE in Determining Virulence

Several workers have investigated the possibility that virulence may to some extent be correlated with enzyme production. The results have been somewhat conflicting. Samsinakova and Misakova (1973) showed that strains of *M. anisopliae* and *B. bassiana* virulent against *G. mellonella* were higher producers *in vitro* of chitinase, protease and lipase than less virulent strains. Similarly, isolates of *B. bassiana* with high virulence against *G. mellonella* had higher lipolytic and proteolytic activity than isolates with low virulence (Pavlyushin, 1978), and isolates of *Beauveria brogniarti* (= *B. tenella*) with the highest lipase activity were more virulent against *Melolontha melolontha* than those with low activity (Paris and Segretain, 1975). In contrast Yanagita (1980) claimed that chitinolytic activity in isolates of *Aspergillus flavus-oryzae* paralleled pathogenicity against *Bombyx mori* but there was no relationship between pathogenicity and activity of lipase and cellulase. The situation is not resolved by consideration of the pathogenicity of 36 isolates of *B. bassiana* against *G. mellonella*. The level of chitinase production for 21 isolates was concordant but production by the remaining 15 was not (Bajan *et al.*, 1979).

Pekrul and Grula (1979) produced UV-induced mutants of *B. bassiana* which possessed varying levels of the three enzyme types. Predictably perhaps some mutants produced very high levels of certain enzymes but were poor pathogens. From this it follows that low entomopathogenicity may not simply be a consequence of the lack of a suitable enzyme "cocktail" but

may derive from a deficiency in some other essential aspect such as the ability to overcome host defences. For a critical study of the importance of cuticle degrading enzymes, point mutants apparently identical to wild type in respects other than enzyme production should be used. Otherwise comparisons between mutants or different isolates for pathogenicity and production of enzymes may only reveal the numerous factors which can influence but be unrelated to cuticle degrading enzyme synthesis. Such an approach has been attempted recently (Al-Aidrous and Seifert, 1980) but the enzyme substrates they used for mutant selection (e.g. starch, milk) have no obvious bearing on cuticle penetration.

It is apparent from the work described above that the involvement of cuticle degrading enzymes in the penetration of insect cuticles remains largely unresolved. It is the aim of the following study to help rectify this situation.

MATERIALS AND METHODS

1) Insect cultures

a) *Schistocerca gregaria*

Stock cultures of *S. gregaria* were maintained in metal cages in a controlled environment insectary at 30°C ± 3°C, 25 - 30% RH and under a photoperiodicity of 11 h light, 13 h dark. The locusts were fed daily with wheat shoots and bran supplemented with dried brewers' yeast.

b) *Calliphora vomitaria*

Wandering larvae of *C. vomitaria* were purchased from suppliers (Bio-serv) and allowed to pupate in sawdust at room temperature (22°C). Emergence of flies was synchronised by refrigerating (for 24 h) pharate adults which were about to emerge. The adults were maintained in wood and gauze cages at room temperature and fed solely on sucrose.

c) *Manduca sexta*

Individual *M. sexta* caterpillars were kept in plastic cups in a controlled insectary at 26°C, 45% RH and under a photoperiodicity of 17 h light, 7 h dark. The caterpillars were fed daily with the synthetic "Manduca diet" of Bell and Joachim (1976).

2) Culture of Fungi

a) Isolation and Maintenance

The sources and original hosts of the nine isolates used are listed in Table 1. *Metarhizium anisopliae* (Metsch.) Sorok, isolates RS 23, RS 298, RS 324, RS 455, RS 543 and RS 549 were kindly supplied by Dr. D.W. Roberts of the Boyce Thompson Institute, New York. Additional isolates of *M. anisopliae* MEI (Bl83O) and *Beauveria bassiana* (Bals.) Vuill, were obtained from Tate and Lyle Ltd., group research and development laboratory, Reading. The *Verticillium lecanii* Nees strain was isolated in this laboratory from a codling moth. The isolates were grown and maintained on Sabourand Dextrose Agar (SDA) at 23°C (*V. lecanii*) or 27°C (*M. anisopliae* and *B. bassiana)* and stored at 4°C. Subcultures were made every 6 months.

b) Growth media

The inorganic salts solution described by Cooper and Wood (1975) was the basis of liquid media for production of inoculum and studies of enzyme production *in vitro*. It contained % (w/v), $NaNO_3$ 0.2, KH_2PO_4 0.1, $MgSO_4.7H_2O$ 0.05; (P.P.M.). $FeSO_4.7H_2O$ 0.2, $ZnSO_4.7H_2O$ 1.0, $NaMoO_4.2H_2O$ 0.02, $CuSO_4.5H_2O$ 0.02, $MnCl_2.4H_2O$ 0.02. In most cases $NaNO_3$ was omitted in cultures containing a carbon source which functioned simultaneously as a source of nitrogen (e.g. chitin or a protein).

Cultures were maintained *c.* pH 6.0 with the non-metabolisable buffer 2-(N-morpholino)ethanesulfonic acid (MES) (0.05 M - 0.1 M)

Table 1. Sources and original hosts of entomopathogenic isolates

Isolate		Host	Origin
M. anisopliae	ME1	Pecan weevil	Brazil
	RS23	Mosquito	U.S.A.
	RS298	Rhinoceros beetle	Western Samoa
	RS324	Austraeris sp.	Queensland, Australia
	RS455	Brown Plant Hopper	IRRI, Philippines
	RS543	Zig Zag Leaf Hopper	" "
	RS549	Commercial preparation of Metabiol B	Brazilia, Brazil
B. bassiana		Pecan weevil	Brazil
V. lecanii		Codling moth	England

unless otherwise stated.

c) Shake cultures

(i) Shake cultures were used to study production of cuticle degrading enzymes (CDE) *in vitro*. 50 ml or 100 ml basal solution containing a carbon source was dispensed into 250 ml Erlenmeyer flasks. After inoculation the flasks were shaken in a rotary incubator (150 rev/min) at 27.5°C for up to 3 weeks.

(ii) The Carbon sources were either supplied in an "unrestricted" manner at 0.2 - 3% (w/v) or the supply was restricted to the approximate rate of utilisation by the fungi by means of diffusion capsules (Pirt, 1971).

These continuous-feed devices consist of nylon cylindrical containers which can be completely filled with a concentrated solution and sealed with a small semi-permeable membrane at one end. When placed in a liquid medium and agitated the rate of diffusion of solute out of the capsule remains almost constant until the internal solute concentration is reduced by *c*. 65%. After sterilisation capsules containing solutions of sugars or amino acids were immersed in shake cultures. Rate of release of nutrients from capsules was controlled by altering solute concentration and/or the number of membranes cut from Visking tubing 8/32". The effect of these factors on release of NAG is illustrated in Fig. 2. Capsules containing monosacch-

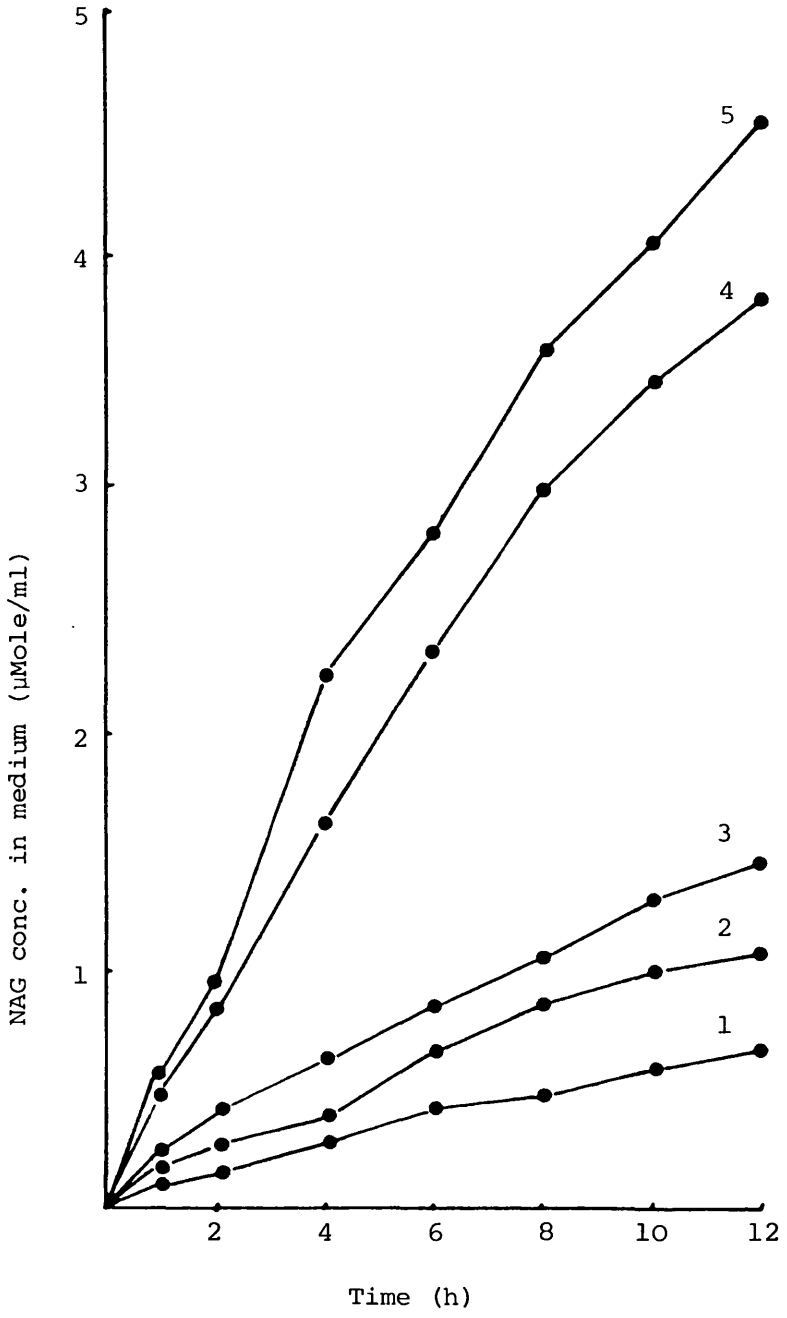


Fig. 2. Release of NAG from diffusion capsules into 100 ml culture volume, shaken at 150 r.p.m. at 27°C. (1) 7½% (w/v) initial NAG concentration in capsule, 3 membrane layers; (2) 15%, 3 membranes; (3) 30%, 3 membranes; (4) 15%, 1 membrane; (5) 30%, 1 membrane. Membrane orifice diameter 4 mm.

arides (30%, w/v) and 3 membrane layers gave satisfactory diffusion over 20 h in 100 ml cultures, so that capsules needed to be changed only once daily.

d) Inoculation of shake cultures

(i) Unrestricted cultures. Spore suspensions for inoculation were prepared from 7-12 d SDA plates incubated at 27°C in the dark. Conidia were introduced *via* a sterile loop into a 50 ml centrifuge tube containing 25 ml sterile distilled water. The contents of the tube were then agitated for several minutes using a vortex mixer to break up aggregations of spores. Mild agitation with glass beads was occasionally necessary to obtain a suspension of single conidia.. Unless stated otherwise, the inoculum per flask was 3×10^6 spores as determined with an improved Neubauer haemocytometer.

(ii) Restricted cultures. Shake cultures containing 100 ml of sucrose 1% (w/v)-basal medium inoculated as above were incubated for 3 d when extensive growth as small (< 1.0 mm) pellets was evident. Mycelium was then removed by centrifugation (1,800 g, 15 min) and washed by a further centrifugation in sterile distilled water. Unless stated otherwise the mycelial pellets from three flasks formed the inoculum for each restricted culture.

The mean dry weight of the pellets from individual flasks

was 31 ± 2.14 mg (n = 9). This level of reproducibility allowed comparisons within and between experiments.

(e) Measurement of fungal growth

Aliquots were taken from each flask at regular intervals and the mycelium in each aliquot was filtered through a dried pre-weighed Whatman 1 filter paper. After washing in distilled water the mycelium and filter paper were dried to constant weight at 70°C and cooled in a desiccator before weighing.

Unless otherwise stated, results are expressed as mg dry wt./flask.

With most media, *M. anisopliae* (MEl) grew as macroscopic spherical masses of hyphae. There was no practical way of accurately measuring fungal growth with insoluble growth substrates (English, Jurale and Albersheim,1971) thus it was estimated using an arbitrary scale ranging from + (poor growth) to +++++ (luxuriant growth).

(f) Enzyme samples

Aliquots from each flask were filtered as described above and after centrifugation (1800 g for 15 mins, at 1°C) the filtrate was analysed for reducing sugars (Nelson, 1944), amino sugars (Reissig *et al.*, 1955) and protein (Bio-Rad). The filtrates were assayed for enzyme activities after dialysis

at 4°C for 24 h in Visking cellulose tubing 8/32" diam. against *c.* 200 vol. of stirred distilled water at pH 6.0.

g) Slide cultures of fungi

In order to examine growth against a glass surface small blocks of SDA (0.5 x 0.5 cm) were cut from a poured plate and transferred to sterile slides. The edges of each block were inoculated and a cover slip applied. The slides were incubated at 27°C in petri dishes supported on glass rods over filter paper soaked in 10% glycerol to maintain high RH.

3) Sterilisation

(I) Except where indicated, all culture media, buffers and salt solutions were sterilised by autoclaving at 121°C for 15 min.

(II) For media which contained more than one carbon source, components were autoclaved separately and mixed after cooling.

(III) Insoluble proteins which could not be filter sterilised were added to a previously sterilised basal medium and autoclaved for 10 min at 115°C.

(IV) Insect cuticle when used as a carbon source in cultures was added to a previously sterilised basal medium and autoclaved for 5 min at 115°C to minimise release of the hot water-soluble fraction.

(V) Where possible proteins, sugars and amino acids were sterilised by membrane filtration (Oxoid membrane filters) under

reduced pressure.

(VI) Diffusion capsules were washed thoroughly in absolute ethanol before addition of solutions, then autoclaved inside plugged boiling tubes at 115°C for 10 min.

4) Measurement of pH

pH readings were taken on a Pye Unicam Model 290 Mk 2 meter.

5) Buffers

a) The following were used in extraction and assay of enzymes and prepared as described by Dawson, Elliot, Elliot and Jones (1969).

Britton Robinson Universal buffer: pH 2.6-12.0

Citric acid-sodium citrate (0.1 M), pH 3.0-6.2

Sodium acetate-acetic acid (0.2 M), pH 3.7-5.6

Succinic acid - NaOH (0.2 M), pH 3.8-6.0

Sodium hydrogen maleate-NaOH (0.2 M), pH 5.2-6.8

K_2HPO_4-KH_2PO_4 (0.2 M), pH 5.8-8.0

Tris-(hydroxymethyl)aminomethane-HCl (0.05 M), pH 7.2-9.1.

b) The non metabolisable buffer 2-(n-morpholino)ethane-sulfonic acid (MES) was used to maintain culture pH. 250 ml of a 0.2 M aqueous solution of the buffer salt was titrated with 0.1 M KOH until the required pH was obtained. The mixture was

then diluted to 500 ml or 1 ℓ with water to give a 0.1 M and 0.05 M solution respectively.

6) Enzyme substrates and inducers of synthesis

a) Insect cuticle

A pure preparation of locust cuticle was prepared by the method of Andersen (1980) and Andersen and Roepstrorff (1978). Locusts were killed by freezing (-20°C) and homogenised in a Waring blender in 1% potassium tetraborate (K borate) (*c.* 100 locusts ℓ^{-1}). The suspension was filtered (1 mm pore diam.) and the retained material was further homogenised and washed several times with 1% K borate. The remaining insoluble material was then stirred overnight in 1-2 ℓ of 1% K borate, filtered, washed with distilled water, and air dried. The dry cuticle was then milled to a fine powder in a Glen Creston-Ball Mill (DEH 48) using the 0.2 mm or 0.5 mm sieve and washed again in 1% K borate and finally distilled water. Any impurities were removed by suspending the powder in a large volume of distilled water and decanting the supernatant once the cuticle had settled. This procedure was repeated several times until a pure cuticle preparation (observed by microscopical inspection) was obtained. When locust cuticle is used this technique provides a well-defined source of cuticle free from contaminating material, but does not extract cuticular proteins or phenols (Andersen, 1980).

Alternative sources of cuticle were the cast exuviae of

locust nymphs and mealworm larvae (*Tenebrio molitor*). These were prepared by grinding in a Creston hammer mill and washing five times in excess distilled water.

b) Haemoglobin (Sigma) was denatured with alkaline urea prior to use as an enzyme substrate. The method was based on Rick (1965). 2.0 g haemoglobin was suspended in *c.* 50 ml deionised water in a 100 ml volumetric flask. 36 g urea and 8 ml of 1 N NaOH was added and the whole was diluted to about 80 ml, with deionised water. It was allowed to stand at room temperature for 1 h for denaturation to occur. Then 10 ml boric solution (6.184 g boric acid + 0.292 g NaCl made up to 100 ml in deionised water) was added with shaking followed by 4.4 ml 5% $CaCl_2$ solution. pH was adjusted to that required (between 3.5 and 9.0) with 1 N HCl and the substrate solution made up to 100 ml with deionised water. The substrate was diluted (final conc. 1% w/v) with an equal volume of Britton Robinson universal buffer for use in protease assays.

c) Casein (BDH, Hammersten) was washed successively with water, 95% ethanal, and ether and dried *in vacuo* (Singh and Vezina, 1971). Casein solution was prepared by suspending 1 g of purified casein in 100 ml of Britton Robinson buffer of the required pH. It was then heated for about 15 min in a boiling water bath until all the casein was denatured and dissolved. After cooling the solution was brought back to 100 mls

with deionised water.

d) Chitin

i) Technical grade crab chitin and purified chitin (suitable for enzyme assays) were purchased from Sigma; both were water insoluble.

ii) Colloidal chitin was prepared by stirring 10 g of Sigma Technical grade chitin in 100 ml conc. (85%) H_3PO_4 for 48 h at 4°C. The resulting liquid was then filtered through glass wool and poured into vigorously stirred 50% (v/v) ethanol to precipitate the chitin in a highly dispersed state. The residue was allowed to sediment and resuspended in H_2O several times to remove excess acid and ethanol then dialysed against running tap water for 48 h to remove chitin oligosaccharides and other low M.W. impurities. The colloidal suspension was diluted down to 5 mg/ml (chitin dry wt) with distilled water and stored at 4°C with a crystal of thymol.

e) Colloidal chitosan was prepared by dissolving 5 g of chitosan (Sigma) in 100 ml of 1 N acetic acid at RT for 30 min with stirring. This preparation was diluted by the addition of 100 ml of distilled deionised water and adjusted to pH 7.0 with 6 N NaOH. The colloidal chitosan formed was dialysed against several changes of K phosphate buffer (0.02 M, pH 7.0) to remove acid and impurities. Final preparations were diluted to 5 mg/ml (chitosan dry wt.) and stored at 4°C.

f) Melanin (Sigma) was solubilised in 0.5 N NaOH at 60°C for 24 h. The clarified solution was adjusted to pH 7.2 or pH 8.2 (0.5 N HCl) and stored at 4°C .

Melanin-substrate complexes were prepared after Bull (1970). Purified locust chitin, colloidal chitin or casein was stirred in an NaOH solution of melanin at pH 8.2 (melanin substrate ratio 1:10). After 60 min 0.2 N HCl was slowly added down to pH 2.0. The substrate melanin complexes were collected by centrifugation and washed successively with 0.2 N HCl (x1), distilled water until near neutral in reaction and acetone (x2).

g) Hide protein azure was purchased from Calbiochem. N-α-Carbobenzoxydiglygyl-L-arginine-β-naphthylamide and N-α carbobenzoxydiglygyl-L-arginine 4-methoxy-β-naphthylamide were purchased from Koch Light. All other enzyme substrates used in both spectroscopic and histochemical assays were purchased from Sigma.

h) Amino sugars and amino acids used as inducers of enzyme production were purchased from Sigma.

7) Analysis of locust cuticle

a) Chitin

For estimations of the proportion (w/w) of chitin in

cuticle, chitin was separated from other material by boiling in 30% KOH for 15 min (Grosscurt, 1978). After repeated washings in ether, ethanol, then water, the residue as pure chitin was dried in an oven at 70°C and the dry weight was determined.

b) amino acids

For amino acid analysis, samples of cuticle were hydrolysed with 6 N HCl in sealed evacuated tubes at 110°C for 24 h. The hydrolysates were evaporated to dryness under reduced pressure and the amino acids in the residue were analysed using a Rank Hilger amino acid analyser.

8) Preparation of cell walls from hyphae (after Lisker *et al.*, 1975; Polacheck *et al.*, 1975).

Mycelium was collected by filtration through Whatman 1 filter paper and washed thoroughly with (≥4 x 100 ml) cold (4°C) distilled water to remove extracellular enzymes. The mycelium was re-suspended in distilled water and comminuted with an overhead homogeniser for 5 min with cooling in an ice bucket. The resulting suspensions were treated in an Ultrasonicator operated at 75 KW and 20 KC until only broken hyphal walls could be seen under the microscope. This generally took ≥ 45 min and the suspension needed to be kept in an ice cooled flask designed to allow good cooling. Changing the ice every 15 min maintained the temperature ≤ 10°C during the treatments.

The sonically-disrupted samples were centrifuged at 120 g for 10 min at 4°C and the very small pellet of coarse particles was discarded. The walls were removed by centrifugation (12,000 g 10 min) and the supernatant was retained and assayed for enzymes. The pellet was washed by repeated centrifugation from cold (4°C) distilled water until no nucleic acids (absorbance at 260 nm) or glucose (glucose-oxidase reagent) could be detected in the supernatant.

9) Preparation of conidial cell walls

Conidia were collected from 7-12 d SDA plates and suspended in distilled water. Spore walls were obtained from cells disrupted either by ultrasonication (75 KW and 20 KC) in the presence of 10 w/v carborundum (100 mesh) or by violent agitation with glass beads (Sigma, Type 5, 450-500 microns) in a vortex mixer. The disrupted spores were centrifuged (15,000 x g, 10 min) and the supernatant retained and assayed for enzymes. The pellet was washed by repeated centrifugations from cold distilled water and/or buffers. Further details are given in the text.

10) Release of cell-bound enzymes

Mycelium was collected by filtration through Whatman no. 1 filter paper and washed thoroughly with (≥ 4 x 100 ml) of cold (4°C) distilled water to remove extracellular enzymes. Cell bound enzymes were extracted by vigorously shaking washed mycelium or cell walls (*c.* 100-200 mg dry wt) for 1 h at 4°C

(or 20°C when SDS was used) in 10 ml of various concentrations of K phosphate buffer to desorb ionically bound protein, mercaptoethanol to release enzyme covalently linked by disulphide bonds and detergents (Tween 80, T80; sodium dodecyl sulphate, SDS; cetyltrimethylammonium bromide (CTAB) to solubilise membranes (Barash and Klein, 1969; Cooper *et al.*, 1981; Lisker *et al.*, 1975). Fungal samples shaken in distilled water at 4°C or 20°C served as controls.

11) Enzyme assays

Except where stated in the text, pH optima of enzymes were determined for *M. anisopliae* (ME1) and used in assays. All assays were performed in duplicate.

a) Proteolytic enzymes

i) Proteases. Protease activity was assayed by measuring:

1. appearance of soluble amino acids produced by the hydrolysis of proteins,
2. release of dye from insoluble protein dye complexes,
3. esterase activity,
4. anilidase activity,

Suitable substrates for the first method included denatured haemoglobin, denatured casein and collagen (Bovine achilles tendon, Sigma).

(i)a Degradation of haemoglobin was determined by a modification of Anson's (1938) method as follows: 1 ml of appropriately diluted enzyme solution was incubated with 5 ml of urea - denatured haemoglobin. After 20 min at 30°C the reaction was terminated by adding 5 ml of 10% trichloroacetic acid (TCA) and the tubes were allowed to stand for 1 h. The residue of undigested protein was removed by filtration through Whatman No. 50 filter paper and the absorbance of the filtrate was read at 280 nm. Enzyme activities are expressed as µg tyrosine equivalents $min^{-1} ml^{-1}$ as determined from a tyrosine standard curve.

(i)b Caseinase activity was routinely assayed as follows. To 1 ml of casein solution was added 1 ml of appropriately diluted enzyme preparation. After 20 min at 30°C the reaction was terminated by adding 5 ml of 10% TCA and the tubes were allowed to stand for 1 h. The experimental procedure was then identical to that described for measuring degradation of haemoglobin.

(i)c Collagenase activity was determined by the method of Mandel *et al.* (1953). The reaction mixture consisted of 0.5 ml enzyme, 0.1 ml toluene and 4.5 ml KPO_4 buffer (0.05 M, pH 8.0) containing 5 mg powdered bovine achilles tendon (Sigma). The mixture was incubated at 30°C for 5 h in a shaking water bath. One unit of activity was arbitrarily defined as the amount of enzyme causing an increase of 0.01 in absorbance at 280 nm per h.

Methods involving measurement of proteolytic degradation products often lack the sensitivity required for detection of

small amounts of enzymes in biological and clinical samples (Rinderkneckt, Geokas, Silverman and Haverbark, 1968). Because of this insoluble protein dye complexes were also used to assay for proteolytic activity.

(i)d Hide protein azure. Assays involving this substrate are reported to be "phenominally" sensitive, being able to detect 1-2 ng of trypsin or elastase (Rinderknecht *et al.*, 1968). 5 ml bottles were charged with 20 mg of insoluble substrate (Calbiochem), 4 ml of Britton-Robinson buffer of required pH and 1.0 ml of appropriately diluted enzyme. The bottles were stoppered, and fastened to a revolving disc to maintain agitation. After incubation at 30°C for appropriate periods of time the reaction was terminated by the addition of TCA (0.25 ml, 500 g/litre). After centrifugation (5,000 g, 10 min) the absorbance was measured at 595 nm. Activities are expressed as OD_{595}-1 units $ml^{-1}h^{-1}$ or as µg trypsin equivalents calculated from a standard curve of trypsin activity against Hide protein azure.

(i)e Keratinase activity was assayed against keratin azure (Sigma). Assay and units are as described for activity against Hide protein azure

(i)f Elastase activity was estimated according to Sachar *et al.* (1955). The reaction mixture consisted of 4.5 ml of KPO_4 buffer (0.05 M, pH 8.0) containing a suspension of 5 mg elastin-cong° (Sigma) and 0.5 ml of enzyme. One unit of activity was arbitrarily defined as the amount of enzyme

causing an increase of 0.01 in absorbance at 595 nm h^{-1} (OD_{450} h^{-1}).

The specificity of a serine protease can often be determined by its relative activity towards simple synthetic substrates containing aromatic or aliphatic amino-acid residues. This is possible even with an impure mixture of enzymes (Walsh and Wilcox, 1970). Most previous studies have used either esters or their corresponding amides which contain a single bond susceptible to enzyme hydrolysis.

(i)h Trypsin-like esterase activity was determined by a modification of the method of Walsh *et al.* (1970) using p-toluenesulfonyl-L-arginine methyl ester (TAME). The reaction mixture (3.1 ml) was measured into a 10 mm quartz cuvette and consisted of 3 ml of Tris buffer (0.04 M, pH 8.1 with 0.01 M $CaCl_2$) containing TAME (1.04×10^{-3} M). After 3 min temperature equilibriation in the spectrophotometer cell compartment 0.1 ml of autoclaved enzyme was added to the reference cuvette and mixed. At time zero 0.1 ml of enzyme solution was added to the sample cuvette and mixed. The increase in absorbance at 247 nm was measured; activities are expressed as $\Delta OD_{247\cdot nm}$ min^{-1} cm^{-1}.

(i)g Chymotrypsin-like esterase activity was determined by a modification of the method of Walsh *et al.* (1970) using N-benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma). The reaction mixture (3 ml) consisted of 1.5 ml of substrate solution (BTEE,

0.001 M in 50% (w/w) aqueous methanol, spectral grade) and 1.4 ml of Tris buffer (0.1 M, pH 7.8, containing $CaCl_2$, 0.1 M) in a 10 mm quartz cuvette. After 3 min temperature equilibriation 0.1 ml of autoclaved enzyme was added to the reference cuvette and mixed thoroughly. The increase in absorbance at 256 nm was measured following addition of 0.1 ml of active enzyme; activities are expressed as $\Delta OD_{256\ nm}\ min^{-1}cm^{-1}$.

(i)h. Trypsin-like anilidase activity was determined against N-benzoyl-L-arginine-p-nitroanilide (BAPNA). 2 ml of Tris buffer (0.1 M, pH 8.0) and 0.5 ml enzyme solution were added to a test tube then equilibriated at 30°C for 10 min before addition of 0.2 ml of substrate solution (1 mM BAPNA in dimethylformamide). After appropriate periods of incubation liberated p-nitroaniline was estimated at 410 nm. Results are expressed as $\Delta OD_{410nm}\ h^{-1}ml^{-1}$.

(i)i. Chymotrypsin-like anilidase activity was determined against N-benzoyl-L-tyrosine-p-nitroanilide (BTPNA). Assay and units are as described for activity against BAPNA.

(ii) Peptide exo-hydrolase activity

(ii)a. Amino peptidase activity was assayed by a modification of the method of Nakadai, Nasuno and Iguchi (1973). The reaction mixture consisted of 0.1 ml enzyme and 1 ml of a 1 mM solution of either L-alanyl-β-naphthylamide (Sigma) or L-leucyl-β-naphthylamide (Sigma) in Britton-Robinson universal buffer (pH 7.0). After incubation at 30°C for appropriate periods of time, the reaction was stopped by the addition of 1 ml of 0.7% (v/v) HCl

in ethanol and 1 ml of 0.06% p-dimethylaminocinnamaldehyde (Sigma) in alcohol. The reaction mixture was allowed to stand for 10 min and the OD was measured at 540 nm. Activity is expressed as μmoles β-naphthylamide released $h^{-1}ml^{-1}$.

(ii)b. Carboxypeptidase A activity was determined by the hydrolysis of carbobenzoxyglycyl-L-phenylalanine (Sigma) according to the method of Appel (1974). The reaction mixture contained 0.5 ml Tris buffer (0.15 M; pH 7.6), 0.5 ml $CoCl_2$ (40 mM) in NaCl (600 nM) solution and 1.0 ml of enzyme. After mixing and preincubation at 35°C for 15 min 1 ml of an aqueous solution of substrate (30 mM, adjusted to pH 7-8 with NaOH) was added. A 0.1 ml sample was immediately (t_o) placed in a stoppered test-tube on ice. A second 0.1 ml sample was taken after 30 min incubation (t_x) at 35°C and the phenylalanine liberated at t_o and t_x measured by the ninhydrin method. Activities are expressed as μmole phenylalanine released $ml^{-1}h^{-1}$. Controls (to determine the spontaneous hydrolysis of the substrate) contained 1 ml distilled water instead of enzyme.

(ii)c. Carboxypeptidase B activity was assayed by measuring the hydrolysis of N-benzoyl-glycyl-L-arginine (hippuryl-L-arginine) according to the method of Appel (1974). The reaction mixture (3 ml) was measured into a 10 mm quartz cuvette and consisted of 2.7 ml Tris buffer (27.5 mM, pH 7.6, with 0.11 M NaCl) containing hippuryl-L-arginine (1 mM). After 3 min temperature equilibriation 0.3 ml of autoclaved enzyme was added to the reference cuvette and mixed. At time zero 0.3 ml of

solution was mixed into the sample cuvette. The increase in absorbance at 254 nm was measured and activities are expressed as $\Delta OD_{254\ nm}\ min^{-1} cm^{-1}$.

(iii) Action on various peptides. The hydrolytic action of fungal enzymes on various peptides was examined by the ninhydrin method. The reaction mixture consisted of 2 ml of Tris buffer (0.1 M, pH 8.0) containing 2 x 10^{-4} M di and tri-peptides or 10^{-3} M other peptides. After incubation at 30°C for appropriate periods of time, the α amino groups in 0.1 ml samples were measured by the ninhydrin method. The results are expressed as µmole alanine equivalents released $ml^{-1} h^{-1}$.

b) Polysaccharidases

(i) Enzymes which catalyse the depolymerisation of crystalline chitin, colloidal chitin and colloidal chitosan were assayed by measuring the release of reducing groups from these substrates using Nelsons (1944) modification of the Somogi method observing the precautions outlined by Marais de Wit and Quickie (1966).

1 ml of enzyme solution was added to 5 mg of substrate suspended in 1 ml of Cit-PO_4 buffer (0.1 M, pH 5.2) and the mixture was incubated for the required period in a shaking water bath at 35°C. The reaction was stopped by adding 2 mls of the Nelson alkaline copper reagent. Enzyme activity is expressed as µmole NAG equivalents released $ml^{-1} h^{-1}$ as

assessed from a standard curve prepared for the amino sugar.

(ii) As colloidal chitin may be heterogeneous, with regions of the polymer comprising low levels of glucosamine, chitinase was assayed in some experiments by direct measurement of NAG using the modification of the Morgan-Elson procedure of Reissig *et al.* (1955).

The reaction mixture contained 1.5 mg colloidal chitin suspended in 0.3 ml citrate-phosphate (Cit-PO_4) buffer (0.2 M, pH 5.2) and 0.2 ml of an appropriately diluted enzyme solution. The mixture was incubated in a shaking water bath at 35^{o}C and the reaction stopped by adding 0.5 ml of Reissig's Reagent A. Enzyme activity is expressed as µmole NAG released $ml^{-1}h^{-1}$ as assessed from a standard curve prepared for the sugar.

(iii) The ability to degrade chitin azure (Sigma) was assessed in a reaction mixture containing 20 mg of substrate suspended in 4 ml of Britton-Robinson buffer (pH 5.0) and 1 ml of enzyme. After appropriate time intervals the insoluble substrate was removed by centrifugation (5,000 g, 10 min) and the absorbance measured at 595 nm. Activity is expressed as ΔOD units (595 nm) h^{-1}.

c) Enzymes which catalyse the hydrolysis of chitobiose, chitotriose and chitotetraose were assayed by measuring the release of NAG from these substrates using the modification of the Morgan-Elson procedure of Reissig *et al.* (1955).

The reaction mixture contained 0.3 ml Cit-PO_4 buffer (0.1 M, pH 5.0), 0.1 ml appropriately diluted enzyme solution and 0.1 ml of an aqueous solution of substrate (0.15 M). After required time intervals at 35°C the reaction was stopped by adding 0.5 ml of Reissig's Reagent A. Activities are expressed as µmoles NAG released $ml^{-1}h^{-1}$ as assessed from a standard curve.

d) Activities of N-acetyl-β-D-glucosaminidase, N-acetyl-β-D-galactosaminidase, β-D-glucosidase and β-D-glucuronidase were determined by measuring the rate of hydrolysis of the corresponding p-nitrophenyl glycoside. The reaction mixture contained 0.5 ml of appropriately diluted enzyme and 1 ml of glycoside solution (1 mg/ml) buffered at pH 5.0 (0.1 M, Cit-PO_4). After required time intervals at 35°C reactions were stopped by adding 1 ml of 1 N NH_4OH containing 2 mM disodium EDTA (English *et al.*, 1971). Absorbance was measured at 400 nm. Activities are expressed as nmoles or µmoles p-nitrophenol released $ml^{-1}h^{-1}$.

e) Lipase activity

The reaction mixture for the determination of lipolytic activity consisted of 1 ml of filtrate, 3 ml of N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer (HEPES, 0.2 M, pH 7.0), 1 ml 0.1 M $CaCl_2$ and 1 ml of a neutralised olive oil (Sigma, special for enzyme assay) emulsion in a 2% solution of polyvinyl alcohol (PVA). After appropriate periods of time with vigorous shaking at 30°C reactions were stopped by adding 15 ml of ethanol. The mixture obtained was titrated with 0.05 N NaOH using the indicator thymolphalein.

Activity was expressed as μmoles of oleic acid equivalents.

f) Esterase activity. Activity against aliphatic esters was determined by measuring the hydrolysis of p-nitrophenyl esters possessing acyl moieties varying from C_2 to C_{18}.

The method was adapted from Staeudinger *et al*. (1973). Substrates (10 mM) were dissolved in dimethyl sulphoxide (spectroscopic grade; Analar). The reaction mixture was contained in a 10 mm quartz cuvette and consisted of 0.1 ml of substrate and 2.9 ml of Britton-Robinson buffer (pH 8.0). After 3 min temperature equilibriation 0.5 ml of autoclaved enzyme was mixed into the reference cuvette. At time zero 0.5 ml of enzyme was added to the assay cuvette and mixed. The increase in absorbancy was measured at 410 nm. Activity is expressed as μmoles p-nitrophenyl released $ml^{-1}h^{-1}$.

g) Polyphenol oxidase. 1 ml of enzyme solution was added to 1.5 ml phosphate buffer (0.1 M, pH 7.0) containing 0.0005 M $CuCl_2$ in a 10 mm quartz cuvette. At time zero, 1 ml of 0.4% catechol was added and thoroughly mixed. The increase in absorbancy at 495 nm was recorded and activities expressed as $\Delta OD_{495} min^{-1}$ (90 sec-30sec) ml^{-1}. Controls were calculated from readings of extract minus catechol and catechol minus extract.

h) Peroxidase

(i) Peroxidase (Pütter, 1974). The reaction mixture was measured into a 10 mm quartz cuvette and consisted of 3 ml

phosphate buffer (0.1 M, pH 7.0), 0.05 ml guaicol solution (20.1 mM) and 0.1 ml of enzyme. After 3 min temperature equilibriation 0.03 ml of H_2O_2 solution (0.042% = 12.3 mM) was added and increase in absorbance was measured at 436 nm. Activities are expressed as $\Delta OD_{\text{units 436 nm}}\ min^{-1} cm^{-1}$.

(ii) Peroxidase (Lobenstein and Linsey, 1961). 0.2 ml of enzyme solution was added to a cuvette containing 2.0 ml of pyragallol reagent, freshly prepared by mixing 10 ml of 0.5 M pyrogallol solution and 12.5 ml of 0.66 M phosphate buffer, pH 6.0, and making the volume up to 100 ml with distilled water. The spectrophotometer was adjusted to the null point at 420 nm, 0.5 ml of 1% H_2O_2 was then added and the contents mixed. The increase in absorbance was recorded at 420 nm. Activities are expressed as $\Delta OD\ min^{-1}$ (90s - 30s) at 420 nm.

i) Nucleases

An agar diffusion technique (Fermor and Wood, 1981) was used to detect DNAase and RNAase. DNA (1 mg ml^{-1}) or RNA (2 mg ml^{-1}) was suspended in a buffered solution of molten agar (Oxoid ion agar No. 1, 11 g ℓ^{-1} in 0.05 M cit buffer, pH 5.0 or 0.05 M phosphate buffer , pH 7.0) containing 0.005% thiomersal to prevent bacterial contamination. The suspensions were spread evenly over glass slides (∿ 5 ml/ slide) and wells cut in the solidified agar with a steel borer (6 mm diam.). 20 µl culture supernatant was added to each well and the slides incubated at 25°C and 100% RH for 18 h (RNA) or 48 h (DNA),

prior to flooding with 1 M HCl to precipitate undigested nucleic acid. Clear zones in the opaque medium indicated nuclease activity.

Results are expressed in terms of mm^2 cleared zone.

j) Enzymic degradation of cuticle

In a typical experiment 30 mg samples of ground cuticle or exuviae was vigorously shaken at 30°C with 2.0 ml of dialysed culture filtrates (5 d cultures) and 4.0 ml of Britton-Robinson buffer at pH 5.0 or 7.5. Samples (0.5 ml) were taken at intervals up to 24 h incubation. Release of amino acids due to proteolytic activity was determined with ninhydrin. Chitinase activity was assayed by following the release of NAG (Reissig *et al.*, 1955). Variations from this method are cited in the text.

Amino acid analysis of samples was conducted by the procedure described for analysis of locust cuticle.

(i) Chitinolytic hydrolysis of cuticle. The amount (w/w) of cuticle chitin hydrolysed by pathogen enzymes was determined by measuring the total amount (in mg) of amino sugars solubilised (Smith *et al.*, 1979).

The percentage of total locust cuticle chitin hydrolysed was estimated with the knowledge that cuticle contains *c.* 25% (w/w) chitin (Sect. I, 2).

(ii) Proteolytic hydrolysis of cuticle. The amount (w/w) of cuticle protein solubilised was estimated by 2 methods.

1) Following enzymolysis the residue of cuticle was washed several times in distilled water and dried to constant weight at 70°C. The balance of weight loss after subtracting that estimated for chitin hydrolysis was assumed to be due to solubilisation of protein (protein and chitin together comprise more than 95% (w/w) of most cuticle types (Neville, 1975). The percentage of cuticle protein hydrolysed was estimated by assuming that the cuticle contains *c*. 75% (w/w) protein (Sect. I, 2).

2) Values obtained from method 1 were checked by calculating the total amount of amino acids (in alanine equivalents) solubilised during hydrolysis (ninhydrin).

Unless stated otherwise, controls for enzyme assays (A-J) contained autoclaved enzyme (121°C for 30 min) in place of unheated enzyme solutions. Whenever the reaction period exceeded 4 h toluene was layered on to each reaction mixture and the contents sealed. At the end of lengthy assays solutions were tested for bacterial contamination. All absorbance readings were made using a Cecil 505 UV spectrophotometer equipped with a CE 500 recorder.

Enzyme activities are not generally expressed as specific activities because of high protein levels in many of the original growth media, e.g. from cuticle.

k) Enzymatic characterisation of entomopathogens with the API-ZYM system

The enzymatic profiles of entomopathogenic isolates were studied using the semi-quantitative API ZYM system (Slots, 1981). The 19 enzyme substrates and activities included in the system are listed in Table 2.

The isolates were grown for 5 d in 1% ground chitin/basal salts medium. 2 drops (0.06 ml) of dialysed culture filtrate were added to each of the twenty cupules of the API ZYM gallery which was placed in a moist chamber at 30°C for 3 h.

After incubation, one drop of each of the API reagents A and B was added to each cupule. The colour reaction was read after 5 min. The composition of the reagents used to detect the enzyme reactions was:

Reagent ZYM A;

Tris(hydroxymethyl-)aminomethane	25 g
Hydrochloric acid	11 ml
Lauryl sulfate	10 g
Distilled water	to make 100 ml

Reagent ZYM B;

Fast blue BB	0.35 g
2-Methoxyethanol	to make 100 ml

The results were recorded by assigning arbitrary values 0-5 to the colours developed as per the colour chart provided by the manufacturers (API). Zero corresponds to a negative reaction; 5 to a reaction of maximum intensity.

In order to demonstrate the reproducibility of results all cultures were examined at least twice and a number were tested three or four times. It was noted that autoclaved filtrate (120°C, 15 min) could occasionally produce a brown discolouration in cupules containing substrates for alkaline phosphatase and phosphoamidase. This did not however resemble the colouration produced by a positive reaction.

L) Detection of enzymes and peroxide using agar medium

The variability among isolates in their production of peroxide and extracellular enzymes was studied by means of solid culture medium in petri dishes. Conidia were point inoculated at 4 equidistant points on to the plates which were then incubated at 27°C (isolates of *M. anisopliae* or *B. bassiana*) or 23°C (V.lecanii).

(i) Proteolytic activity. Initially, proteolytic activity was detected in a medium containing 15 g of Oxoid agar No. 1 plus 24 g of non-fat powdered milk (skimmed milk) per litre of basal salt solution adjusted to pH 5.5.

In most later experiments the agar No. 1 was replaced by nutrient agar (22.8 g) following the method of Gabriel (1968).

Table 2. Enzymes detectable by the API ZYM test

No.	Enzyme assayed for	Substrate
1	Control	
2	phosphatase alkaline	2-naphthyl phosphate
3	esterase (C4)	2-naphthyl butyrate
4	esterase lipase (C8)	2-naphthyl caprylate
5	lipase (C14)	2-naphthyl myristate
6	leucine arylamidase	L-leucyl-2-naphthylamide
7	valine arylamidase	L-valyl-2-naphthylamide
8	cysteine arylamidase	L-cystine-2-naphthylamide
9	trypsin	N-benzoyl-DL-arginine-2-naphthylamide
10	chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide
11	phosphatase acid	2-naphthylphosphate
12	phosphoamidase	naphthol-AS-β1-phosphodiamide
13	α-galactosidase	6-Br-2-naphthyl-βD-galactopiranoside
14	β-galactosidase	2-naphthyl-βD-galactopyranoside
15	β-glucuronidase	naphthol-AS-β1- βD-glucuronic acid
16	α-glucosidase	2-naphthyl-αD-glucopyranoside
17	β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
20	α-fucosidase	2-naphthyl-αL-fucopyranoside

The nutrient agar and skimmed milk were prepared at double strength and autoclaved separately (nutrient agar 15 min at 121°C, and the skimmed milk 5 min at 110°C).

Clearing of the medium around the colonies indicated proteolytic activity. Results are expressed in terms of mm diam. cleared.

(ii) Chitinase activity. Chitinase activity was initially determined with a medium comprising basal salts solution, 0.02% yeast extract, 0.2% dry weight colloidal chitin and 2% Oxoid agar No. 1 adjusted to pH 5.5.

Plates were prepared in the manner described by Hankin and Anagnostakis (1975) with an underlay of 1.5% agar and a 3 mm overlay of the chitin medium. The test medium was prepared at double strength without agar and autoclaved separately (15 mins, 121°C). The components were cooled to about 50°C before mixing. Clearing of the medium around the colonies indicated chitinase activity.

(iii) Peroxide formation. Peroxide formation was detected on a heated haemoglobin agar medium (Koenigs, 1974). The basal medium consisted of 2% malt extract, 2% glucose, 0.1% peptone (MEL) and 2% Oxoid Agar No. 1 (MEA) in 900 mls distilled water. After autoclaving (15 min, 120°C) it was cooled to 80°C and a suspension of bovine haemoglobin powder (Sigma) (11% w/v) was added slowly with stirring. The medium was held at 80°C for 10 min, cooled to 45°C and adjusted to pH 6.5 with 1 N

NaOH before pouring into plates. Peroxide production was inferred when a green discolouration developed beyond or under colonies (Koenigs, 1974).

Of the 47 compounds which form coloured complexes with methemoglobin only sulfhemoglobin (absorbance maximal at 618 nm) alkaline hematin (absorbance maximal at 610 nm) and the discolouration due to H_2O_2 are green (Miale, 1967). To check that the green discolouration was due to H_2O_2,MEL with 5.5 g Haemoglobin powder ℓ^{-1} was prepared (Koenigs, 1974). The medium (HL) was autoclaved stirred and distributed 50 ml/flask. Flasks were inoculated and after 7 d of still culture incubation at 25°C, culture filtrate was carefully withdrawn and an absorbance spectrum determined from 360-660 nm against a blank of MEL. The spectrum for each strain was checked for absence of absorption peaks at 610 and 618 nm.

The criterion for measuring peroxide or enzyme production on plates were 1) the time interval between inoculation and the first signs of clearing or discolouration, and 2) the size of haloes. Enzyme and peroxide production was expressed semi-quantitatively by determining the colony diameter/halo ratio.

12) Carbohydrase Estimation

a) Total reducing sugars. Reducing sugars were determined with Nelson's (1944) modification of the Somogyi method observing the precautions outlined by Marais, De Wit and Quickie (1966).

Samples to be analysed were generally diluted to give a concentration of < 100 μg ml^{-1} NAG equivalents. A standard curve 0-500 μg ml^{-1} was prepared for NAG.

b) Total amino sugars. Soluble amino sugars were determined by the method of Smith and Gilkerson (1979) using 3-methyl-2-benzothiazolone hydrazolone hydrochloride (MBTH, Sigma). Samples to be analysed were generally diluted to give a concentration of < 50 μg ml^{-1} NAG equivalents. Standard curves 0-150 μg ml^{-1} were prepared for NAG and glucosamine.

c) N-acetylglucosamine determination. NAG was determined with Reissig *et al.*'s (1955) modification of the Morgan-Elson procedure. Samples to be analysed were generally diluted to give a concentration of < 150 μg ml^{-1} NAG. A standard curve 0-300 μg ml^{-1} was prepared.

13) Protein estimation

a) Protein in culture filtrates and extractions was estimated by the Bio-Rad method. This is reported to be more convenient and less liable to interference than other assay techniques. (Bio-Rad Laboratories Manual; Bradford, 1976).

Stock Bio-Rad dye reagent (Bio-Rad Laboratories) was diluted with 4 volumes of distilled water and filtered (Whatman No. 1) prior to use.

Procedure: 5 ml of diluted dye reagent was added to test-tubes containing 0.1 ml of the sample to be assayed. After shaking, the tubes were stood for 15 min then the absorbance was read at 595 nm.

Samples to be assayed were generally diluted to give a concentration of < 1 mg ml^{-1} protein. A standard curve 0-1.4 mg ml^{-1} was prepared from bovine gamma globulin which provides a typical response with the dye reagent (Bio-Rad).

b) Protein levels in column eluates were usually estimated directly by measuring absorption at 280 nm.

14) Amino-acid estimation

Amino acids in solution were estimated by the ninhydrin method (Moore and Steine, 1948). Stock ninhydrin reagent was prepared by mixing equal volumes of a 4% (w/v) solution of ninhydrin in methyl cellosolve with citrate buffer (0.2 M, pH 5.0) containing $SnCl_2.H_2O$ (0.2%, w/w).

Procedure: 1 ml of ninhydrin reagent was added to 0.1 ml of the solution to be assayed and then heated at 100°C for 20 min. 5 ml of a mixture of equal volumes of n-propanal and water was added and the tubes were allowed to stand for 15 min. The absorbance was read at 570 nm. Samples to be analysed were generally diluted to give a concentration of <100 µg alanine equivalents ml^{-1}. Standard curves were prepared for alanine, glycine, phenylalanine and tyrosine.

15) Paper Chromatography

Paper chromatography was used routinely to detect mono- and oligo-saccharides in enzyme digests.

Whatman No. 1 paper was adapted for continuous descending chromatography by cutting the bottom edge of the paper into a series of uniform points. This enabled the solvent to drip evenly off the paper when the boundary reached the serrated edge (Partridge, 1949). Developing tanks were lined with filter paper saturated in the solvent for several hours before chromatograms were developed (at *c.* 23°C).

Two solvent systems were used to develop chromatograms:

A. Butanol-ethanol-water-NH_3 4:10:49:1 (v/v) (Berger and Reynolds, 1958)

B. Pyridine-Pentan-2-ol-water 1:1:1 (v/v) (Waterhouse Hackman and McKellar, 1961).

Although system B gave the best separation in ascending chromatography both solvents gave similar degrees of resolution with the descending system.

10 µl test samples were applied to the paper along with reference samples of NAG (2% w/v), glucosamine (2% w/v), chitobiose (0.5%, w/v), chitotriose (0.5%, w/v) and chitotetraose (0.5%, w/v). The area of application was reduced by rapid drying in a stream of hot air. Buffer salts in test samples caused tailing of the spots and slight lowering of Rf values. This problem was reduced by keeping buffer salts to a minimum and

where possible performing controls with reference samples incorporated into enzyme digests (co-chromatographed).

After developing with solvent for up to 32 h papers were sprayed with aniline hydrogen phthalate reagent (Partridge, 1949) (prepared by adding 0.93 g aniline and 1.66 g phthalic acid to 10 ml of water saturated butanol, and colour developed by heating for 5 min at 105°C. Mobilities of the oligomers relative to that of NAG (R_{NAG}) were, respectively, 0.54 for the dimer, 0.34 for the trimer and 0.13 for the tetramer.

16) Enzyme purification

a) Fractional precipitation with ammonium sulphate

Ammonium sulphate was used to salt out proteins from the extracellular fluid of fungal cultures grown on either cuticle, ground chitin or casein.

The method was modified from Cooper (1975). Solid $(NH_4)_2SO_4$ was added stepwise to stirred filtrates (pH 6.0, 21°C) to give the following concentrations (% w/v): 20, 40, 60, 80, 100. At each concentration the precipitated protein was collected after 2 h by centrifugation (23, 000 x g 20 min, 8°C). The supernatant was used for the next precipitation and each precipitate was dissolved in a minimum volume of water (pH 6.0). The fractions were then dialysed (24 h, 4°C, vs 300 vol distilled H_2O) and made up to equal volumes before enzyme assay or storage at -20°C.

b) Iso-electric focussing

i) Column focussing. Protein obtained from the ammonium sulphate precipitation step were exhaustively dialysed before fractionation by iso-electric focussing in a 110 ml column (LKB 8101). The pH gradient (pH 3-10) was provided by carrier ampholytes at a final concentration of 1% (w/w). The apparatus was set up as described in the manufacturer's instructions and operated at 4°C. The following solutions were used:

Cathode solution: 1 pellet (*c.* 0.1 g) of NaOH in 10 ml distilled water;

Anode solution: 0.2 ml conc. H_3PO_4, 15 g sucrose and 12 ml distilled water;

Light gradient solution: 1.3 ml pharmacia "pharmalyte" ampholine pH 3-10 in 51.7 ml sample and distilled water;

Dense gradient solution: 27 g sucrose, 1.4 ml ampholine and 35.7 ml distilled water.

Procedure: The sucrose gradient was formed and introduced into the column as described by the manufacturers. *M. anisopliae* cultures contain large amounts of acidic proteins (see plate 6) so the cathode was placed at the top of the column (Cooper and Wood, 1975) ensuring that any precipitation did not disrupt the density gradient in the rest of the column.

The power pack (LKB 2103) was set initially at 3 KV, 3W. The voltage was increased to 10 KV after the current had fallen to *c.* 5mA. The run was then continued until the current stabilised at *c.* 3 mA

The column was emptied by an LKB peristaltic pump and the eluant collected as 4 ml fractions (LKB redirac). The pH gradient was determined immediately at 4°C. Before enzyme analysis fractions were exhaustively dialysed to remove sucrose and most of the carrier ampholytes.

ii) a. Flat bed gel iso-electric focussing. Iso-electric focussing was performed at 8°C with an LKB 2117 multiphor on ready-prepared LKB thin-layer (1 mm) polyacrylamide gels (PAG plates) which contained ampholine carrier ampholytes to give pH range 3.5 to 9.5. The PAG plates and multiphor were handled according to the manufacturer's instructions. 19 µl samples containing 1-5 mg protein/ml were applied on the gel near the cathode electrode with small pieces (*c.* 10 mm) of chromatography paper. Over 1 h the voltage was gradually increased from 2.5 KV up to 12 KV and maintained at that for 30 min. The sample application papers were removed after 30 mins to prevent tailing of the bands. The pH gradient was determined at points along the gel with a surface pH electrode. The zones were refocussed for 10 min after measuring the pH.

ii)b Detection of proteins on gels. The method is based on that described in the manufacturer's (LKB) instructions.

Prepared solutions:

A) Fixing solution: 57.5 g trichloroacetic acid and 17.25 g sulphosalicylic acid were added to 500 ml distilled water.

B) Destaining solution: 500 ml ethanol and 160 ml acetic acid were mixed and diluted to 2 litres with distilled water.

C) Staining solution: 0.46 g Coomassie Brilliant Blue R-250 was dissolved in 400 ml destaining solution (B).

D) Preserving solution: 40 ml of glycerol was added to 400 ml of the destaining solution.

Procedure:

Immediately after focussing the PAG plates were lowered into solution A for 45 min and washed (5 min) in solution B. The destaining solution was then removed and plates soaked for 10 min at 60°C in solution C. Following this plates were washed and soaked with several changes of solution B until the background was clear. To make a permanent preparation the destained PAG plates were placed in solution D for 1 h and allowed to dry on a glass plate.

(ii)c. Detection of esterases on gels. Immediately after focussing PAG plates were lowered into a 100 ml solution of 0.1 m K phosphate buffer at pH 7.0,containing 50 mg fast Blue BB and 8 mg of each naphthyl ester dissolved in 2 ml of acetone. The esters used either separately or together were α naphthyl acetate, β naphthyl acetate, α naphthyl propinate, α naphthyl caprylate, and α naphthyl myristate enzyme activity was detected after 1-60 min.

(ii)d. Detection of aminopeptidases on gels. Immediately

after focussing the gels were lowered into a 100 ml solution of 0.1 M K phosphate buffer at pH 7.0 containing 50 mg Fast blue BB to which was added 10 mg L-alanyl 4 methoxy-β-naphthylamide and/or 10 mg L-leucyl-4-methoxy-β-naphthylamide. Enzyme activity was detected after 1-60 min.

c) Sephadex G-100 gel column chromatography

Proteins obtained from ammonium sulphate precipitation were fractionated by Sephadex-G100 descending chromatography using a 60 x 2.5 cm column (LKB) at 4°C, set up as described in the manufacturer's instructions. The top adaptor of the column was connected *via* a three way valve to a Mariotte flask which served as the buffer reservoir. The bottom adaptor was connected *via* an LKB peristaltic pump to an LKB Redirac fraction collector.

The general procedure for performing gel filtration was that described by Pharmacia in their Manual "Gel Filtration, theory and practice". The sephadex G-100 superfine was supplied as a dry powder. Before use it was allowed to swell in an excess of K phosphate buffer (0.15 M, pH 6.5) for 7 h at 95°C. The gel was then cooled (4°C) and allowed to settle for 30 min. The surface layer was decanted so that the suspension that remained was a fairly thick slurry - though not so thick as to retain air bubbles.

The gel bed was poured in one continuous operation and stabilised by allowing 2-3 column volumes of degassed K phosphate buffer to be passed down the column. The flow of eluant was limited by the peristaltic pump to 8.5 ml/h. When the gel

was settled the bed height was 48.7 cm giving a volume of 239.05 cm^3. The homogeneity of thebed was checked before use by running through a 2 ml solution of blue dextran 2000 (2 mg/ml) applied to the top of the gel *via* the 3-way valve and eluted with K phosphate buffer at 7 ml/h. The dextran moved through the bed as a narrow band indicating that the gel was uniform. In addition the large polymer (detected by absorbance at 280 nm in 3.3 ml fractions) allowed determination of the void volume (V_o = 66 ml).

Protein solutions (2 ml samples containing *c.* 1% protein) were added to the gel and 3.3 ml fractions were collected. Each fraction was assayed for protein (absorbance at 280 nm) and (following dialysis) enzyme activity. The K phosphate buffer (0.15 M, pH 6.5) used for gel swelling and elution was always degassed under vacuum before use, and contained Merthiolate (0.005%) to prevent microbial contamination.

d) Purification of chitinase by adsorption on colloidal chitin

The procedure was adapted from that of Cornelius., Dandrifosse and Jeuniaux (1976) and is based on the specific adsorption of chitinase to its insoluble substrate (Sect II, 9a).

Those fractions from gel filtration which contained high chitinolytic activity were pooled and concentrated by dialysis against polyethylene glycol. Colloidal chitin (1 mg chitin/5 chitinase units) was added and the suspensions were stirred

at 23°C for 15 min. The chitin was removed by centrifugation (6,000 g, 10 min) and fresh aliquots of chitin were introduced sequentially to achieve total adsorption of the enzyme. After removal of each chitin aliquot by centrifugation the sediments were suspended separately with stirring for 15 min in K phosphate buffer (0.2 M, pH 7.0) to remove ionically-bound enzyme. The suspensions were centrifuged and the washed residues were resuspended in the buffer, then incubated at 30°C in the presence of toluene. Every 3 h the suspensions were centrifuged and the sediments were resuspended and incubated in fresh buffer at 30°C until complete solubilisation of chitin as a result of enzyme action was obtained. All the supernatants with high chitinolytic activity were pooled and dialysed against polyethylene glycol.

17) Determination of molecular weights and Stokes radius (R_s)

a) Molecular weights

The molecular weight of enzymes was estimated by gel filtration with Sephadex G-100 (Andrews, 1964) and the following marker proteins: ribonuclease (Mw 13,700, Stokes radius R_s Å 16.4), Chymotrypsinogen A (Mw 25,000 R_s 20.9), ovalbumin (Mw 43,000 R_s 30.5), albumin (Mw 67,000, R_s 35.5) and aldolase (Mw 158,000,R_s 48.1). The standards were dissolved in 0.15 M K phosphate buffer, pH 6.5 and loaded (2 at a time) on a column of Sephadex G-100 (2.5 x 48.7 cm) equilibriated with the same buffer. Elution was made at a flow rate of 7 $ml^{-1}h^{-1}$. For each protein standard and enzyme the diffusion coefficient $K_{av} = V_e - V_o/V_t - V_o$) was calculated (Laurent and Killander,

1964), where V_e is the protein elution volume and V_o and V_t the void and total volumes, respectively, of the gel bed (see previous section). For the standards K_{av} was plotted against M.W. on semi log paper (Fig. 3) and from the curve the MW of each enzyme was read.

The standard proteins formed a straight line over the molecular weight range 13,700 to 67,000 daltons; the useful range of the gel extended up to at least 158,000 daltons. The results provided were obtained with one column, but data from a second column fitted almost identical curves.

b) Stokes radius (R_s)

Stokes radius (R_s),"the radius of a sphere of equal area to the enzyme", was calculated by gel filtration using Sephadex G-100 (siegel and Monty, 1966) and the marker proteins listed above. For each standard and enzyme $\sqrt{-\log (K_{av})}$ was calculated, where K_{av} is the diffusion coefficient. For the standards the resulting values were plotted against R_s (Fig. 4) and from the curve the R_s of each enzyme was calculated.

18) Pathogenicity and symptomatology of *M. anisopliae* (ME1).

Adult flies, *Calliphora vomitaria* 24 h after emergence from the pupae were used in this study.

To confirm the pathogenicity of *M. anisopliae* (ME1)(the isolate used throughout most of this study), flies were distributed

Fig. 3. Calibration curve using molecular weight markers on Sephadex G-100 fine.

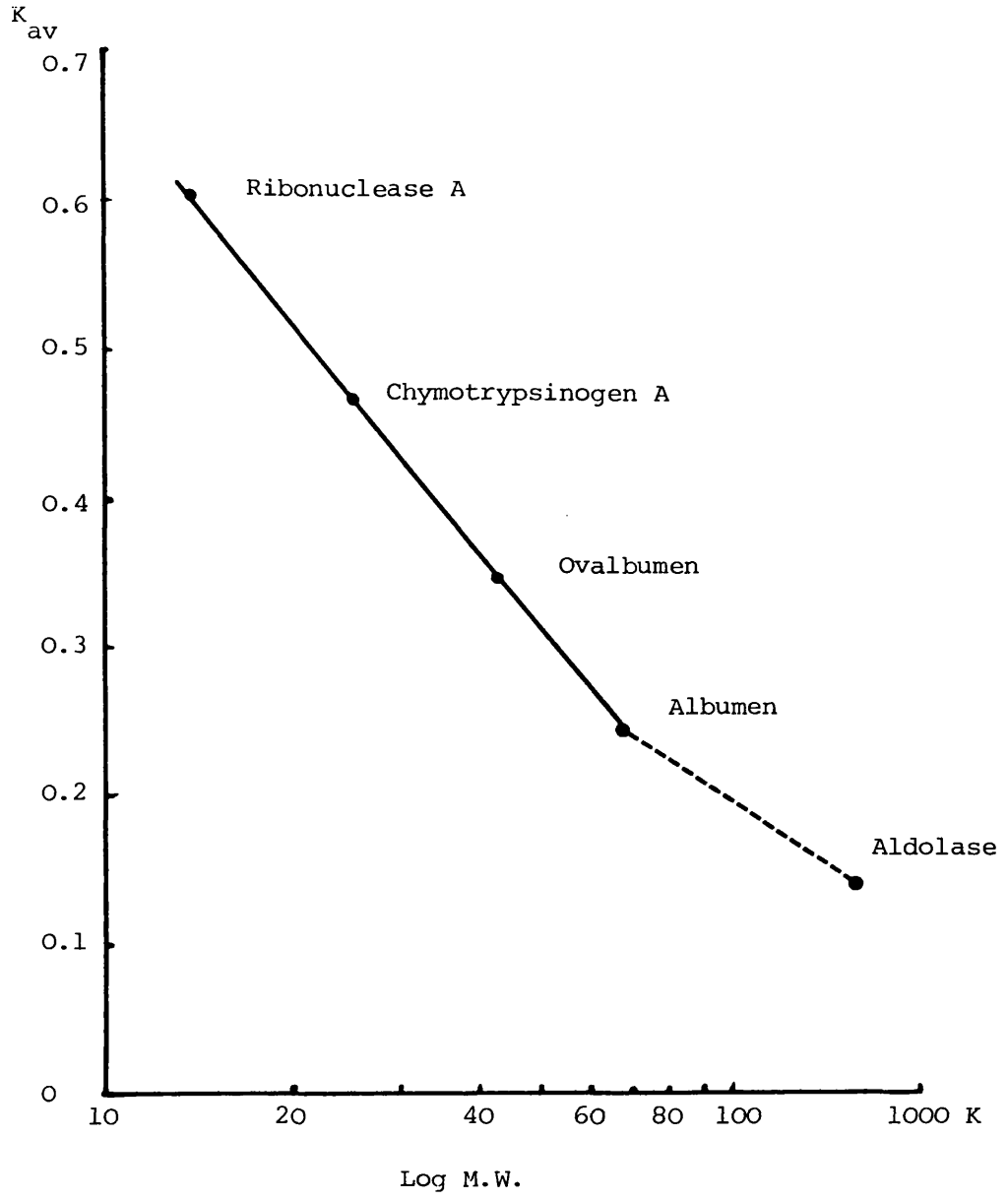
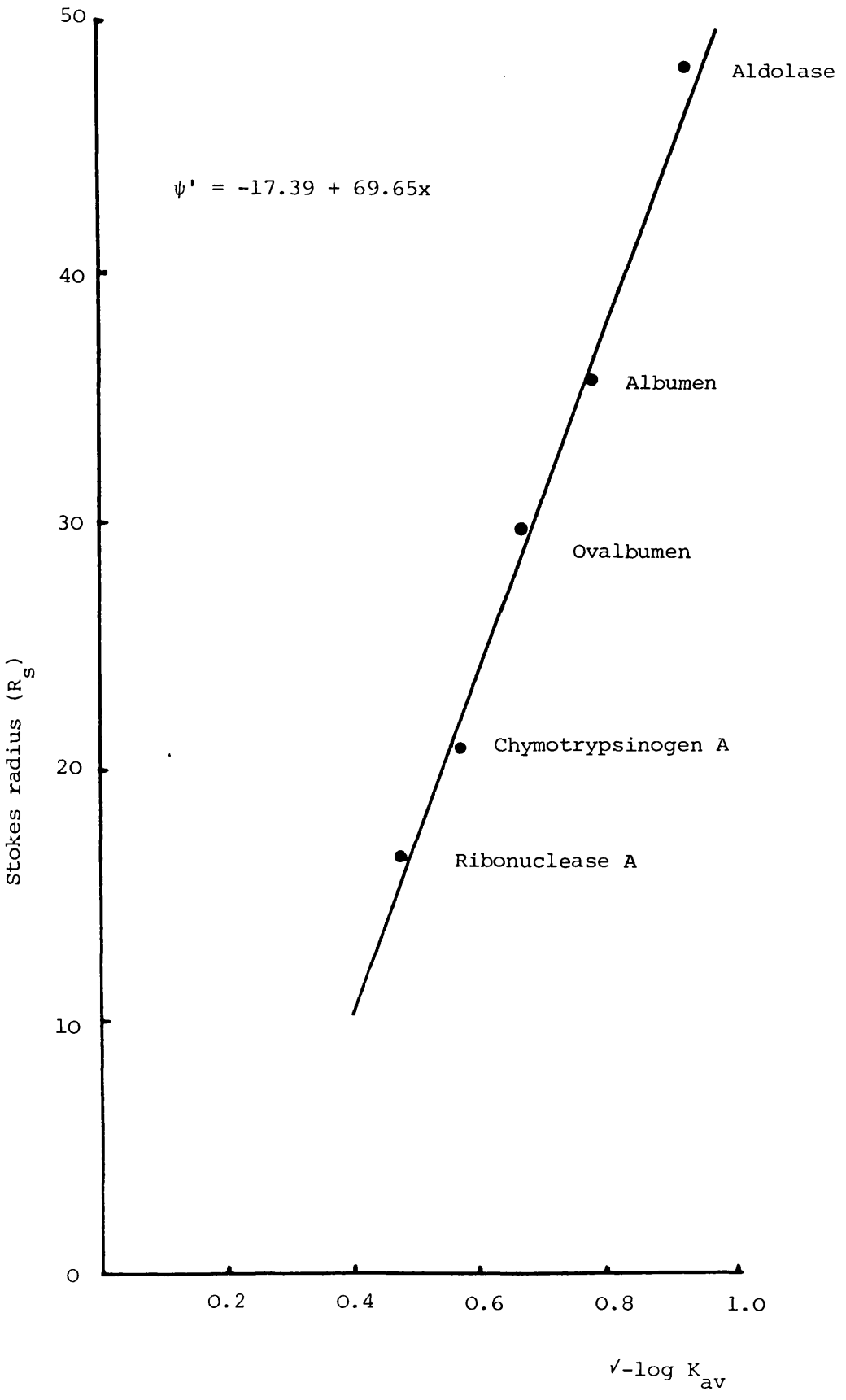


Fig. 4. Calibration curve using R_s markers on Sephadex G-100 fine.



in beakers each of which contained cotton wool moistened with a suspension of conidia (10^4/ml) in sterile tap water-sucrose (1%). The beakers were kept in daylight at room temperature. Control insects were kept under identical conditions except that they were not exposed to conidia.

At the onset of symptoms samples of flies were taken for dissection and examination to look for fungal growth. Mortality was recorded daily and dead insects were dissected.

19) Conidial behaviour on cuticle

The behaviour of conidia on the surface of cuticle was studied by applying conidia to blowfly wings which are relatively transparent and thereby allow use of conventional light microscopy.

Whole insects were surface sterilised in 5% sodium hypochlorite (5 min) and rinsed with 4 changes (5 min each) of sterile distilled water. Wings were then dissected away and placed on the surface of water agar plates. For most experiments 10 µl containing *c.* 400 conidia was pipetted on to the exposed upper surface of each wing. When the inoculum was from a suspension more concentrated that 10^4 spores or when spores were applied dry, there was a tendency for clumping to occur, especially along the wing veins.

The plates were incubated at 27°C for about 30 h. Infection sites were examined periodically, stained with 1% lactophenol

cotton blue and the % germination determined.

20) Extraction of enzymes from blowfly wings

Blowfly wings were extracted at various times after infection (c.1,500 spores/wing) using K phosphate buffer (0.2 M, pH 7.0) in the proportion 2 wings ml^{-1} buffer. After vigorous shaking for 1 h at $4^{o}C$ the preparation was centrifuged (5,000 g, 10 min $4^{o}C$) and the supernatant dialysed (200 vol dist water, pH 6.0). In most cases the supernatant was then concentrated by dialysis against polyethylene glycol (M.W. 20,000) before assaying for enzyme activity.

In some experiments frozen wings were finely comminuted using a pestle and mortar with liquid nitrogen. The ground tissue was extracted in the same way as whole wings.

21) Histological techniques

a) Light microscropy

(i) Samples were viewed and photographed through a Leitz-orthoplan Weitzlar microscope. Monochrome and colour prints were made with Panatomic X and Kodachrome 64 respectively.

(ii) Thin sections of unfixed cuticle for enzyme histochemistry were prepared using a cryostat. This consisted of a Cambridge rotary rocking microtome contained within a refrigerated cabinet maintained at $-17^{o}C$.

Infected insects were killed and frozen in dry ice and < 3 mm transverse sections of the body were cut with a cleaned razor blade. Chosen sections were mounted in a small quantity of 1% glucose on the object holder and frozen into place by the manual application of two pieces of dry ice either side of the holder. The object holder was attached to the microtome and the tissue trimmed to its full face with one end of the knife. The blade was then moved to give a fresh cutting edge and sections (7-10 µm) cut with the anti-roll guide plate in position. A thoroughly cleaned slide was carefully touched flat against each section which adhered to the slide as it thawed. No adhesive was necessary.

For enzyme histochemistry the incubation medium was delivered directly on to the unfixed section on the slide.

b) Enzyme Histochemistry

(i) Lipase

(i)a. The Tween method. Water-soluble esters which on hydrolysis give proeucts having insoluble Ca salts have been used for the detection of lipases (Gomori, 1945, 1949). These conditions are fulfilled by the Tweens which are fatty acid esters of polyglycols and polysorbital.

True lipases hydrolyse the esters of both saturated and unsaturated fatty acids, while typical esterases only attack the esters of saturated fatty acids (Gomori, 1949). To distinguish between the two activities Gomori recommended use of

Tween 80 (an unsaturated oleic acid ester) as substrate for "true lipase" and Tween 60 (a saturated stearic acid ester) or Tween 40 (a saturated palmitic acid ester) as substrates for lipase/ esterase.

Incubating medium comprised 5 ml of Tris buffer (0.5 M, pH 7.3), 2 ml of calcium chloride solution (10% w/v), 2 ml Tween solution (5% (w/v), T_{40}, T_{60} or T_{80}) and 40 ml of distilled water.

Procedure (modified from Gomori, 1945).

1. preparations of infected cuticle were fixed in ice cold formalin (3 h) and incubated for 3-12 h at 30°C;
2. washed thoroughly in distilled water;
3. placed in 1% lead nitrate for 15 min;
4. washed in running water for 5 min;
5. treated with 1% ammonium sulphide for 1 min;
6. washed thoroughly in distilled water, mounted on slides and observed immediately.

A positive result was indicated by a brownish black deposit of lead sulphide.

(i)b. Naphthol AS-nonanoate method for lipase. Broadly speaking the esters of short chain fatty acids (C_2-C_4) are acted upon by esterases, and the long chain esters (C_8 and upwards) by lipases (Shnitka, 1974). Abe, Kramer and Seligman, (1964) reported the use of the long chain ester naphthol AS-nonanoate (C_9) in the histochemical detection of lipase.

Procedure: (modified from Abe *et al.*, 1964) Preparations of infected cuticle were incubated at 30°C for 1 to 4 h in a medium which comprised: 5 ml 0.4 M Tris buffer (pH 7.4), 4.9 ml distilled water, 0.1 ml 2% naphthol AS-nonanoate in dimethyl acetamide and 10 mg fast blue BB salt . The turbid solution was filtered before use. After incubation cuticle preparations were washed thoroughly in distilled water for 10 min, mounted on slides and observed immediately.

A positive result was indicated by a blue/violet colouration.

ii) α-Naphthyl ester method for esterase (after Gomori, 1950)

Esters of short chain fatty acids were used as substrates for esterase activity. A reagent comprising 10 mg α-naphthyl acetate (C_2) or α-naphthyl proprionate (C_3) in 0.25 ml acetone was added to 20 ml of 0.1 M K phosphate buffer, pH 7.4 and shaken vigorously until most the initial cloudiness had disappeared. 75 mg fast blue BB was then added, shaken, and the medium filtered directly on to preparations of infected cuticle. After incubation for 1-15 min at R.T. the cuticle was washed thoroughly in water, mounted on slides and observed immediately. A positive result was indicated by a violet colouration.

iii) Naphthylamide method for proteases. Trypsin-like activity was determined by naphthylamidase activity against N-benzoyl-L-arginine β-naphthylamide HCl, N-benzoyl-diglycyl-L-arginine β-naphthylamide and N-benzoyl-diglycyl-L-arginine 4-

methoxy-β-naphthylamide. Chymotrypsin-like activity was determined with N-benzoyl-DL-phenylalanine β-naphthylamide.

Procedure: preparations of infected cuticle were incubated for up to 2 h at R.T. in 20 ml tris buffer (0.1 M, pH 7.4) containing 20 mg substrate and 10 mg Fast garnet GBC or Fast blue BB. The preparations were washed thoroughly in distilled water and then transferred for 5 min to a solution of 0.1 M cupric sulfate. After rinsing in water they were mounted on slides and observed immediately. A positive result was indicated by red azo dye precipitates.

iv) Naphthylamide method for aminopeptidase. Aminopeptidase was localised by activity against L-leucyl-4-methyoxy-β-naphthylamide HCl (LMN) or L-alanyl-4-methoxy-β-naphthylamide (AMN) by an adaption of the method of Monis, Wasserkrug and Seligman (1965).

Preparations of infected cuticle were incubated for 5 min to 4 h at 30°C in the following medium:

Phosphate buffer (0.1 M, pH 7.0)	5.0 ml
Substrate solution (LMN, 10 mg ml^{-1}; AMN, 8 mg ml^{-1})	0.5 ml
Saline, 0.85%	4.0 ml
Fast blue BB salt	10 mg

After incubation the preparations were rinsed in saline and

then transferred for 5 min to a solution of 0.1 M cupric sulfate. After washing in water they were mounted on slides and observed immediately. A positive result for aminopeptidase was indicated by a pink or red azo dye precipitate.

v) Naphthol AS-Bl-Garnet GBC method for Acetyl-β-glucosaminidase activity (after Hayashi, 1965). A solution comprising 3 mg naphthol AS-Bl-N-acetyl-β-glucosaminide in 0.5 ml ethylene glycol monomethyl ether was added to 9.5 ml of citrate buffer (pH 5.0, 0.1 M), and shaken vigorously. 10 mg of Fast Garnet GBC salt was added and the medium filtered directly on to preparations of infected cuticle. After incubating for up to 4 h at 30°C, the cuticle was washed thoroughly in water, mounted on slides and observed immediately. A positive result was indicated by a brick red precipitate.

Controls for each histo-enzymological test were produced by heating infected cuticle for 15 min at 80°C to inactivate enzyme. Additional controls were provided by omitting an essential factor from the incubation medium such as the substrate or diazonoium salt or $CaCl_2$ for the Tween media.

Whenever the incubation period exceeded 4 h toluene was added to reaction mixtures which were tested subsequently for bacterial contamination.

c) Detection of β-glucans and chitin by fluorescence staining

(i) Calcofluor M2R New. Fungi and cuticles were stained

at R.T. for 1 min in a 0.05% (w/v) solution of calcofluor white M2R New. The stained material was then waashed 5 times for 1 min each in 0.1 M K phosphate buffer pH 7.0 (Kritzman, Chet, Henis and Hüttermann (1978). Stained and unstained material was observed with a Leitz orthoplan Wetzlar fluorescence microscope using an excitation filter with 390 - 490 nm transmission and a barrier filter of 515 nm.

ii) Fluorescein isothiocyanate conjugated wheat germ agglutinin (FITC-WGA) (after Mirelman, Galun, Sharon and Lotan, 1975). Fungi and cuticles were stained at RT for 10 min with 200 µg FITC-WGA (Sigma) in 0.4 ml phosphate-buffered saline (0.02 M K phosphate buffer, pH 7.4 containing 0.85% NaCl). The preparations were then washed with saline and observed under UV illumination as for Calcofluor.

d) Scanning electron microscopy

Direct observations by light microscopy of infection of blowfly wings by conidia was supplemented by scanning electon microscopy using cuticle from *S. gregaria* and *M. sexta*.

Sections of cuticle were removed from 5th instar larvae of *M. sexta* or *S. gregaria* and the inner surface scraped free of host tissue with a cleaned scalpel blade. The excised cuticle was surface sterilised in 5% sodium hypochlorite (5 min) then washed with four changes of sterile distilled water. After drying the cuticle was placed in a sterile perspex cell (Robinson, 1966) so that part of it was stretched across a small aperture (2 mm diameter). A 10 µl suspension of conidia (∿ 400 spores) of

M. anisopliae (ME1) was placed on the exposed upper surface and the cells were incubated at 27°C and 100% RH. In some experiments the lower chamber of the cell was filled with isotonic locust saline (Appendix I).

After incubating for up to 72 h, segments of cuticle were removed from cells, fixed for 12 h by 3-4% glutaraldehyde in cacadoylate buffer (0.1 M, pH 7.2), then washed and further fixed for 1 h in 1% osmium tetroxide in cacadoylate buffer (0.1 M, pH 7.2). The samples were dehydrated in a graded series of acetone, then critical-point dried in a Polaron E3000, and coated with gold in a Polaron sputter coater. Upper and lower surfaces of the cuticle were viewed and photographed (FP4) through a JEOL J5M-35C scanning microscope at 15 KV.

22) Chemicals

All chemicals used were of analytical grade unless otherwise stated, and were supplied by Sigma or BDH Ltd.

RESULTS AND DISCUSSIONS

Section I

Regulation of enzyme production in culture by entomopathogenic fungi

1. General growth characteristics of *M. anisopliae* (ME1)

As most of this investigation centred on *M. anisopliae* (ME1) its general physiology was investigated in shaken cultures to obtain information about the effect of cultural conditions on growth and as a prerequisite to more critical studies on enzyme production.

The pathogen had no special growth factor requirements and grew in a 1% sucrose/basal salts medium containing ammonium, nitrate or urea as sources of nitrogen. Proteins, peptides, amino acids, chitin, chitosan and amino sugars were utilised as both carbon and nitrogen sources. The organism grew maximally at pH 5.5 to 6.5 but low levels of growth still occurred at pH 4.0 and pH 8.5. Growth on SDA plates was more rapid at 27°C than 23°C (*c.* 70% of growth at 27°C) or 35°C (*c.* 42% of growth at 27°C).

Various carbohydrates supported the growth of ME1. Rapid growth was obtained on glucose, dextrose, arabinose, cellobiose and several polysaccharides containing β-glucosidic bonds. Starch and glycogen were also utilised which shows that the fungus possess amylase. *M. anisopliae* presumably produces β-galactosidase as growth occurred on lactose, albeit comparatively slowly.

Growth on carbohydrates did not result in "appreciable" acid production in shake cultures (initial pH 6.0). With nitrate, cultures generally reached a higher pH value (8.0 - 8.5) than with growth on ammonium salts (*c.* 7.5). Presumably the alkalinity of the medium was reduced as ammonium ions wereutilised. Other carbon sources such as lanolin (a wax), olive oil, coconut oil, lecithin (a phospholipid) and long chain fatty acids also supported growth. Most fatty acids are virtually insoluble but growth occurred on the crystals themselves. Growth on carbohydrates, proteins and amino acids will be described in more detail later (Sect. I.3.6a, 7b).

The form of growth varied with cultural conditions and inoculum level e.g. with lactose as sole carbon source growth comprised a tangle of hyphae dispersed through the medium. In most "simple" media (i.e. containing only one carbon source) growth was in the form of macroscopic, roughly spherical pellets, the morphology of which depended on the carbon source. With sucrose,pellets were small (< 1.0 mm) and compact, and were of similar dimensions on proteins and N-acetylglucosamine (NAG), but more diffuse. Pellets were large and very diffuse and there was some associated filamentous growth with pectin and oils. When the media contained large amounts (> 2% w/v) of utilisable insoluble carbon sources (e.g. chitin or cuticle) growth was as a mass of mycelium interspersed between and binding onto the particles. In medium containing smaller quantities of chitin or cuticle (< 2%) growth was mainly as pellets.

The effect of inoculum level on growth form again depended on the carbon source, but generally with < 10^5 spores cultures were characterised by a small number of large pellets of up to 3 mm diam. (in 7 d). Higher inoculum loads resulted in a larger number of small pellets.

With complex media such as that described by Samsinakova (1966) for *B. bassiana* and in a locust homogenate growth was mainly as blastospores and short hyphae. With this form of growth it was possible (with a haemocytometer) to accurately measure growth even in the presence of an insoluble substrate such as chitin. Unfortunately a medium was not found which both resulted in growth in this form and allowed high chitinase activity.

As growth with chitin and locust cuticle (< 2% w/v) was largely as pellets, the way they were formed was studied. The medium used contained 1 or 2% chitin as sole carbon source. Soon after inoculation many spores aggregated into small clumps. Germination usually began about 10 h after inoculation but was not synchronous. Each conidium generally produced one or occasionally two germ tubes which elongated to form branched hyphae. By about 48 h loose pellets were visible which by 72 h had reached *c.* 1.2 mm diam.

Pellet formation seemed to result from two factors: aggregation of conidia, and the entanglement of the long branched hyphae produced from conidia. The addition of Tween 20 to the medium (0.2% v/v) prevented the aggregation of conidia and this

resulted in an increased number of very small pellets (see Table 9).

Although the pellets continued to increase in diameter up to about 3 weeks their centres showed signs of lysis and death (change in colour from tan to grey and appearance of large vacuoles) after only 7 d. Obviously this growth form could lead to large differences in the physiology of the outer and inner hyphae of pellets. With some carbon sources such as cuticle and certain protein complexes the small colonies were so numerous that the rotary action of the shaker was reduced by the increased viscosity. This presumably would have affected the rate of diffusion of nutrients and gases.

After several days cultures often became light yellow to dark brown depending on the amount of growth and culture conditions. The pigment(s) appeared loosely bound to the fungal mycelium as it was partly released into the medium during growth, and could be washed out of the mycelium completely by centrifugation.

Other isolates of *M. anisopliae* and isolates of *B. bassiana* and *V. lecanii* were also investigated. The pellet growth form of ME1 appeared to be typical of *B. bassiana* and *M. anisopliae* isolates when these were grown in NAG and casein. In contrast young cultures (< 4 d) of *V. lecanii* contained large numbers of blastospores (*c.* 10^6-10^7 spores/ml) but pellets were produced in older cultures.

2. Composition of locust cuticle

It was considered necessary to determine the composition of locust cuticle before critical studies concerning growth on cuticle, regulation of cuticle degradation and penetration of cuticle could be conducted.

Ground cuticle prepared with potassium tetraborate (Materials and Methods) contained *c.* 26% w/w chitin. Amino acid analysis of ground cuticle, of the cuticle fraction solubilised into basal media by heat sterilisation, and of exuviae (5th instar nymphs) revealed that the same 15 amino acids were present but in differing proportions (Table 3). Alanine was the major amino acid in both exuviae (35.3%) and ground cuticle (27.1%); in this and other respects protein composition resembles that found in alternative sources of sclerotized cuticle (Andersen, 1973). The higher proportion of glycine in K borate prepared cuticle (16.5%) *cf* exuviae (10.7%) presumably derives from endocuticle in which this amino acid predominates (Andersen, 1973). The abundance of non-polar residues like glycine and alanine with small side chains may permit close packing of protein molecules (Andersen, Chase and Willis, 1973). Proline (*c.* 10.5%) and valine (*c.* 9.2%) were also reasonably abundant but the sulphur containing amino acids cysteine and methionine were not detected. Tyrosine (*c.* 3%) would be available for cross-linking of protein chains if oxidised *in situ* (Neville, 1975).

Amino acids solubilised by heat sterilisation probably come largely from endocuticle which their composition resembles,

particularly as regards the relative proportion of alanine and glycine (Table 3).

Table 3. Amino-acid composition (in number of residues per 100) of (I) finely powdered exuviae (II) finely powdered cuticle derived using 1% K tetraborate (Andersen, 1980) and (III) amino acids solubilised during heat sterilisation of (II)

	(I)	(II)	(III) [a)]
Aspartic acid	3.9	4.0	4.5
Threonine	2.2	2.6	2.8
Serine	3.6	4.5	5.1
Glutamic acid	3.0	5.2	5.7
Proline	10.2	11.5	16.2
Glycine	10.7	16.5	17.0
Alanine	35.3	27.1	18.6
Valine	9.6	8.9	7.9
Isoleucine	4.0	2.8	3.2
Leucine	6.8	6.2	5.8
Tyrosine	3.3	2.8	2.9
Phenylalanine	1.1	1.2	0.3
Histidine	2.2	2.2	5.1
Lysine	1.4	1.7	1.8
Arginine	2.7	2.8	3.1

a) The concentration of amino acids in freshly heat-sterilised media was 0.12 mM (as determined by the ninhydrin method).

3. Growth on Cuticle Components

As well as assiting penetration by solubilising cuticle polymers, enzyme action could release amino-acids and other compounds for utilisation by the fungi during infection (Leopold *et al.*, 1970: Samsinakova *et al.*, 1971). The ability of *M. anisopliae* (ME1) to grow on NAG and each of the amino acid components of locust cuticle as sole C and N source was measured in cultures containing 0.5 g/50 ml of buffered basal medium (Table 4). In addition cultures containing 1% beeswax and 1% oils were used to determine whether the lipoid epicuticle might be a potential source of nutrients. Good growth was supported by NAG and several of the amino acid components of cuticle (Table 4). The oils and wax also allowed good growth but only in the presence of 0.2% $NaNO_3$ as nitrogen source.

4. Synthesis of cuticle-degrading enzymes in culture on cuticle

A. The range of cuticle-degrading enzymes produced by entomopathogenic fungi.

To induce production of the potential spectrum of cuticle degrading enzymes, pathogenic isolates of *M. anisopliae*, *V. lecanii* and *B. bassiana* were inoculated (3×10^6 conidia/100 ml) into buffered liquid cultures containing 1% ground locust cuticle as sole carbon and nitrogen source.

The pathogens grew in this medium and produced a range of extracellular cuticle-degrading enzymes corresponding to the

Table 4. Growth of *Metarhizium anisopliae* (MEl) after 4 d in 50 ml of buffered basal medium containing 1% solutions of NAG or L-amino acids.

Carbon source	$\bar{x}$ weight (mg)
NAG	180.2 (197.7 - 166.9)[a]
Glutamic acid	134.3 (154.3 - 123.2)
Alanine	94.7 (108.0 - 83.4)
Aspartic acid	87.0 (95.2 - 81.6)
Serine	69.5 (77.3 - 61.4)
Valine	41.8 (48.5 - 37.2)
Proline	35.2 (37.4 - 32.8)
Phenylalanine	34.9 (37.3 - 33.4)
Lysine	24.5 (26.4 - 21.5)
Histidine	16.9 (20.5 - 11.3)
Isoleucine	14.9 (15.4 - 14.0)
Leucine	14.3 (15.8 - 12.5)
Glycine	13.2 (14.4 - 12.1)
Threonine	11.8 (12.1 - 11.2)
Arginine	11.5 (11.8 - 11.2)
Chitin	++++[b]
Tyrosine	++++
Beeswax[c]	+++
Olive oil[c]	+++
Tricaproin[c]	++
Trimyristin[c]	++

a) $\bar{x}$ dry wt of mycelia for 3 replicates. The range is given in parenthesis.

b) when the carbon source was insoluble or only partially soluble direct measurement was precluded, growth was estimated using an arbitrary scale ranging from + (poor growth) to +++++ (luxuriant growth)

c) 0.2% $NaNO_3$ added as nitrogen source

major components of insect cuticles *viz.* protein,chitin and lipid. Enzyme activities in 5 d culture filtrates are shown in Table 5. At 5 d, variation in proteolytic or chitinolytic enzymes between the 3 replicate flasks of each isolate was less than 14%. However a marked variation in enzyme levels in different isolates of *M. anisopliae* as well as between different species was found. This variation may inpart be accounted for by differences in growth rates and the sequential phasing of enzyme production (4B).

Endoproteases were exceptional in being produced in large amounts by all the isolates. Figs. 5 to 7 show pH activity curves from the 9 isolates. Maximum activity varied between pH 8.0 and 9.0 but *V. lecanii, B. bassiana* and *M. anisopliae* strains ME1, RS324, RS543 and RS23 showed a second minor peak of activity between pH 4.5 and 6.0. The activity curves in *M. anisopliae* isolates RS455, RS298 and RS549 showed only one peak but were asymmetrical with a plateau in the acid pH region. These results suggest that at least two proteolytic enzymes are produced on cuticle which together give activity over any pH likely to be encountered during parasitism. In contrast with the endo-proteases production of peptide exo-hydrolases showed far greater isolate variation. Thus there is a 100 fold difference in aminopeptidase activity from filtrates of *M. anisopliae* RS549 and *V. lecanii*.

Chitinase and NAG'ase activities were produced in varying amounts by all the pathogens. There was a tendency for high producers of chitinase to also be good producers of NAG'ase

but the ratio of the two activities covered a wide range between isolates. The greatest chitinase and NAG'ase activities were found with RS324; RS23, *B. bassiana* and *V. lecanii* also possessed high extracellular activities.

Lipase was assayed with two substrates, tributyrin and olive oil. All filtrates showed tributyrase activity but *M. anisopliae* RS543 after 5 d had no extracellular activity against olive oil. This result throws doubt on the usefulness of tributyrin as a screening agent for lipase activity. Further investigation revealed that while a commercial preparation of lipase (Sigma) degraded tributyrin, so did a purified protease (*M. anisopliae* ME1, pH 8.2 opt.). Tributyrin like tweens are not by definition true lipase substrates and there have been previous reports of their degradation by non lipolytic enzymes including proteases and carboxypeptidases (Sierra, 1964; Brokerhoff and Jensen, 1974). The production of esterase activity by the pathogens did not appear to be related to that of lipase.

All the isolates produced varying amounts of activity against pyrogallol. This activity was absent when H_2O_2 was omitted from the reaction mixture confirming it was peroxidative in nature. No activity however was found against guaiacol, the preferred substrate for peroxidase (Pütter, 1974). There have been reports of catalase having a peroxidative reaction against pyrogallol (Pütter, 1974) and until it is further characterised it will be tentatively assumed that the enzyme detected was catalase.

Table 5. Extracellular cuticle degrading enzymes in 5 day culture filtrates from isolates of 3 entomopathogenic species.

Isolate	Growth[a]	Protease (assayed at alkaline pH optima)	Aminopeptidase	Carboxypeptidase	Lipase (olive oil)	Lipase (Tributyrin)	Esterase	Chitinase	NAGase	Catalase	Polyphenol oxidase	Deoxyribonuclease	Ribonuclease
M. anisopliae													
ME1	++++	10.03	2.70	3.27	0.63	8.83	4.31	0.54	1.51	6.6	1.2	14	56
RS324	+++++	11.28	0.79	2.86	0.28	12.33	8.11	3.23	12.63	60.0	0	33	17
RS455	+++	8.13	3.13	6.61	0.73	8.00	1.61	0.32	0.25	2.5	0.8	94	47
RS543	++	5.59	2.43	0.28	0	5.00	0.46	0.09	0.30	12.0	0	12	43
RS549	+++	11.01	9.30	1.32	0.165	7.0	0.89	0.12	0.12	1.0	0	38	74
RS23	++++	9.89	1.54	3.25	0.48	10.0	7.27	0.82	2.18	3.5	1.7	38	13
RS298	+++	7.18	0.99	0.54	1.11	6.0	0.12	0.70	0.63	31.0	0	25	64
B. bassiana	+++	8.38	2.09	0.52	0.02	8.0	0.62	0.80	4.29	16.0	0	160	84
V. lecanii	++++	7.79	0.09	3.41	0.35	9.5	0.92	1.18	1.06	20.0	0	34	87

a) growth was estimated using an arbitrary scale ranging from + (poor growth) to +++++ (luxuriant growth)
b) Enzyme activities expressed as units (see Materials and Methods). Each result is the mean of 3 replicates.

Using catechol as substrate polyphenol oxidase activity was found either to be absent or present in trace amounts only.

DNA'ase and RNA'ase were formed by all isolates but are unlikely to be involved in cuticle degradation. They have however a potential role as "toxins" and may affect the epidermal cells during invasion.

B. Sequential production of cuticle-degrading enzymes

The pattern of extracellular enzyme production in cultures containing locust cuticle was studied with *M. anisopliae* (ME1) *B. bassiana* and *V. lecanii* (Figs. 8, 10 and 11). Essentially similar results with ME1 were obtained using ground exuviae (Fig. 9), but chitinase production was comparatively low *cf.* whole cuticle (< 30% after 7 d).

In all the cultures enzymes appeared sequentially and showed distinct phases of rapid production. Emphasis was placed in this study on enzyme production by physiologically young mycelia as *prima facie* this has greater relevance to cuticle penetration *in vivo*. In young cultures the sequence of enzyme production by each species was similar with some variation in individual cases. Esterase activity and enzymes of the proteolytic complex *viz.* protease (assayed at pH 8.0), aminopeptidase and carboxypeptidase, appeared first (< 24 h) and increased rapidly after 48 h. The sole exception to this was in *V. lecanii* where aminopeptidase did not appear until after 72 h. The proteolytic enzymes were followed by NAG'ase which, particularly

Fig. 5. pH activity curves for proteolytic activity of (■—■) *M. anisopliae* RS23, (▲—▲) *M. anisopliae* RS549, (●—●) *M. anisopliae* RS298

Fig. 6. pH activity curves for proteolytic activity of (■—■) *V. lecanii*, (▲—▲) *M. anisopliae* RS455, (●—●) *M. anisopliae* RS543

Fig. 7. pH activity curves for proteolytic activity of (■—■) *B. bassiana*, (●—●) *M. anisopliae (ME1)*, (▲—▲) *M. anisopliae* RS324

Maximum activities are shown in Table 5.

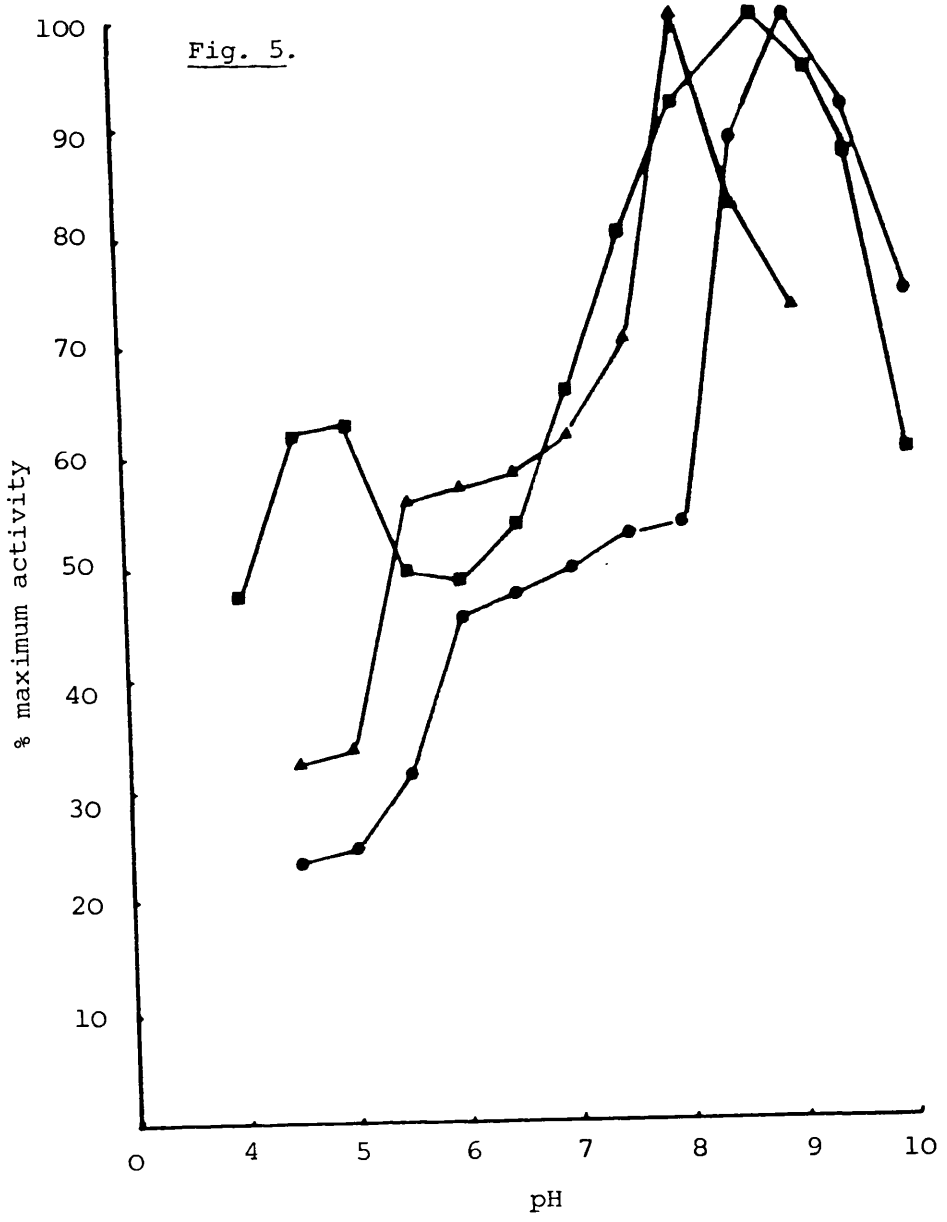


Fig. 6.

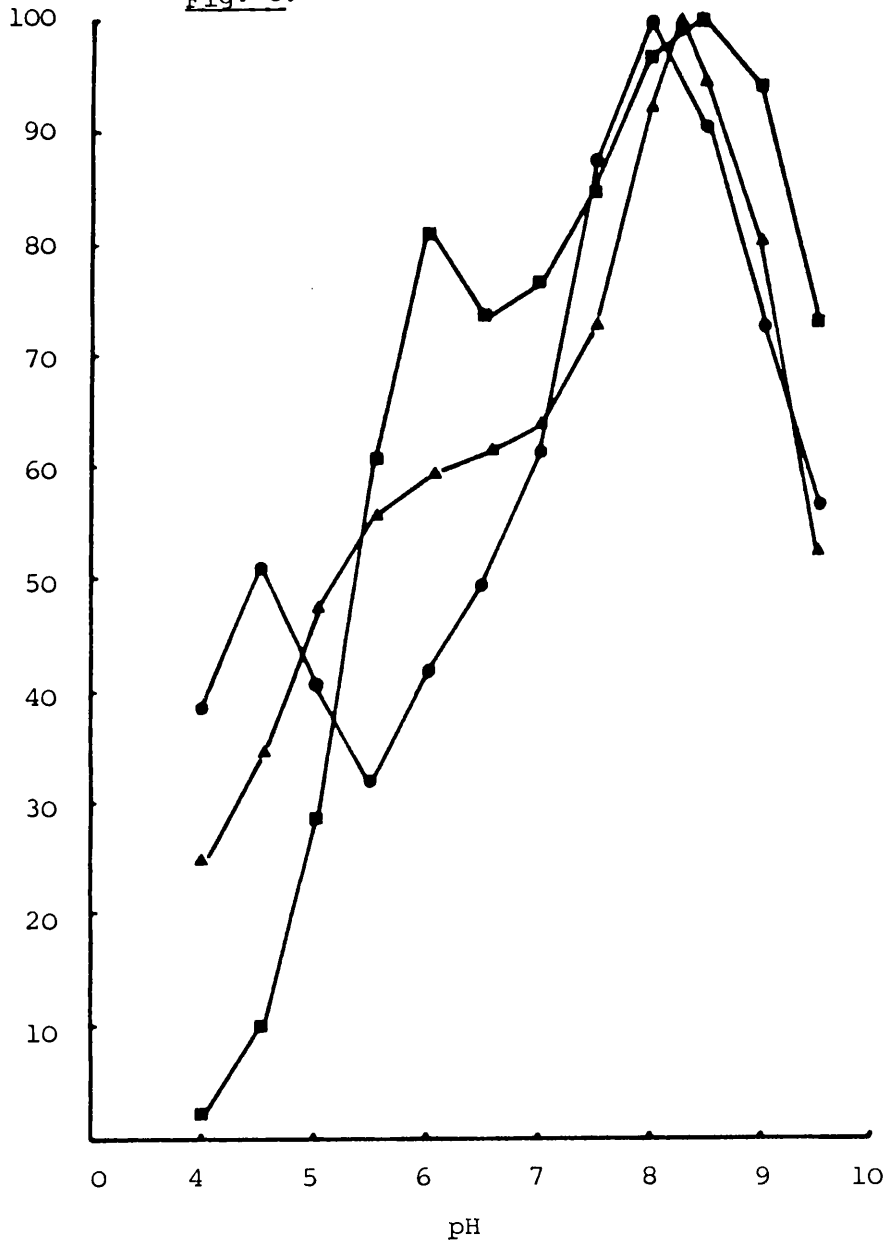
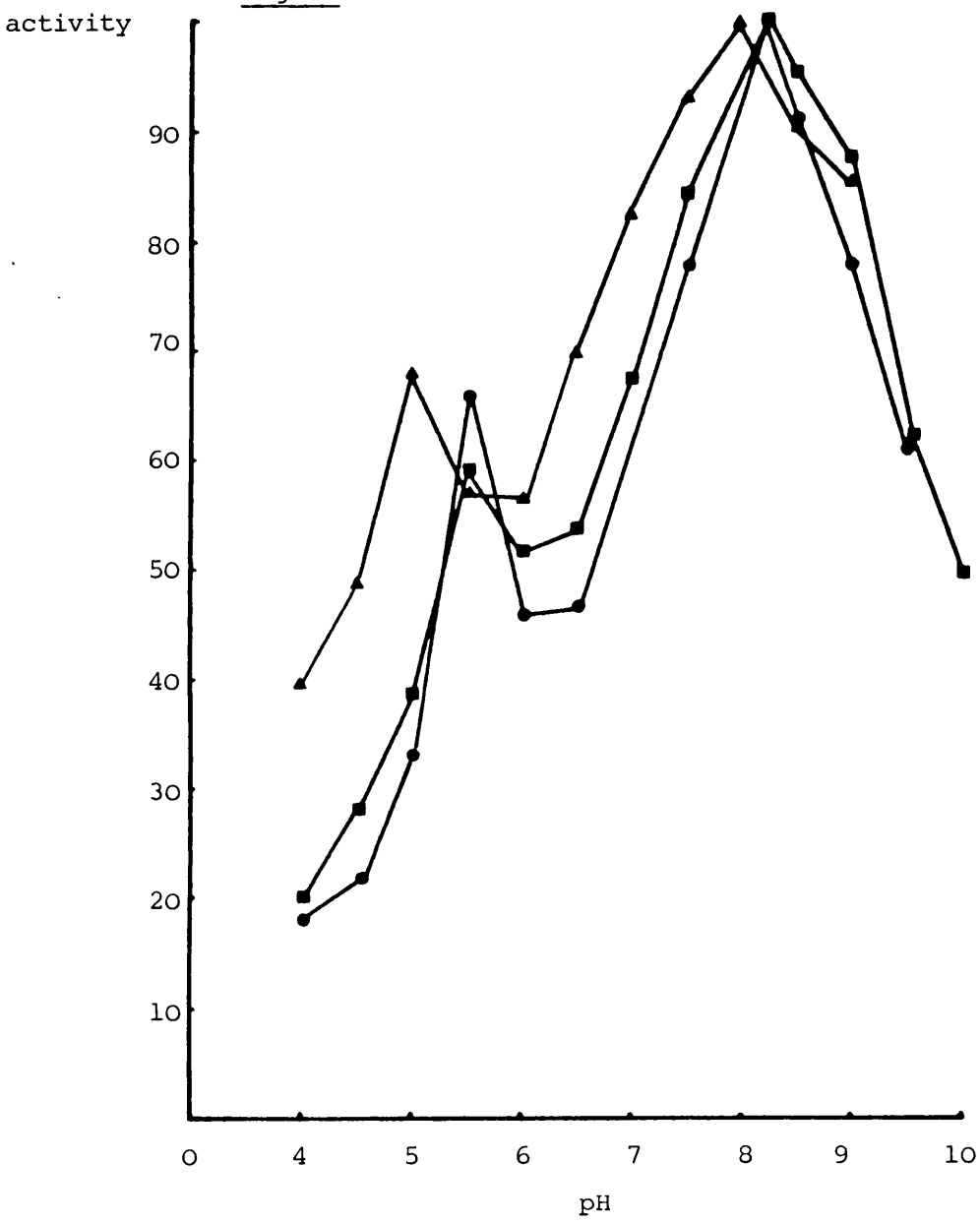

% maximum activity
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

Fig. 7


% maximum activity
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

evident in MEl, increased independently of chitinase. The timing of chitinase appearance varied more than that of proteases. It was produced at low basal levels up to 3 to 5 d after which activity increased rapidly. The most marked difference in timing of enzyme production between the three fungi was shown by catalase (peroxidase?) activity which appeared after day 1 in *V. lecanii* and increased rapidly up to day 3, but which did not appear until day 3 in *M. anisopliae* and *B. bassiana*. Lipase was not detected until day 5 in any filtrate.

C. Influence of cuticle particle size on enzyme production

To determine whether particle size could influence enzyme production basal medium was prepared as described but with either 1% unground (> 0.5 mm) or ground (< 0.2 mm) cuticle. Flasks were inoculated with 3×10^6 spores.

Altering the accessibility of cuticle to the fungi in this way had a strong influence on enzyme yields (Fig. 12) and growth; both were considerably greater with ground cuticle.

D. Soluble degradation products in cuticle fluids during growth

The levels of extracellular protein, amino acids and amino-sugars in culture fluids of *M. anisopliae* (MEl) grown on 1% ground locust cuticle are shown in Fig. 13. In comparison with culture fluids from fungi growing on sucrose or olive oil (Table 6), 5 d cuticle cultures contained higher levels of proteins

Figs 8-11. Each point is the mean of 3 replicates. The results are representative of two similar experiments.

Fig. 8. Production of extracellular enzymes by *M. anisopliae* (ME1) growing on 1% ground locust cuticle/basal salts medium. Enzymes followed by maximum activities detected are: (△—△) protease, 13.45 μg trypsin units; (■—■) aminopeptidase, 3.0 units; (●—●) carboxypeptidase A, 3.80 units; (✕—✕) esterase, 4.92 units (▼—▼) lipase, 1.70 units; (□—□) chitinase, 0.7 units; (○—○) NAG'ase, 1.75 μmole ml/h; (▲—▲) catalase (peroxidase?), 15 OD units/min; (▽—▽) polyphenol oxidase, 3.6 OD units/min.
Chitobiase activity (not shown) followed NAG'ase closely, maximum activity 2.3 units.
Designation of units given in Materials and Methods

Fig. 9. Production of extracellular enzymes by ME1 growing on 1% ground locust exuviae/basal salts medium. Maximum enzyme activities are: protease, 14.23 μg trypsin units; aminopeptidase 2.42 units; carboxypeptidase 4.60 units; chitinase 0.18 units; NAG'ase 1.42 units; lipase 0.62 units; esterase 5.14 units
Symbol designations are given in Fig. 8.

Fig. 8.

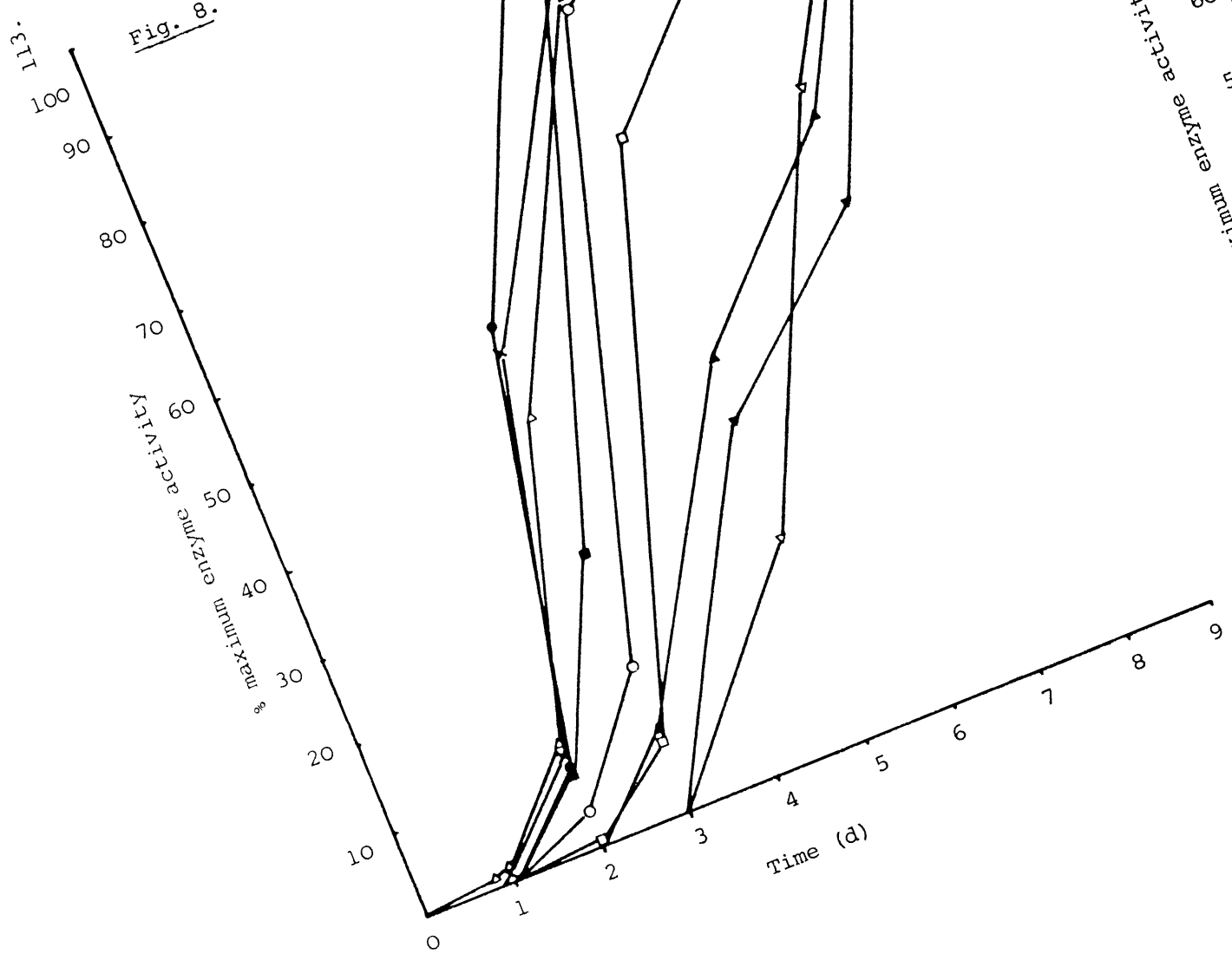

% maximum enzyme activity
Time (d)
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7
8
9

Fig. 9

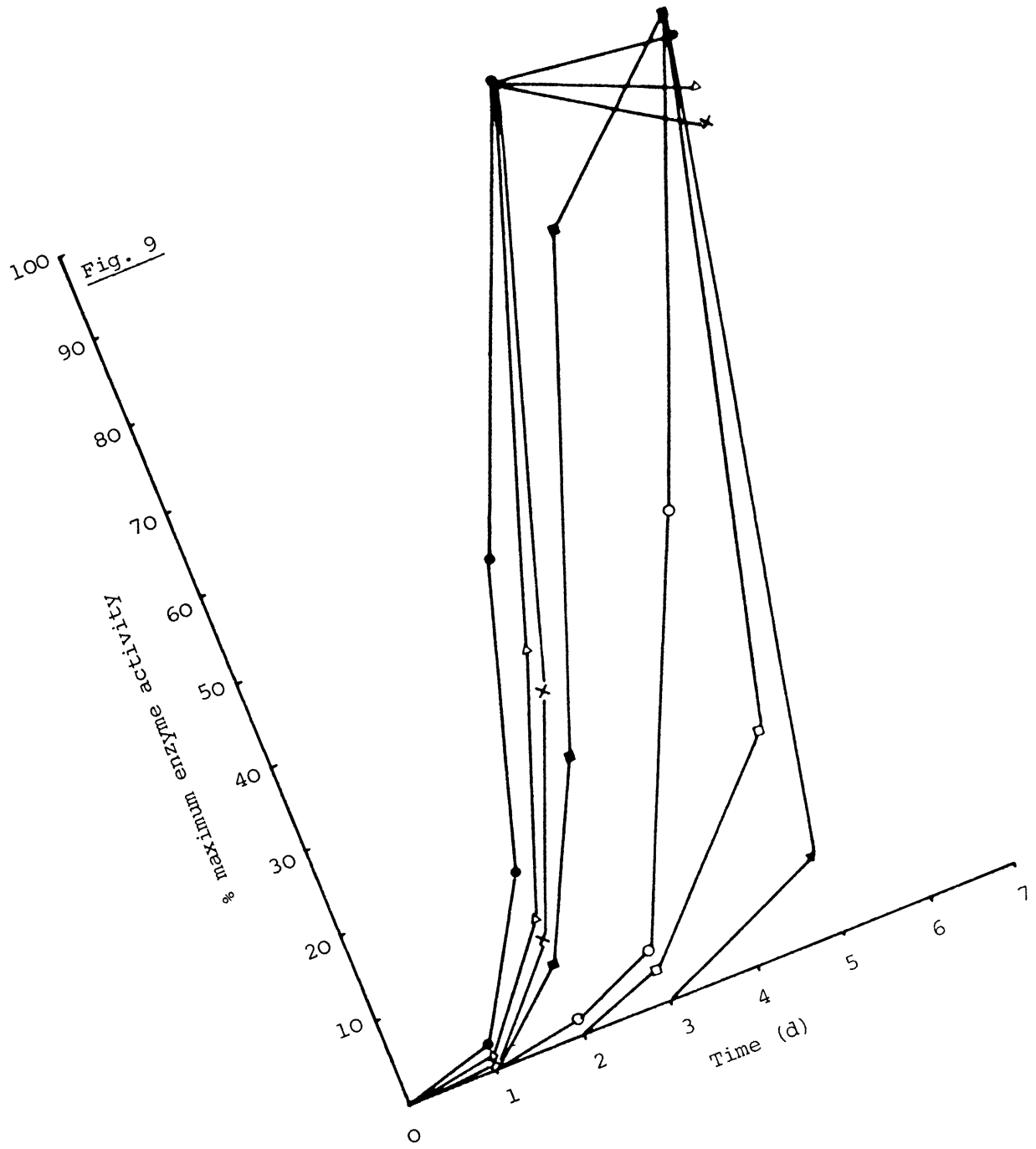

% maximum enzyme activity
Time (d)
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7

Fig. 10. Production of extracellular enzyme by *V. lecanii* growing on 1% ground locust cuticle/basal salts medium. Maximum enzyme activities shown in Table 5 . Symbol designations are given in Fig. 8.

Fig. 11 Production of extracellular enzyme by *B. bassiana* growing on 1% ground locust cuticle/basal salts medium. Maximum enzyme activities are shown in Table 5 . Symbol designations are given in Fig. 8.

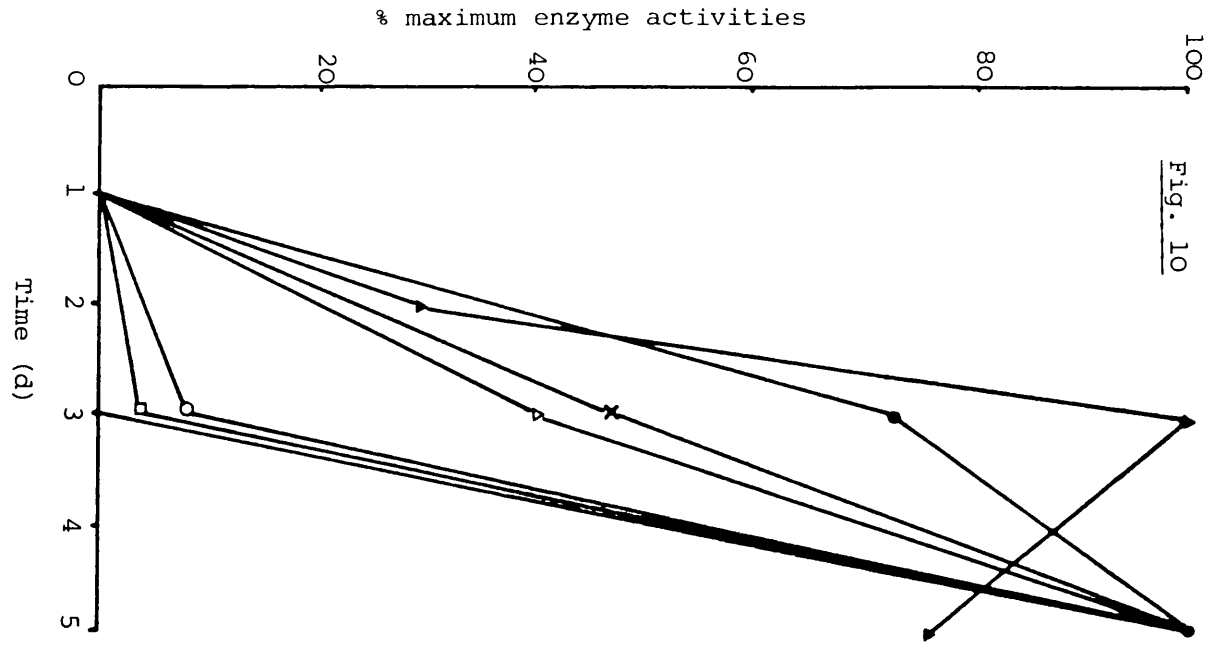

% maximum enzyme activities
0
20
40
60
80
100
Time (d)
0
1
2
3
4
5

Fig. 10

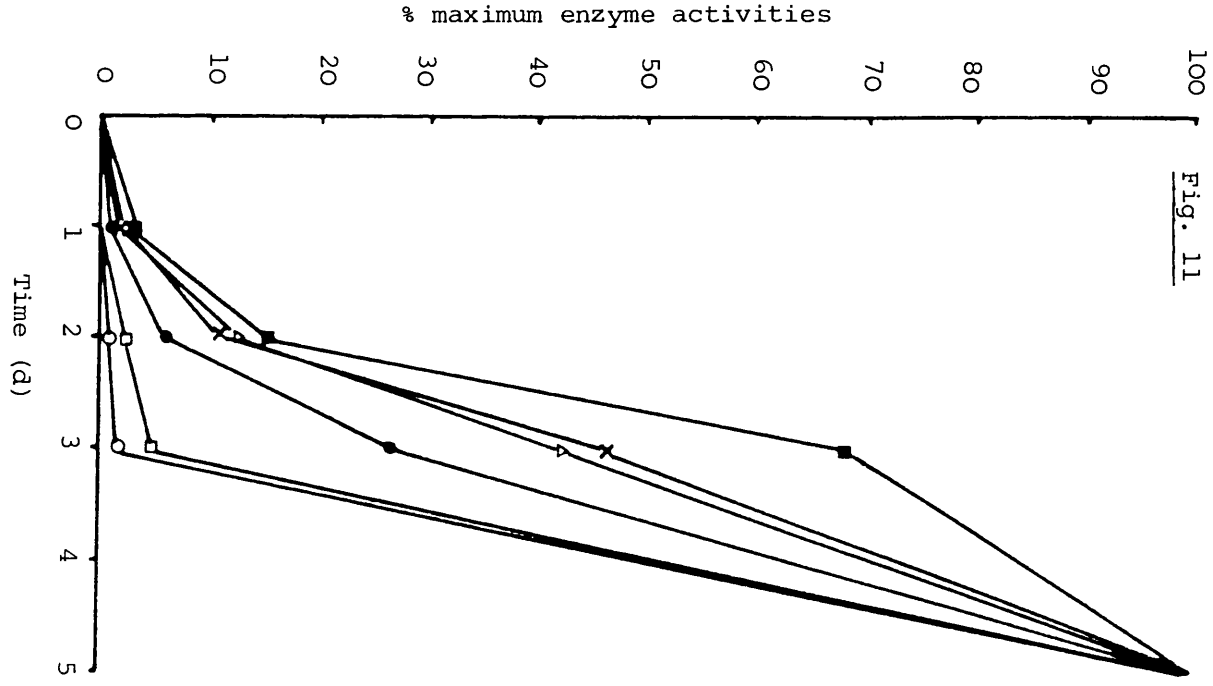

% maximum enzyme activities
0
10
20
30
40
50
60
70
80
90
100
Time (d)
0
1
2
3
4
5

Fig. 11

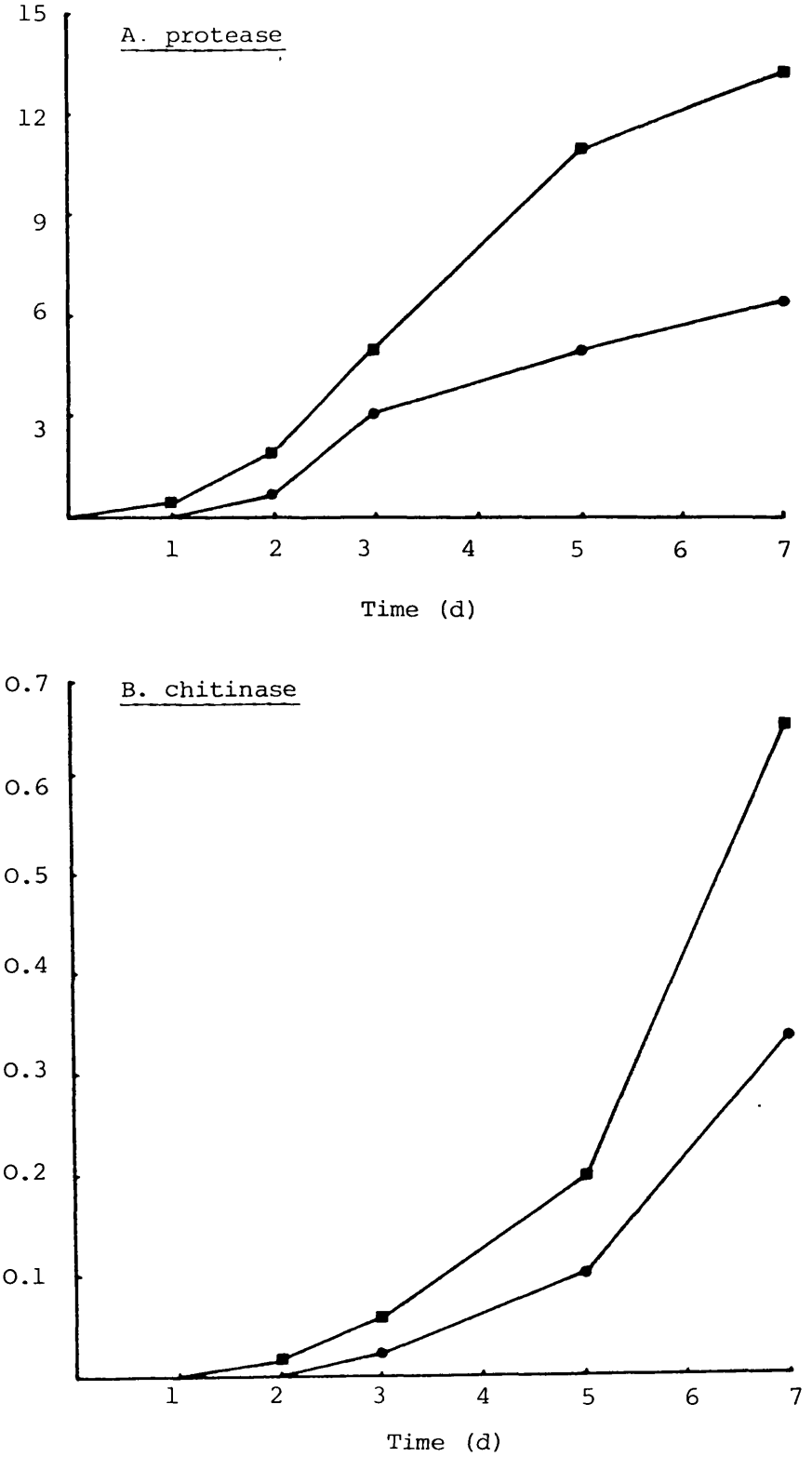


Fig. 12. Production of extracellular protease (A) and chitinase (B) by *M. anisopliae* (MEl) growing on unground (●—●) (> 0.5 mm) and ground (■—■) (< 0.2 mm) cuticle. Each result is the mean of 3 replicates. The results are representative of 2 similar experiments.

(*c* x 2.5) and very much higher levels of amino acids (*c* x 42). Soluble amino sugars were only found in culture fluids containing cuticle.

These results suggest that amino acids and amino sugars derive mainly from cuticle degradation rather than from the fungi during growth. The source of soluble protein is more ambiguous. Growth of ME1 on other polymeric substrates (e.g. chitin and starch, Table 8) prcduced similar levels of protein to growth on cuticle. This protein will consist partly of extracellular enzymes whose production may be higher on polymers *cf.* sucrose, which could exert catabolite repression (Tables 8 and 9).

Time course studies with cuticle cultures (Fig. 13) showed that low levels of cuticle constituents were solubilised by the initial heat sterilisation; amino-sugars presumably were derived from exposed chitin at the cut edges of cuticle fragments. In uninoculated controls these compounds remained at similar levels throughout the incubation period (10 d). In inoculated flasks the concentration of amino acids increased markedly over 24 h, followed by a sharp increase in amino-sugars after 72 h of growth. This finding coincides with the sequential appearance of protein and chitin degrading enzymes.

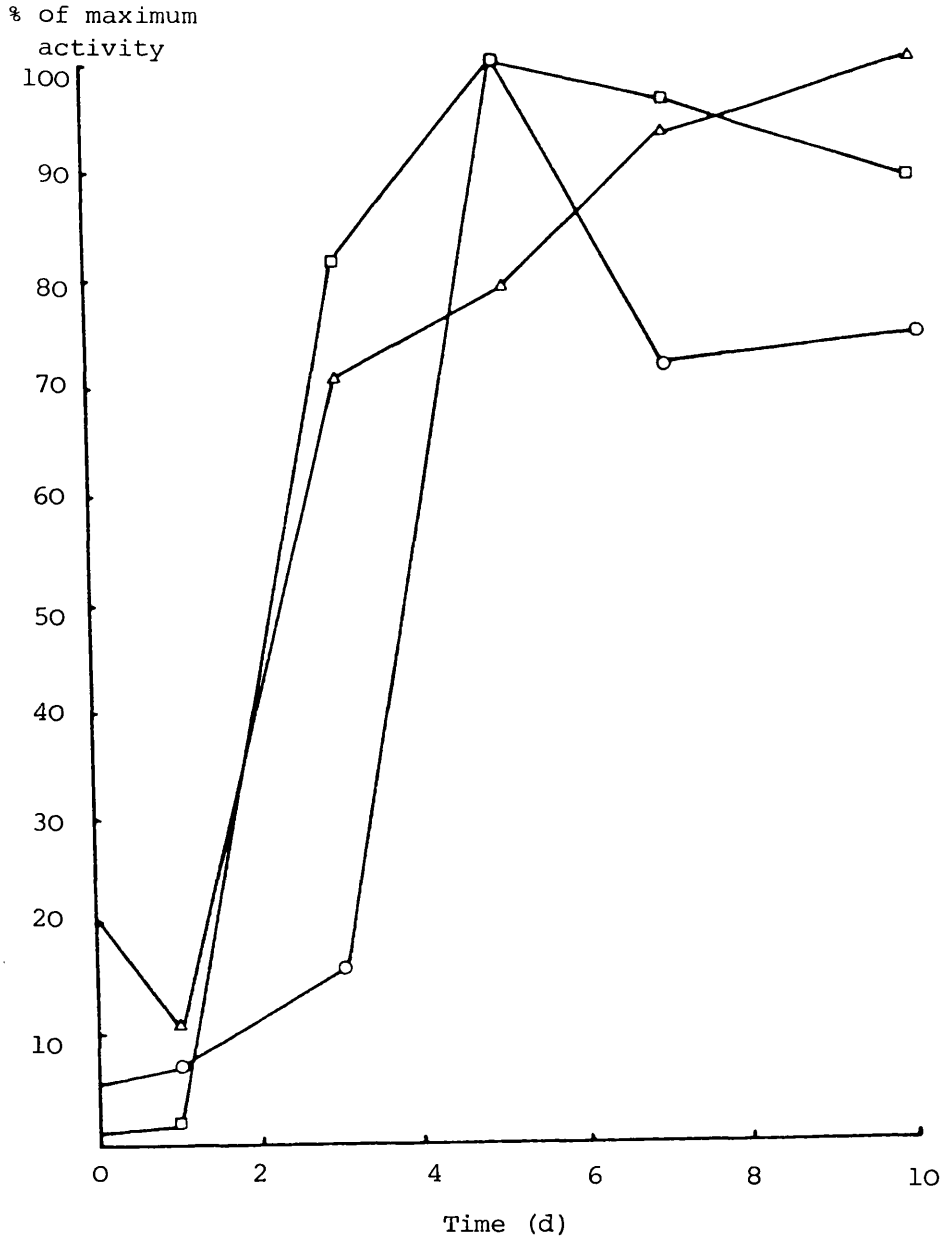


Fig. 13. Appearance of soluble degradation products in cultures of *M. anisopliae* (MEl) grown on cuticle.

Type of molecule and maximum amount formed are: (Δ—Δ) protein, 54.63 µg/ml; (□—□) amino acids (4.32 µmoles/ml; (O—O) amino sugars 0.024 µmoles/ml.

Each point is the mean of 3 replicates. The results are representative of 2 similar experiments.

Table 6. Soluble degradation products in fluids from 5 d cultures of *M. anisopliae* (MEl)

	1% cuticle	1% exuviae	1% sucrose[a)]	1% olive oil[a)]
protein (μ g ml^{-1})	54.63	57.22	22.31	20.71
Amino acids (μmole ml^{-1})	14.32	8.96	0.33	0.15
Amino sugars (μmole ml^{-1})	0.024	0.015	0	0

Amino acids (ninhydrin), amino sugars (Smith *et al.*, 1979) and protein (Bio-Rad) were measured and the figures compensated for release of cuticle degradation products during heat sterilisation.

a) $NaNO_3$ (0.2%) sole nitrogen source.

5. Enzymatic characterisation of entomopathogens with the API ZYM system

The enzymatic profiles of 9 isolates grown on 1% chitin/basal salts medium were studied using substrates included in the API ZYM system (Materials and Methods). The results are summarised in Table 7 and Plate 1.

All isolates tested exhibited varying levels of activity against substrates for alkaline and acid phosphatase, esterase (C_4), esterase lipase (C_8) and leucine arylamidase. With the exception of *M. anisopliae* (RS 549) all isolates produced phosphoamidase. Isolates were generally inactive or showed weak activity against substrates for β-glucuronidase, α-glucosidase, α-fucosidase and lipase (long chain esterase). *M. anisopliae* (RS324) was exceptional in lacking cysteine aminopeptidase and being the only isolate to produce more than trace levels of α-mannosidase activity.

The API ZYM pattern obtained for *V. lecanii* was clearly distinct from that of the other species with respect to:

1) the higher production of acid rather than alkaline phosphatase;
2) the higher activity against caprylate (C_8) than butyrate (C_4) esters;
3) possessing trace levels only of arylamidase (aminopeptidase) activity;
4) possessing higher activity against the chymotrypsin than the trypsin substrate;
5) the absence of β-galactosidase activity.

In spite of all culture filtrates possessing high levels of proteolytic activity against hide protein azure, they only weakly hydrolysed amide substrates for trypsin or chymotrypsin. This may reflect a preference of fungal proteases for longer chain peptides (see Sect. III, Discussion).

There were notable differences between isolates in activity profiles against the three aryl amide (aminopeptidase) substrates. Valine naphthylamide was preferentially hydrolysed by 5 isolates (*B. bassiana*, *M. anisopliae*; MEl, RS549, RS23 and RS298). Cysteine naphthylamide by 2 isolates (RS543 and RS455) and leucine naphthylamide by 1 isolate (RS324). The distinctive aminopeptidase profiles suggest that their substrate specificity may be a way of distinguishing closely related isolates. However it would be necessary to investigate a larger number of substrates and isolates from each species to establish this possibility. It was noteworthy that valine aminopeptidase production by *M. anisopliae* (RS549) varied from moderate to strongly positive in different culture flasks. This was the only reaction which varied substantially (by more than one grade) on repeated testing with any of the isolates.

In spite of the differences in carbon source, assay substrate and the semi-quantitative nature of the API ZYM technique, variations between isolates in production of aminopeptidase and NAG'ase on chitin medium showed similar trends to those determined quantitatively in cuticle culture filtrates (Table 5). The most significant difference was the inability of the API ZYM

technique to detect NAGase production by RS543 on chitin medium; low levels of activity were found in cuticle cultures.

The API ZYM technique revealed comparatively low levels of esterase activity against naphthylcaprylate in chitin cultures of *B. bassiana* and *M. anisopliae* RS23 (*cf* levels detected in Table 5) but in other respects variability between isolates resembled that found in cuticle cultures assayed quantitatively with PNP caprylate.

The results of this study indicate that the API ZYM technique provides a rapid and reproducible method for distinguishing between isolates and potentially therefore could be of use in their identification. However its semi-quantitative nature suggest its major use is for preliminary screening before further testing by more precise procedures.

Synthesis of enzymes on defined substrates

Before a role *in vivo* can be assigned to enzymes produced *in vitro* it is necessary to understand how their synthesis is regulated. This will give an insight into how production is likely to be affected by cuticle components encountered during pathogenesis.

It will also enable culture conditions to be established for selective biosynthesis of specific enzymes as the first stage of purification. The results are summarised in Table 14.

Table 7. Reactions of isolates in the API ZYM test

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
V. lecanii	0	5	2	4	0	<1	0	<1	1	2	3	3	<1	0	0	0	1	4	0	<1
B. bassiana	0	2	1	<1	0	1	5	2	<1	0	3	4	0	2	0	0	1	4	<1	0
M.anisopliae																				
RS324	0	4	4	3	<1	1	<1	0	<1	0	4	5	1	4	0	0	2	4	2	0
RS549	0	1	3	2	<1	1	3	2	1	<1	4	0	<1	1	0	0	1	1	0	1
RS455	0	1	3	2	1	4	4	5	1	<1	4	1	0	1	0	0	1	1	0	0
RS23	0	1	1	1	<1	4	5	4	1	1	4	1	<1	<1	0	0	<1	2	0	1
ME1	0	1	2	2	1	4	5	3	2	1	3	4	<1	2	0	0	<1	4	<1	0
RS298	0	3	2	1	<1	3	4	3	1	<1	3	5	<1	2	<1	<1	1	3	<1	<1
RS543	0	1	1	1	<1	4	4	5	1	<1	4	5	0	3	0	<1	<1	0	0	0

Tests: 1) control, 2) phosphatase alkaline, 3) esterase (C_4) 4) esterase lipase (C_8), 5) lipase (C_{14}), 6) leucine aminopeptidase, 7) valine aminopeptidase, 8) cysteine aminopeptidase, 9) trypsin, 10) chymotrypsin, 11) phosphatase acid, 12) phosphoamidase, 13) α-galactosidase, 14) β-galactosidase, 15) β-glucuronidase, 16) α-glucosidase, 17) β-glucosidase, 18) β-glucosaminidase, 19, α-mannosidase, 20), α-fucosidase. Number values 1 to 5 indicate increasing colour intensity.

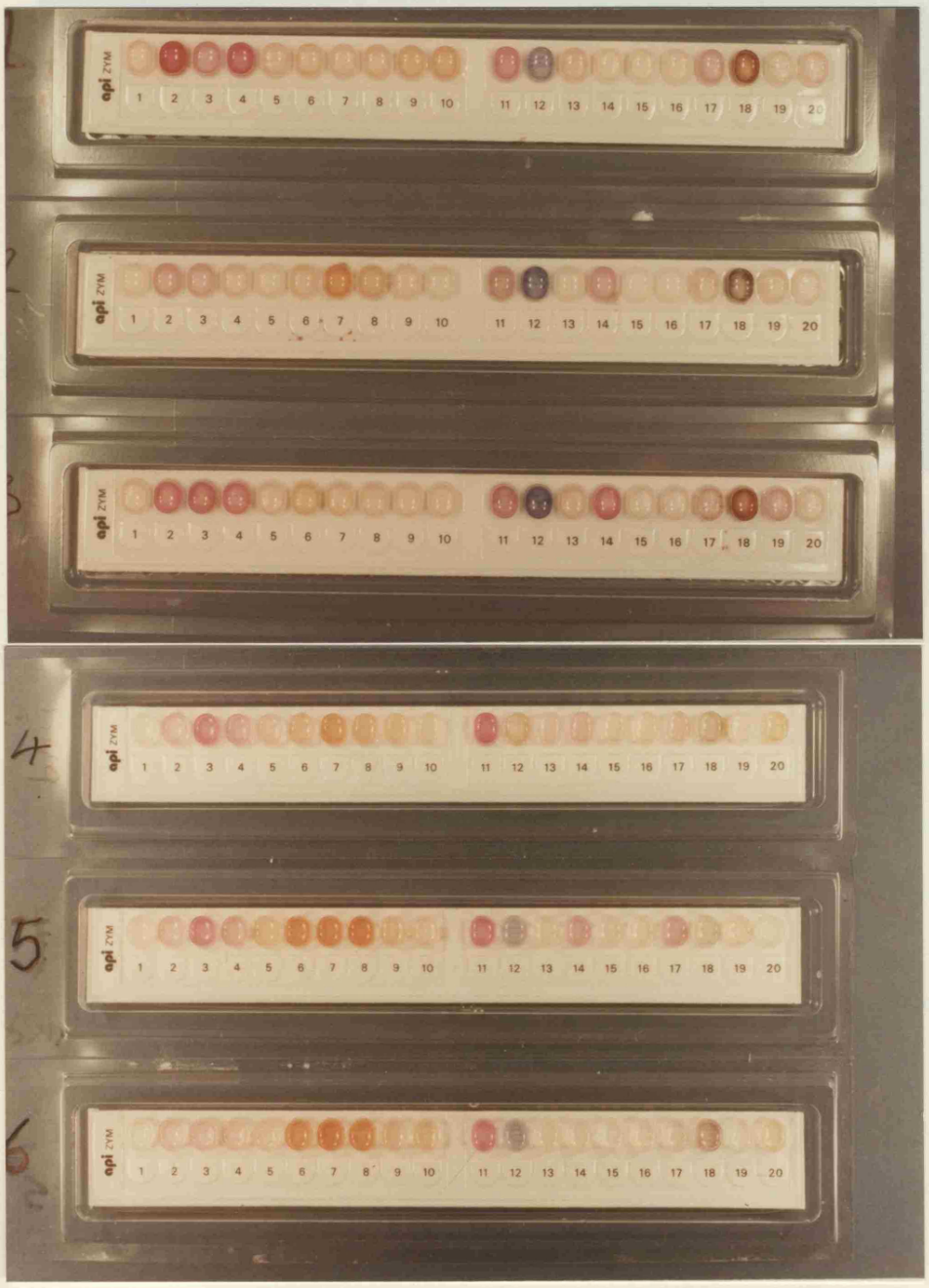
Plate 1. Reactions of isolates in the API-ZYM test.

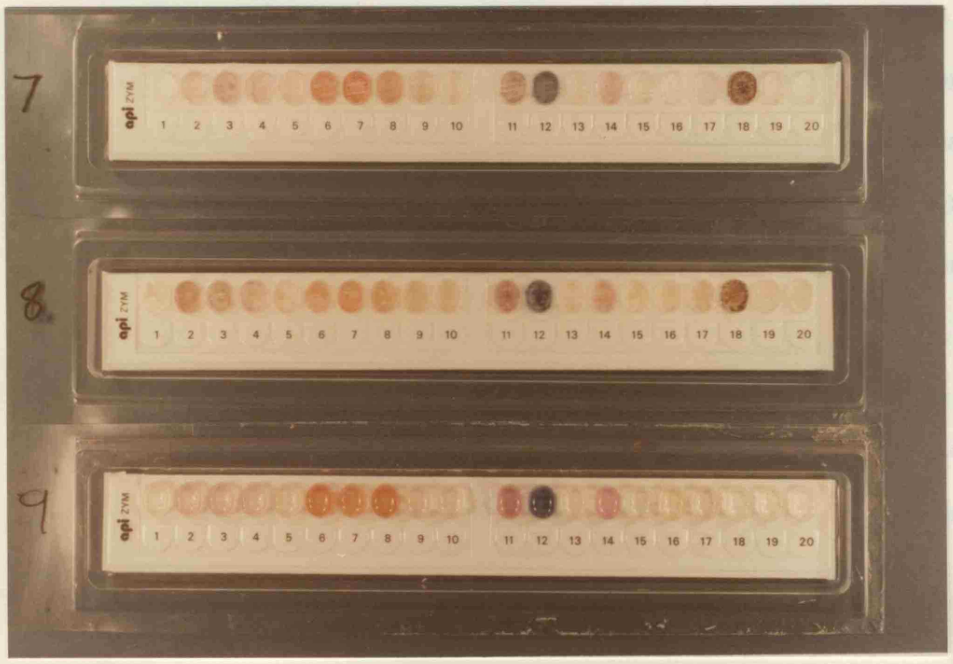
1) *V. lecanii*, 2) *B. bassiana*, 3) RS324,

4) RS549, 5) RS455, 6) RS23, 7) ME1, 8) RS298,

9) RS543. The 19 enzyme substrates and

activities are listed in Tables 2 and 7.




7
8
9
api ZYM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

6. Regulation of Production of Chitinolytic enzymes

In contrast to the critical studies on regulation of cell wall degrading enzymes (CWDE) of plant pathogens (e.g. English *et al.*, 1981; Cooper and Wood, 1975) much of the work on regulation of chitinase production by bacteria and fungi have involved long term trials in batch cultures in which such fundamental considerations as the effects of catabolic repression(CR) growth rate, autolysis and changes in pH have been ignored or insufficiently allowed for (see Literature Review).

Because of the difficulty in interpreting these results a more discriminating approach was applied in this study to determine the mode of chitinase regulation in *M. anisopliae* (MEl).

A. Chitinase synthesis on various carbon sources in batch cultures.

Initially the effect of different carbon sources on growth and chitinase production was tested in unbuffered basal medium supplemented with chitin, glucose, NAG or one of the other carbon sources listed in Table 8. After 5 d growth cultures were harvested and analysed for growth, pH and, following dialysis, for chitinase activity.

Appreciable growth occurred on all the carbon sources tested but high chitinase activities were only found in cultures supplied with chitin. Thus chitinase appears to fulfil the usual criterion of inducibility (Cooper, 1977; Stirling, 1979). Lack of chitinase production with other polymers such as pectin and cellulose is particularly indicative of induction, as a repressible constitutive enzyme should appear in higher yields on polysaccharides

cf monomers if the gradual rate of degradation of polymers fails to effect CR (Cooper, 1977). Similarly when screening potential inducers (e.g. monosaccharides) in batch cultures, enzyme synthesis could remain at a basal level if inducing sugars also repress synthesis at the high levels employed to sustain growth. It is interesting therefore that growth on NAG (the monomer of chitin) when at concentrations less than 0.5% resulted in slightly more enzyme than was obtained with other non-chitinous substrates. At higher NAG concentrations chitinase production was repressed. Chitinase production was also repressed when additional carbon sources such as carbohydrates,olive oil or proteins were added to a medium containing chitin (Table 9). This occurred even though growth was enhanced.

Small amounts of chitinase were produced in media not containing chitin. Fig. 14 shows the growth and extracellular chitinase activity in cultures containing sucrose as the sole carbon source. Growth declined after 5 d whereas chitinase activity increased gradually throughout, suggesting that activity might result from autolysis. Microscopic examination revealed hyphal lysis particularly in the centre of the pellets. As it is possible that autolysis of some hyphae might have provided additional nutrients for the maintenance of growth of some of the remaining hyphae, the activity of chitinase with fungal mycelium as substrate was examined. The action of filtrate from a 10 d culture containing 2% chitin on boiled or autoclaved mycelium resulted in release of NAG ($N = 5$, $\bar{x} = 9.34$ µg ± 1.62 mg^{-1} mycelial dry wt). but none was liberated when live mycelium or autoclaved filtrate

were used. Microscopic examination showed that boiling caused some disruption of hyphae. Presumably this exposed the chitin component of the wall to enzymic degradation. In *Neurospora crassa*, chitin microfibrils are found in the innermost layers of the wall embedded in a proteinaceous material (Hunsley and Burnett, 1970). The chitinase produced in non-inducing media may not be solely the product of autolysis as inducible enzymes are often found in small amounts in the absence of inducer - *viz* basal synthesis. If sufficient NAG is released to initiate induced synthesis of chitinase, the process of wall degradation should become autocatalytic.

B. Influence of chitin concentration and particle size on chitinase production

For studying the effect of chitin concentration and particle size, basal medium was prepared as described but with different concentrations and preparations of chitin *viz* 1% unground chitin, 1%, 2% and 3% ground chitin and 1% colloidal chitin. Altering the accessibility of chitin to the fungi in this way had a strong influence on growth and chitinase production. Highest amounts of growth were obtained at 3% ground and 1% colloidal chitin. However, the highest chitinase activity < 14 d after inoculation occurred with 2% ground chitin (Figs. 16 and 17). Media with 1% unground chitin induced less enzyme than did 1% ground, but 1% colloidal (the most accessible form) induced only comparatively low chitinase. Fig. 15 shows the change in pH and formation of chitinolytic enzymes in one experiment with altered forms of chitin. In all cultures there was a close correlation in patterns of appearance of activities against crystalline and colloidal chitin, indicating

Table 8. The effect of different carbon sources on growth, pH and chitinase production.

Inoculum level	Carbon source	pH	Extracellular protein ($\mu g\ ml^{-1}$)	Chitinase activity ($\mu mole\ NAG\ ml^{-1} h^{-1}$)	Reducing groups in media ($\mu g\ ml^{-1}$)	Mycelial dry wt. (mg/ 50 ml)
$3x10^6$	1% chitin	7.05	59.3	1.34	5	fair
$1x10^4$	1% chitin	6.85	13.5	0.24	90	fair
$3x10^6$	2% chitin	6.89	83.4	1.47	11	quite good
$3x10^6$	3% chitin	7.49	97.4	0.69	70	good
"	1% coll. chitin	8.14	134.2	0.10	176	good
"	1% chitosan	7.62	32.6	0.54	38	good
"	0.2% NAG	6.82	15.3	0.14	294	36
"	0.5% NAG	6.44	20.5	0.10	385	84
"	1.0% NAG	6.50	30.6	0.03	802	163

Table 8 (continued)

Inoculum level	Carbon source	pH	Extracellular protein ($\mu g\ ml^{-1}$)	Chitinase activity ($\mu mole\ NAG\ ml^{-1} h^{-1}$)	Reducing groups in media ($\mu g\ ml^{-1}$)	Mycelical dry wt. (mg/50 mls)
$3x10^6$	1% cellulose	6.15	8.0	0	-	poor
"	1% carboxy-methyl cellulose	6.80	17.2	0.01	-	fair
"	1% pectin	6.00	95.8	0.01	0	fair
"	1% sucrose	6.18	41.3	0.04	> 1,000	28
"	1% lactose	6.11	33.0	0.01	> 1,000	15
"	1% xylan	6.05	13.2	0	6	9
"	1% soluble starch	6.73	73.4	0.02	41	17.4

Table 9. The effect of chitin and additional carbon sources on chitinase production

Sources of carbon	pH	Extracellular protein ($\mu g\ ml^{-1}$)	Chitinase activity ($\mu mole\ NAG\ ml^{-1} h^{-1}$)	No. of pellets (ml^{-1})	growth
1% chitin	7.05	59.3	1.34	2.25	fair
" " + 0.2% NAG	6.76	35.3	0.07	2.15	fair
" " + 1.0. NAG	6.77	64.2	0.03	3.87	good
" " + 0.2% peptone	7.48	74.3	0.49	4.14	good
" " + 0.01M cysteine	6.43	90.3	0.24	8.80	v. good
" " +0.5% casamino acids	7.81	112.4	0.11	3.50	v. good
" " +0.2% T_{80}	6.72	43.2	1.06	17.80	fair
" " +0.5% Olive oil	6.88	68.4	0.21	2.03	good
" " +0.5% sucrose	6.32	56.7	0.06	2.50	good

Each result is the mean of 3 replicates. The experiment was repeated once.

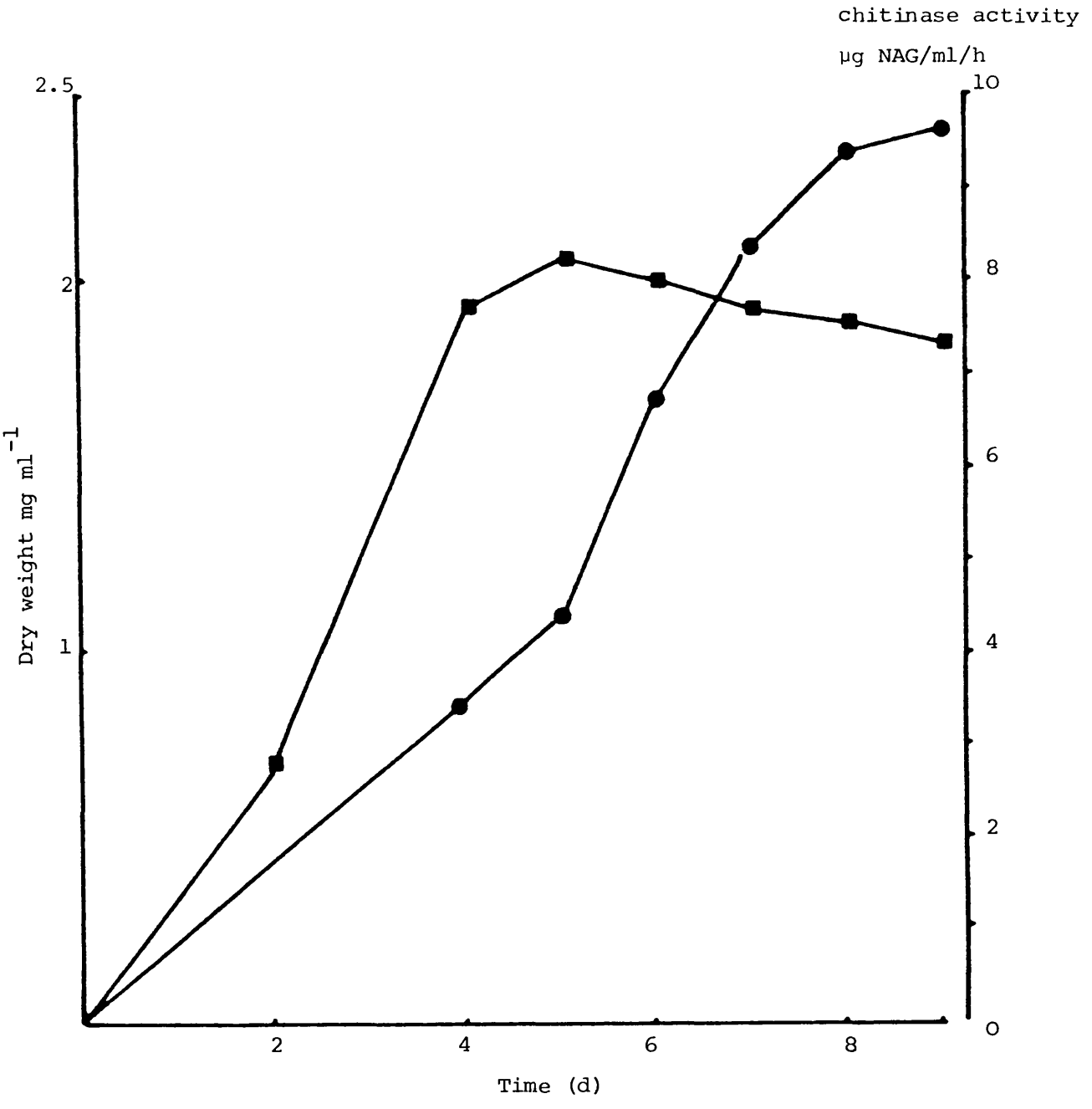


Fig. 14. The mycelial dry weight and chitinase activity in 1% sucrose/ basal salts medium: (■—■) mycelial dry weight; (●——●) chitinase activity.

Each point is the mean of 3 replicates. The results are representative of 2 similar experiments.

Fig. 15. Chitinolytic enzyme production in a basal salts medium containing (A) 2% ground crystalline chitin or (B) 1% colloidal chitin. Chitinase was assayed with colloidal chitin (□—□) or crystalline chitin (△—△). Chitobiase (○—○) was assayed with chitobiose. Reducing sugars in the media (●——●). pH (- - - - -). Each point is the mean of 3 replicates. The results are representative of two similar experiments.

Fig. 15

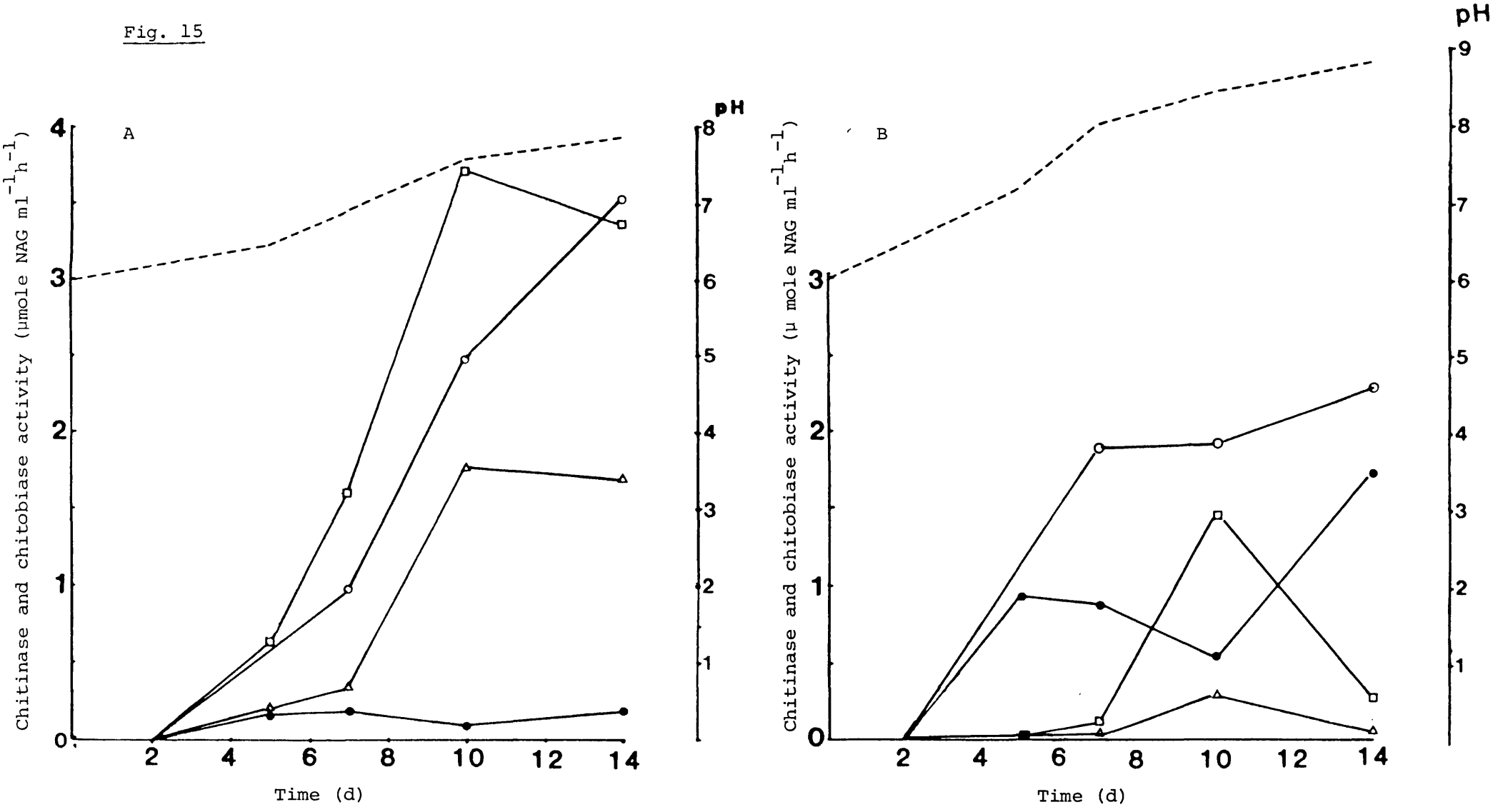

A
B
pH
Chitinase and chitobiase activity (µmole NAG ml^{-1}h^{-1})
Chitinase and chitobiase activity (µ mole NAG ml^{-1}h^{-1})
Time (d)
Time (d)
0
1
2
3
4
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9

Fig. 16. Effect of chitin concentration and form on the production of chitinase. Growth media contained: 1% ungrounid chitin (□—□); 1% ground chitin (■—■); 2% ground chitin (▲——▲); 3% ground chitin (○——○); 1% colloidal chitin (△——△).

Fig. 17. Effect of chitin concentration and form on specific activity of chitinase.

Figs. 16 & 17. Each point is the mean of 3 replicates. The results are representative of two similar experiments.

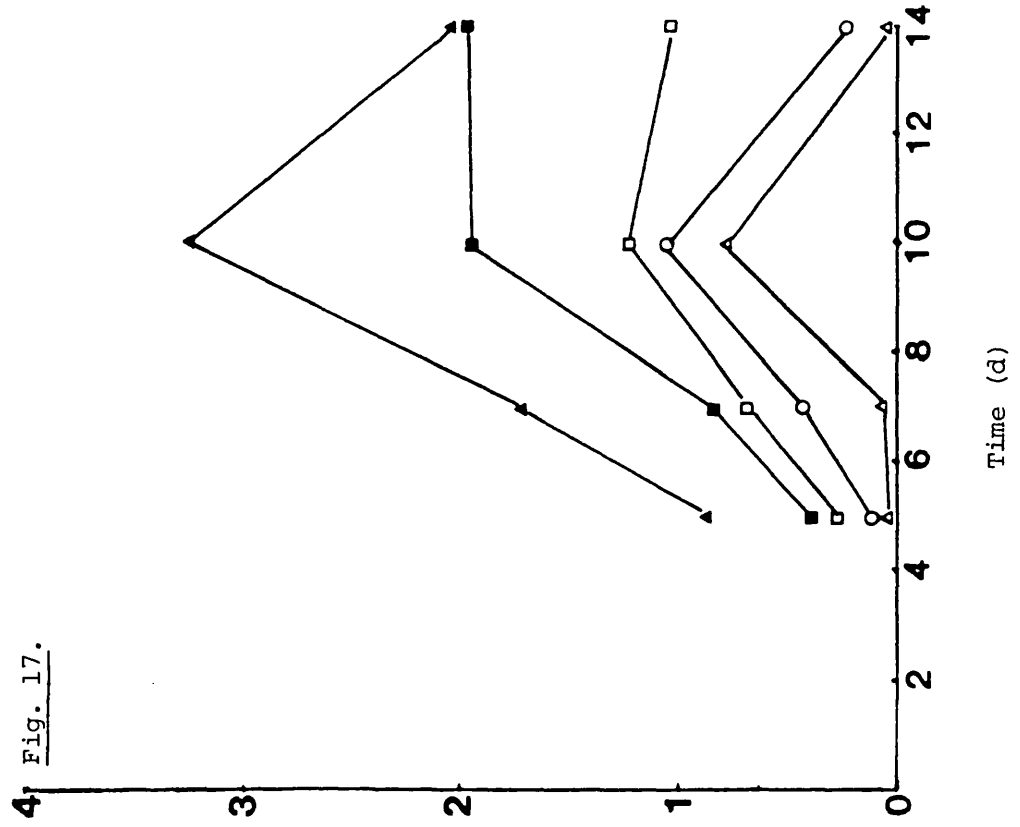

Specific chitinase activity (units/mg protein)
0
1
2
3
4
2
4
6
8
10
12
14
Time (d)

Fig. 17.

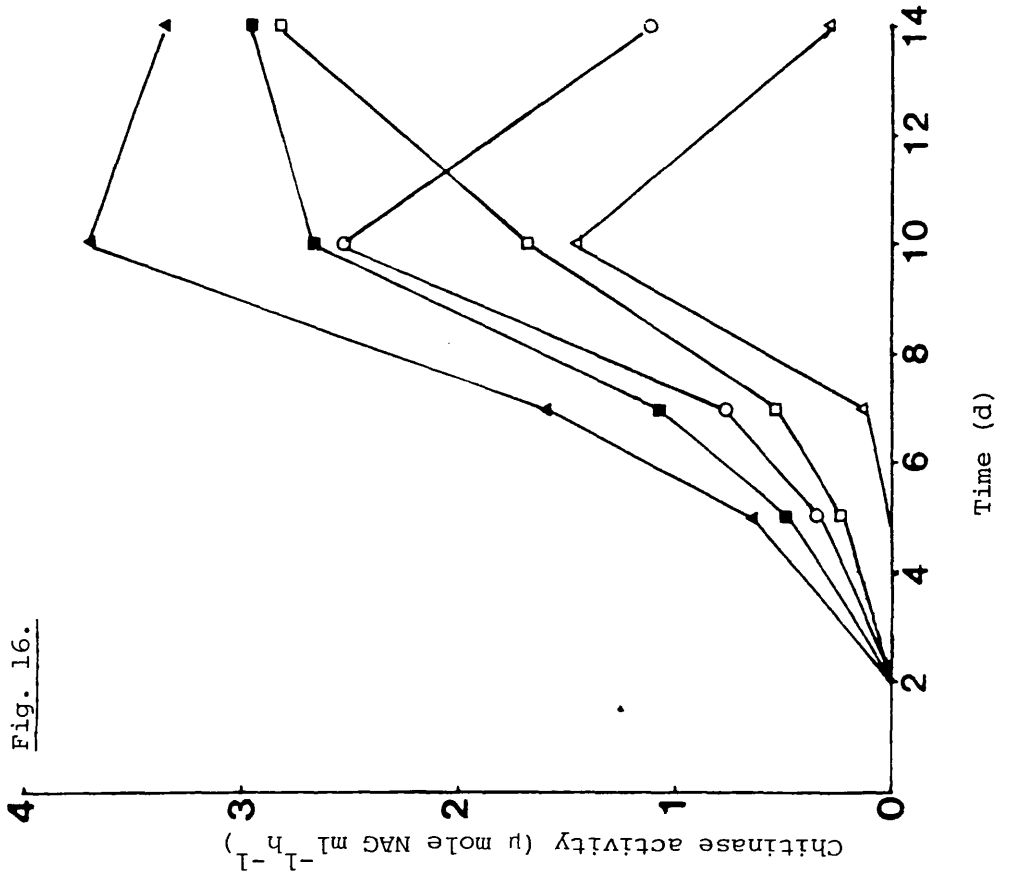

Chitinase activity (μ mole NAG $ml^{-1}h^{-1}$)
0
1
2
3
4
2
4
6
8
10
12
14
Time (d)

Fig. 16.

that a single enzyme is probably responsible. However, activity against chitobiose was not related to production of chitin-degrading enzymes and evidently results from a different enzyme.

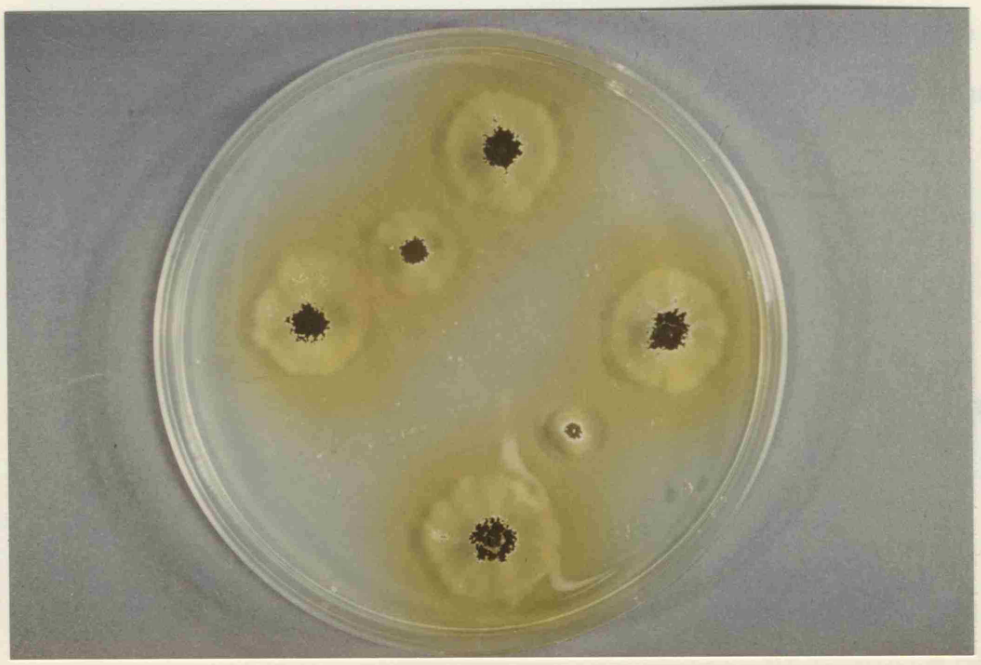
High amounts of amino sugars were detected in flasks containing 3% ground and 1% colloidal chitin. Presumably therefore the comparatively low levels of chitinase in these flasks is the result of catabolic repression. In all cultures there was a rapid increase in pH during production of chitinolytic enzymes. The pH of the medium would probably affect the stability and release of extracellular enzymes (Sect. II), therefore MES buffered media (pH 6.0 - 6.5) was used in later experiments with restricted carbon source supply.

C. Detection of chitinases on solid media

Experiments involving growth on agar media containing insoluble chitin substrate were conducted to collaborate some of the above results. Chitinase activity (indicated by clearing zones around colonies) was detected in agar media containing yeast extract (0.02%) and a suspension of colloidal chitin (0.2%). Addition of alanine (1%) or NAG (1%) severely inhibited chitinase production or activity in the three strains of *M. anisopliae* tested (ME1, RS324, and RS23).

The plate technique was adapted for the detection of NAG'ase by incubating the plates with PNP-β-N-acetylglucosamine (1 mg/ml; Cit PO_4 buffer, 0.1 M, pH 5.0) for 1 h and colour development with 1 N NH_4OH (Plate 2). The resulting yellow halo usually coincided with the chitinase clearing zone.

Plate 2. NAG'ase production by *M. anisopliae* (MEl), grown for 6 d at 27°C on chitin agar (Materials and Methods). Enzyme was detected by incubating with *p*NP N-acetyl-β-D-glucosamine as described in the text.



D. Effect of cyclohexamide on chitinase and NAG'ase production by *M. anisopliae* MEl

Chitinolytic enzymes induced by chitin could result from activation of inactive precursors or alternatively by *de novo* synthesis of the enzymes. To investigate this, cyclohexamide (10 μg ml^{-1}), an inhibitor of protein synthesis was added to 3 day old cultures grown in unbuffered 1% chitin/basal salts medium (Figs. 18 and 19).

The addition of cyclohexamide prevented any increase in the level of chitinase for 3 days, after which there was some recovery from the effects of the inhibitor. N-acetylglucosaminidase was also inhibited, but here the recovery began after only 1 day. Possibly chitinase production was additionally inhibited by thelarge amounts of reducing sugars which built up over 3 or 4 days after the introduction of cyclohexamide. However irrespective of this it seems clear that cyclohexamide inhibits, at least temporarily, the production of chitinolytic enzymes and that therefore *de novo* synthesis is involved in their accumulation.

E. Induction of chitinolytic enzymes in restricted feed cultures

As described above chitinase activity was only found in large amounts if chitin but not other polymers or monomers (including NAG), was present in the media. Thus chitinase fulfils the usual criterion of an enzyme regulated by induction and CR. It is unlikely however that chitin itself is the inducer as its insolubility and large size would prevent its entry into fungal cells. Mono or oligosaccharides which would result from

Figs. 18-19. Each point is the mean of 3 replicates. The results are representative of 2 similar experiments.

Fig. 18. Effect of cyclohexamide on the production of extracellular chitinase by *M. anisopliae* (MEl) growing on 1% chitin culture. Cyclohexamide (lO µg/ml) was added 3 d. (↓) after inoculation (□—□). The graph also shows unamended controls (O——O) and reducing sugars in media (▲- - -▲).

Fig. 19. Change in pH (□) and extracellular protein (O) in control flasks (——) and flasks with cyclohexamide (lO µg/ml) added after 3 d (- - - - -).

Fig. 18.

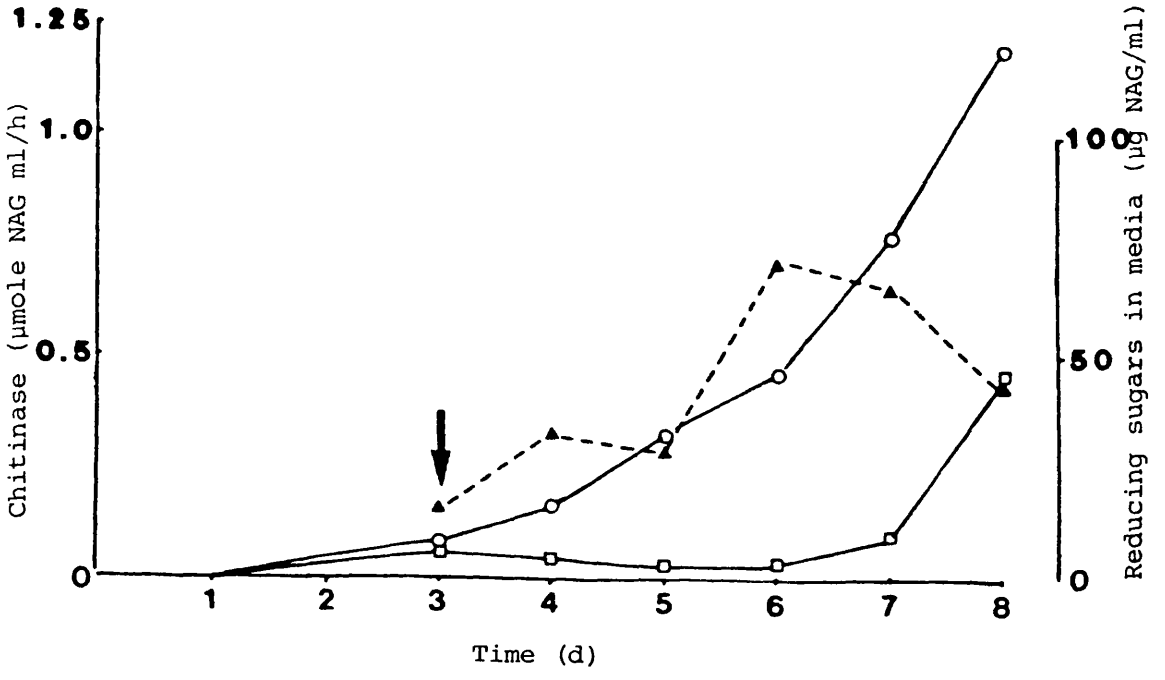
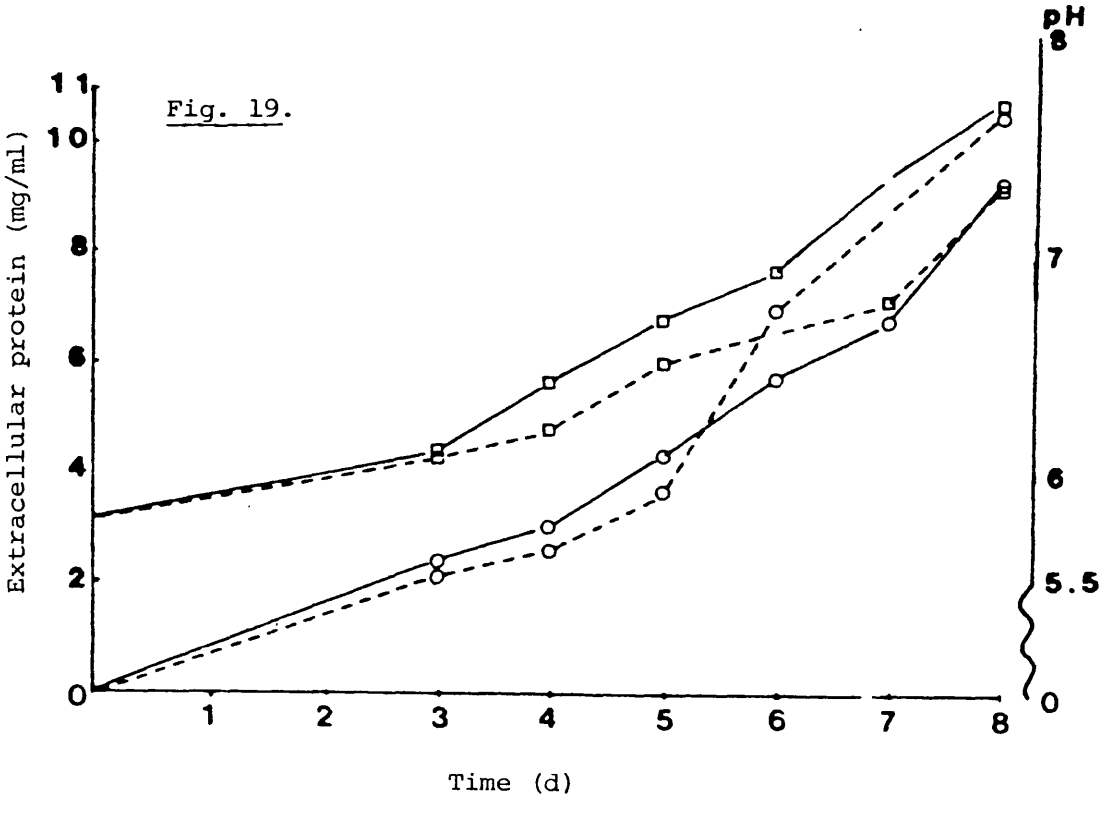

1.25
1.0
0.5
0
Chitinase (µmole NAG ml/h)
100
50
0
Reducing sugars in media (µg NAG/ml)
1
2
3
4
5
6
7
8
Time (d)

Fig. 19.


11
10
8
6
4
2
0
Extracellular protein (mg/ml)
pH
8
7
6
5.5
0
1
2
3
4
5
6
7
8
Time (d)

chitin breakdown are more likely effectors. Previous experiments with shaken cultures containing between 0.2% and 1% substrates had resulted in only small amounts of chitinase activity. However it is known with fungal phytopathogens that inducing sugars can repress synthesis at very low concentrations, e.g. Cooper *et al.* (1975); also the results with batch cultures demonstrated an inverse correlation between chitinase production and concentration of NAG or reducing sugars in cultures (Figs. 15 and 16). To avoid any possible CR it is necessary to supply potential inducers at rates similar to those at which they would be released during growth on a polysaccharide. The most effective method of doing this is to use the diffusion capsules developed by Pirt (1971). They provide a means of supplying established cultures with inducing sugars at constant controlled levels, thereby allowing growth rates to be restricted and repression by sugars minimized or prevented (Pirt, 1971; Cooper and Wood,1975).

Sugars were fed from diffusion capsules at linear rates of *c.* 2 mg/100 ml to shake cultures of *M. anisopliae* (MEl) containing standardised mycelial inocula (Materials and Methods). The concentration of supplied sugars in the medium did not rise above 0.07 mg 100 ml^{-1} indicating that they were being utilised by the fungi at approximately the rate of diffusion. Consequently, growth was limited by the restricted rate of supply of nutrient sugars and was considerably less (1/7th in the first 2 d) than that shown in media containing "unrestricted" amounts of a carbon source (Fig. 21).

The results obtained using diffusion capsules containing

various sugars or alanine are summarised in Figs. 20 and 22, and Table 10 . If synthesis of chitinolytic enzymes is constitutive with CR the main or only form of control, then the enzymes should appear in cultures with restricted supplies of glucose. Under these conditions only low levels of chitinase (5% of maximum activity) were produced which presumably represents basal synthesis. Production of chitinase was induced by NAG, the monomer predominant in chitin, and to a lesser extent by glucosamine which is a minor component of many natural chitins (Hackman and Goldberg, 1965). The amount of chitinase obtained after 4 or 7 d with restricted supplies of NAG was never as great as in batch cultures with chitin as sole carbon and nitrogen source. This could be due to the limited growth conditions the fungi were growing under.

Results (Table 10) indicate that *M. anisopliae* (ME1) produces at least two enzymes, distinct from chitinase, that degrade chitosan and chitobiose respectively. The chitosanase was induced by glucosamine and also to a lesser extent by NAG. Basal levels of enzyme (3% of max.) were produced when mycelium was supplied with restricted glucose. The production of NAG'ase paralelled that of chitobiase indicating that these activities may reside in the same molecular species (see Sect. II, IJ). The highest levels of NAG'ase occurred on NAG or glucosamine but significant amounts were also produced with glucose or alanine (> 36% of max.); evidently therefore NAG'ase is less adaptive than chitinase or chitosanase.

Fig. 20. Production of chitinase by *M. anisopliae* (ME1) with NAG at initial concentration of 1% (●—●) or in restricted supply (■—■); diffusion rate *c.* 2 mg/100 ml/h.

Fig. 21. Growth of *M. anisopliae* (ME1) with NAG at initial concentrations of 1% (●—●), unrestricted cultures, or in restricted supply (■—■); diffusion rate *c.* 2 mg/100 ml/h.

Figs. 20-21. Each result is the mean of 3 replicates. The results are representative of two similar experiments.

Fig. 20

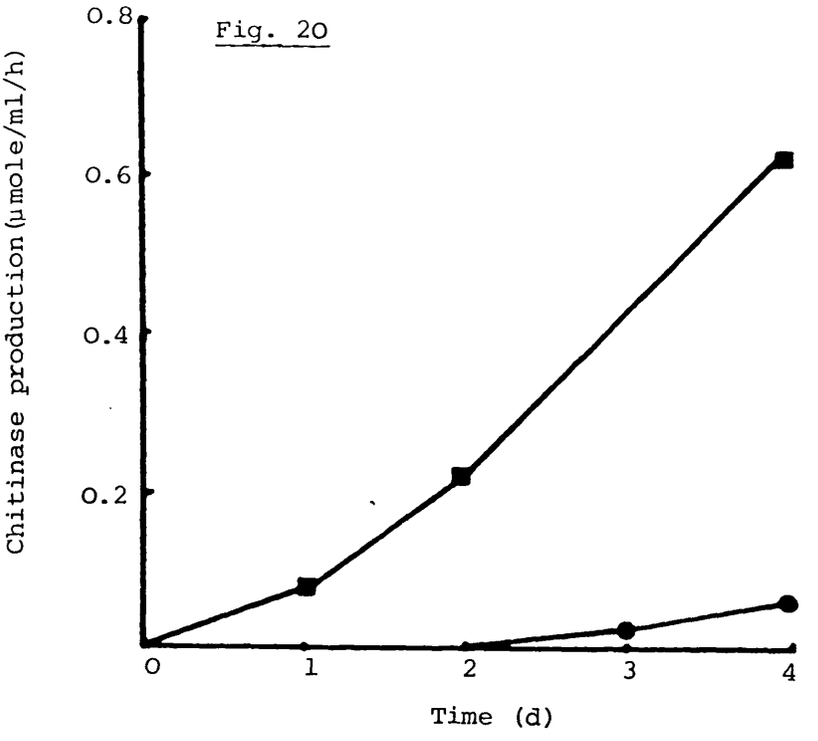

Chitinase production (µmole/ml/h)
0.8
0.6
0.4
0.2
0
1
2
3
4
Time (d)

Fig. 21.

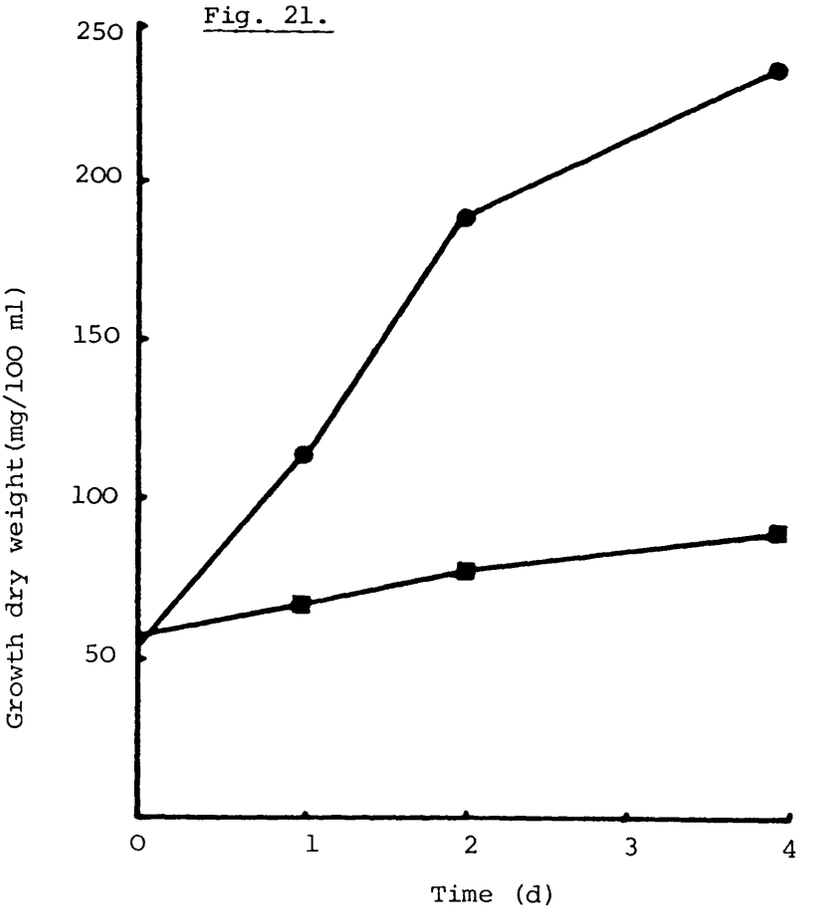

Growth dry weight (mg/100 ml)
250
200
150
100
50
0
1
2
3
4
Time (d)

Table 10. Induction of synthesis of chitin degrading enzymes of *M. anisopliae* (ME1) by a restricted supply of sugars.

Inducer[b]	Enzyme activities (% of maximum[a])			
	Chitinase	Chitosanase	Chitobiase	NAGase
NAG	100	87	100	100
glucosamine	73	100	88	92
glucose	5	3	41	43
galacturonic acid	5	4	32	36
dextrose	6	4	63	67
alanine	3	3	48	52

a) Enzyme activities expressed as a % of the maximum activities attained after 4 d exposure to different carbon sources. 100% activities detected were, chitinase, 0.61 µmoles NAG $h^{-1}ml^{-1}$, chitosanase, 0.43 µmoles G $ml^{-1}h^{-1}$. NAGase, 0.61 µmoles PNP $ml^{-1}h^{-1}$, chitobiase, 1.35 µmoles NAG $ml^{-1}h^{-1}$.
b) inducers were fed from diffusion capsules at linear rates of *c.* 2 mg per 100 ml per hour.
c) The results were calculated from the means of at least 5 replicates.

F. Effect of rate of supply of NAG on chitinase synthesis

To test for CR, supplies of NAG were increased in restricted cultures so that supply exceeded the requirements of the organism and sugars accumulated to high levels in the media. Cultures of *M. anisopliae* MEl were grown for 4 d with NAG supplied from diffusion capsules at *c.* 20 µg $ml^{-1}h^{-1}$ (2.0mg 100 $ml^{-1}h^{-1}$) and *c.* 95 µg $ml^{-1}h^{-1}$ (9.5 mg 100 $ml^{-1}h^{-1}$).

Increasing the rate of supply decreased the synthesis of chitinase by *c.* 66% while causing a four-fold increase in growth (Fig. 22B). Reducing group determination showed that NAG when supplied at 20 µg $ml^{-1}h^{-1}$ never exceeded 25 µg ml^{-1}. At the higher supply rate however NAG accumulated in the culture to > 100 µg ml^{-1}.

These figures suggest that CR of the chitinase may be effected by NAG when in excess of the immediate growth requirements of the organism. In comparison the effect of CR on chitobiase (NAG'ase) production was considerably less. Increasing the rate of supply increased synthesis of chitobiase by *c.* 140%. This is however a smaller increase than would be expected if levels of extracellular enzyme were related entirely to mycelial growth.

7. Regulation of Protease Production

Many micro-organisms are capable of producing large amounts of extracellular proteases under varying conditions of growth. The relatively few critical investigations conducted on regulation

Fig. 22. Formation of chitinase and chitobiase by *M. anisopliae* (MEl) with NAG supplied at (A) *c.* 2 mg/lOO ml/h and (B) *c.* 9.5 mg/lOO ml/h.

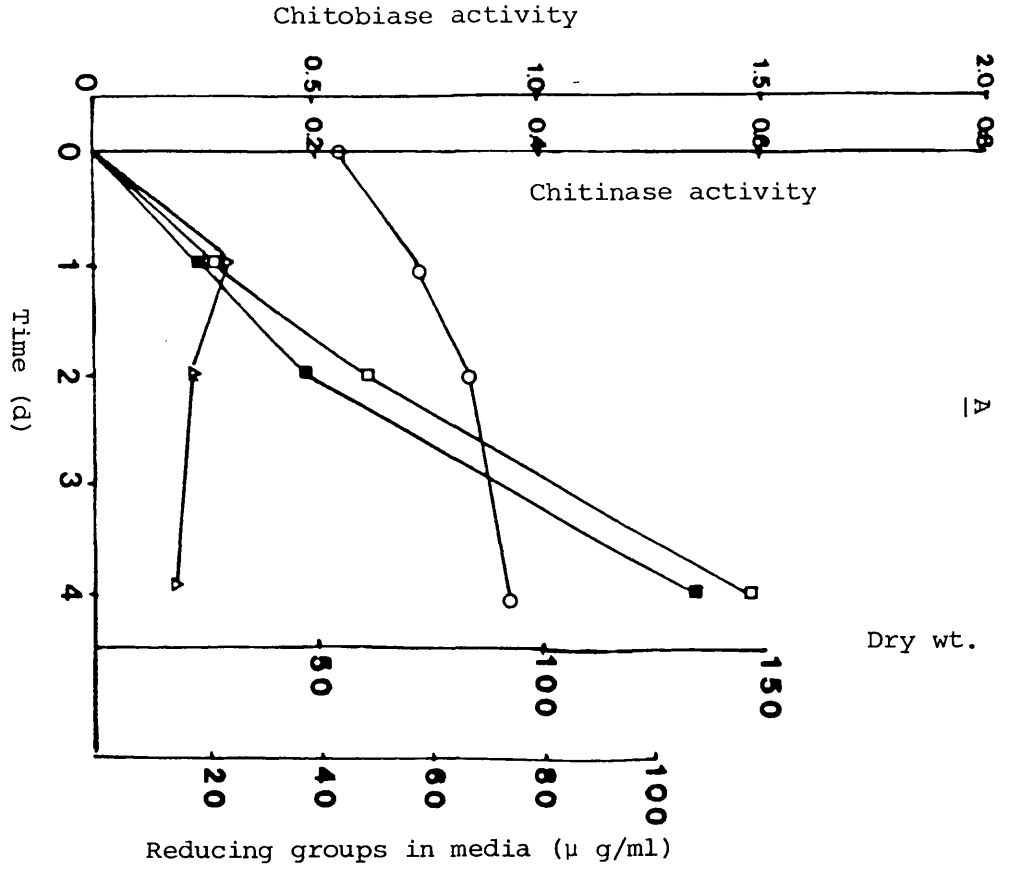
(■—■) chitinase activity, µmole NAG/ml/h

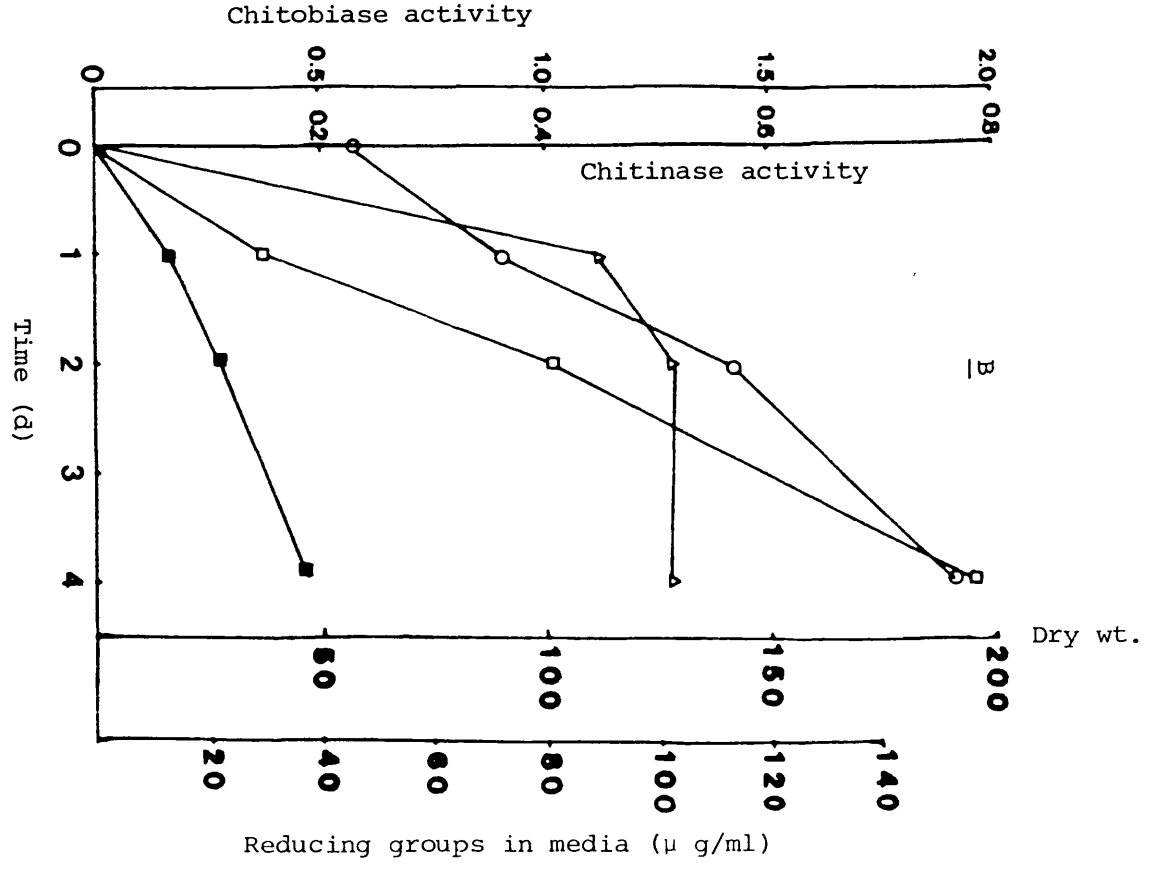
(□—□) chitobiase activity, µmole NAG/ml/h

(○—∩) growth, mg/lOO ml dry wt.

(△—△) reducing groups in media (µ g NAG equivalents ml^{-1}).

Each result is the mean of 3 replicates. Tbe results are representative of 2 similar experiments.


Chitobiase activity
Chitinase activity
Time (d)
Dry wt.
Reducing groups in media (μ g/ml)
A


Chitobiase activity
Chitinase activity
Time (d)
Dry wt.
Reducing groups in media (μ g/ml)
B

of production have emphasised the importance of CR whether the protease is constitutive e.g. several *Aspergillus* spp. (Cohen, 1981), or induced by protein, e.g. *Neurospora crassa* (Drucker, 1972). Unfortunately most other studies, including those involving entomopathogens (e.g. Kucera *et al.*, 1968; Kucera,1981),have not considered the effects of CR.

Because of the difficulty in interpreting these results a more rigorous approach was applied in this study to determine the mode of protease regulation in *M. anisopliae*.

A. Detection of protease production on solid media

Protease production by 8 *M. anisopliae* isolates was initially evaluated with skimmed milk/basal salts agar medium (pH 6.5). Protease activity as revealed by clearing of particulate protein occurred in all strains within a period of 5 d at 27°C. 3 strains were chosen for further studies (ME1, RS324 and RS23).

Solid media were particularly useful in screening for repressors of protease production. Initially this involved the addition of carbon and nitrogen sources to a simple agar medium containing a basal salts solution and skimmed milk (Materials and Methods). Subsequently it was found that the inclusion of nutrient agar as well as skimmed milk (Gabriel, 1968a) encouraged growth and protease production in all strains, and reduced the effect of additional amino acids and other carbon and nitrogen sources on growth. This facilitated the comparison of colonies in different media for proteolytic activity. The use of nutrient agar does

however mean that the standard test media before modification already contains a considerable amount and variety of low molecular weight organic and inorganic nitrogen.

All the amino acids tested (alanine, proline and phenylalanine) and the simple peptide glycyl glycine greatly suppressed protease activity at 0.1 M. The nature of the repression (small indistinct haloes with only partial clearing) precluded quantitative measurements. Easily metabolisable sugars such as NAG and glucose also suppressed proteolytic activity (plates 3-5), but lactose which is poorly metabolised by MEl (Table 8) allowed the production of protease in amounts similar to that in controls. Unlike with added amino acids the reduced haloes formed in the presence of sugars showed complex clearing and the enzymatic index (Table 11) could be accurately determined. Inorganic nitrogen sources, urea and NH_4Cl (20 mM) had little effect on protease activity (Table 11).

The extracellular protease of MEl is not subject to end product inhibition (Sect. II, 5a) so readily utilised metabolites (e.g. alanine or NAG) presumably affected enzyme production rather than activity. Solid media therefore could have considerable value in screening for mutants with altered production or regulation of protease.

B. Protease production in liquid media - batch cultures (unrestricted)

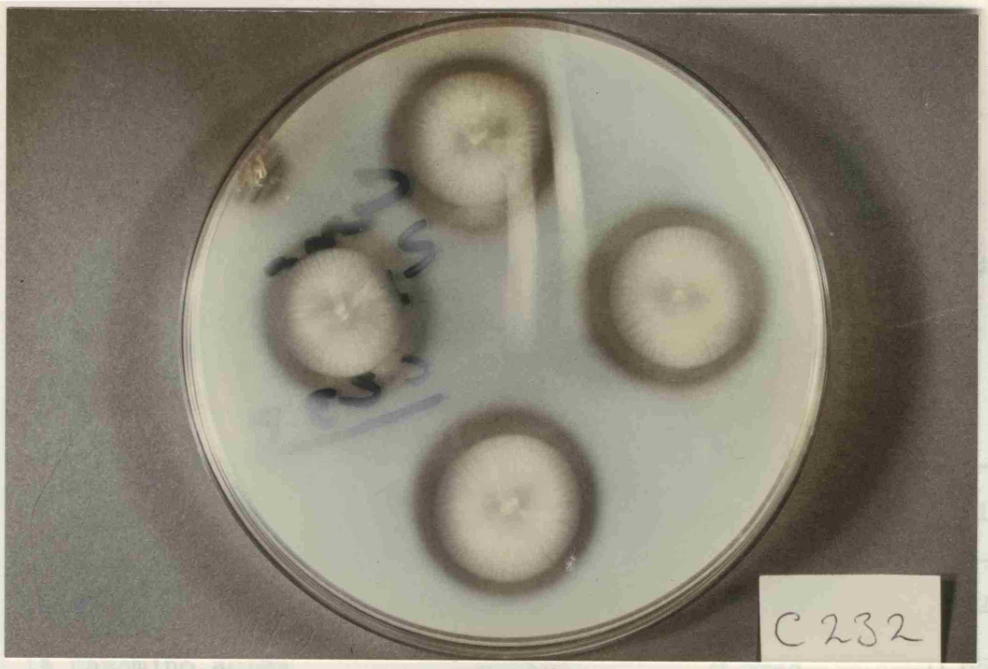
After the preliminary tests with plates the nature of the protease complex and its mode of regulation were studied in liquid

Plates 3 to 5. Comparison of protease production by RS 23, grown for 6d at 27°C on control plates (skimmed milk/nutrient agar medium, see Materials and Methods) and on media supplemented with 1% glucose or 1% NAG.

Plate 3. Control plate. Note, clearing zones indicating enzymic degradation of the skimmed milk.

Plate 4. 1% NAG plate. Note, inhibition of protease activity.

Plate 5. 1% glucose plate. Note, inhibition of protease activity



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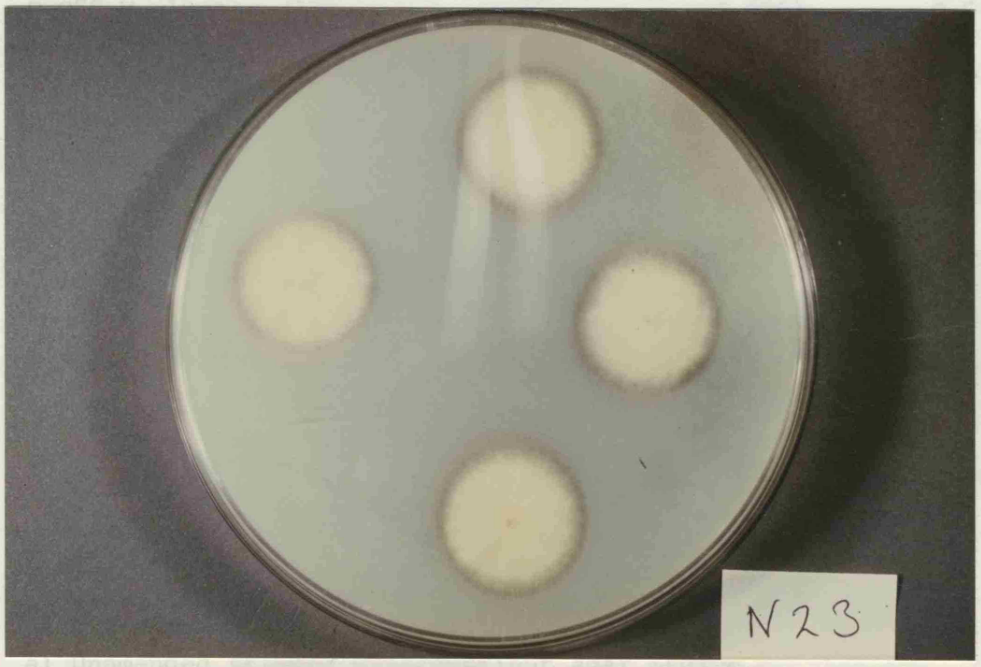

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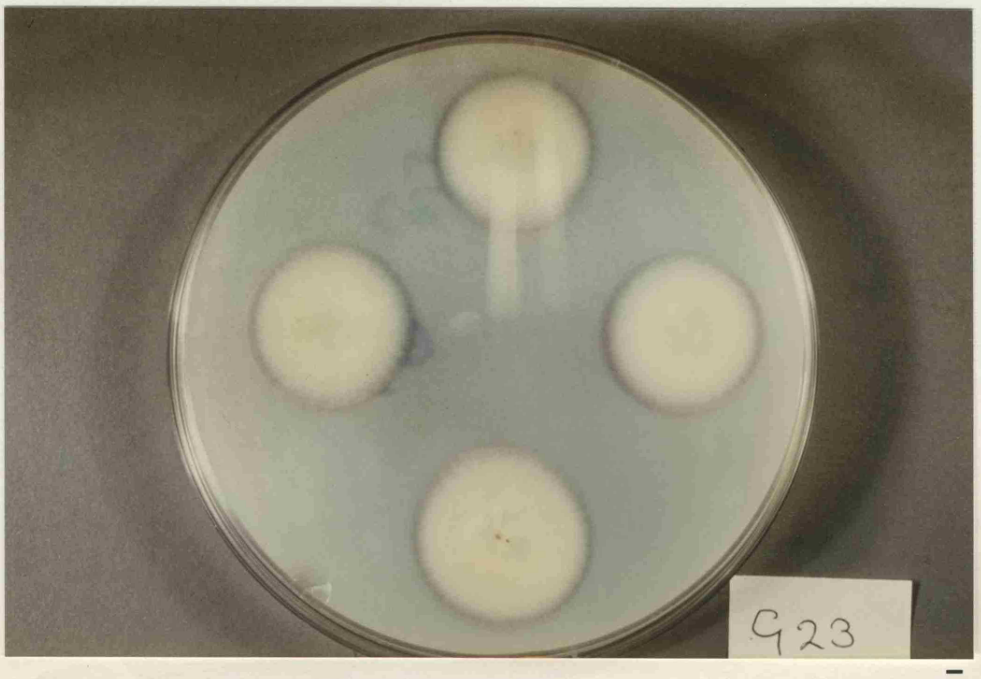


5

Table 11. Comparison of enzymatic index in 3 isolates of *M. anisopliae* grown on skimmed milk/nutrient agar medium with added carbon/nitrogen sources (pH 6.5).

Isolate:	RS23	RS324	ME1
Control[a)]	0.8125	0.7132	0.8234
1% casamino acids	0.8164	0.5944	0.6596
0.055 M glucose (1%)	0.9636	0.9261	0.9477
NAG (1%)	0.9542	0.7432	0.8339
0.055 M NAG (1.22%)	0.9687	0.9112	0.9245
0.055 M lactose	0.8545	0.7431	0.8662
20 mM Urea	0.8199	0.7014	0.8274
20 mM NH_4Cl	0.8741	0.7078	0.8306

Enzymatic index obtained by the colony diam./halo diam. ratio. Means of 12 colonies. NB a value of 1.0 indicates no clearing zone.

a) Unamended skimmed milk/nutrient agar medium.

media. Initially this involved unbuffered unrestricted growth in cultures comprising basal salts containing various carbon and nitrogen sources. After 4 d growth cultures were harvested and analysed for growth, pH and following dialysis, for protease activity.

It appears as evidenced by an assay based on the hydrolysis of haemoglobin (Materials and Methods) that *M. anisopliae* (MEl) produces at least two proteases, with peaks of activity at pH 5.5 and 8.2 (Fig. 22). Evidently the ratio of production of the two proteases varies with the nitrogen sources, but in most cases the major activity is at pH 8.2. Production of this major protease appears to be constitutive as it was produced with all carbon and nitrogen sources tested. However a medium containing 1% glucose and 0.2% $NaNO_3$ did not allow production of detectable amounts of the "minor" protease (pH optimum, 5.5). Organic non protein nitrogen sources such as peptone and casamino acids allowed slight production of the minor form. Greater production occurred when proteins were present, and in a medium containing a crude homogenate of locusts, maximum proteolytic activity was at pH 5.5.

These results indicate that production of the minor protease may be inducible although they could also be consistent with control by repression effected by readily metabolisable compounds such as peptone or glucose.

Repression of protease synthesis was studied in cultures containing 1% protein (e.g. casein or bovine serum albumen,BSA)

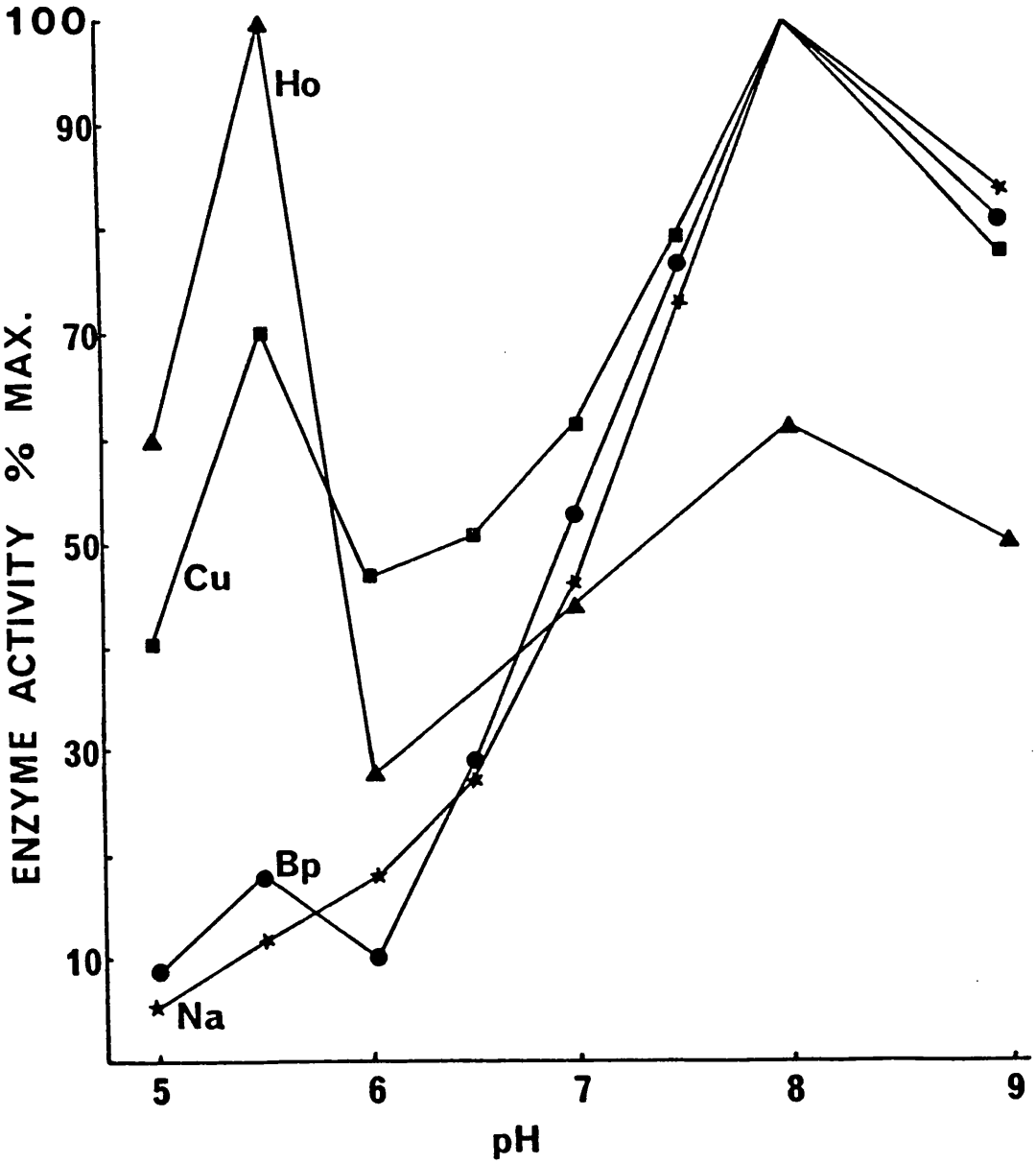


Fig. 22. pH optima of the proteolytic complex of *M. anisopliae* (MEl) produced on different nitrogen media. (Ho) 1% whole locust homogenate; (Cu) 1% locust cuticle, (Na) 1% $NaNO_3$ (+ 1% sucrose) (Bp) 1% bactopeptone.

or complex of proteins (e.g. skimmed milk) with the addition of various carbon and nitrogen sources. The pH rose rapidly in some of these cultures producing conditions unfavourable either for production or stability of acidic protease (unpublished results). The results largely parallel those obtained with agar plates, and are summarised in Tables 12 and 13.

Time course studies of alkaline protease production in control (1% bovine serum albumin, BSA) or repressing media (1% BSA supplemented with additional C and N sources) revealed that measurable activity appeared 1-2 d after inoculation (i.e. during rapid growth; Fig. 23). In media containing repressors there was a tendency for protease production to reach a plateau earlier than in control media. The results in Fig. 23 show clearly that repression by L-alanine is a function of its concentration.

C. Effect of cyclohexamide on protease synthesis

The effect of cyclohexamide, an inhibitor of protein synthesis (see Sect. I, 6D), was examined by adding 10 μg ml^{-1} to 2 and 3 d old cultures, otherwise designed to encourage protease production. The results shown in Fig. 24 illustrate that extracellular protease probably results from *de novo* synthesis.

D. Production of proteolytic enzymes in restricted feed cultures

As described above, proteolytic enzymes would appear to be regulated by CR. To confirm this possibility, production of enzymes in batch (unrestricted) cultures was compared with that in cultures supplied with glucose or amino acids at rates limiting to growth.

Table 12. The effect of addition of various carbon and nitrogen sources to unbuffered basal salts medium on growth and protease production in *M. anisopliae* (ME1)

	Dry wt. (mg ml^{-1})	Protease (pH 8.0) (g trypsin equivalents $ml^{-1}h^{-1}$)	protease (specific activity units mg^{-1} dry wt.)	pH
Casein	2.1	9.45	4.50	7.60
" + 0.055 M lactose	2.35	8.64	3.68	7.45
" + 0.055 M glucose	3.73	2.61	0.70	6.60
" + 0.055 M NAG	3.89	2.88	0.74	7.05
" + 0.01 M alanine	2.21	8.82	3.99	7.40
" + 0.05 M alanine	2.41	7.12	2.95	7.85
" + 0.1 M alanine	2.75	4.52	1.64	8.21
" + 0.1 M glucine	2.42	3.39	1.40	8.85
" + 0.1 M glycylglycine	2.38	3.18	1.34	8.35
" + 0.1 M tyrosine	- a)	4.00	- a)	8.40
" + 0.05 M NH_4Cl	2.17	7.97	3.67	7.12
" + 0.1 M urea	1.94	5.48	2.83	8.33

a) tyrosine partially insoluble

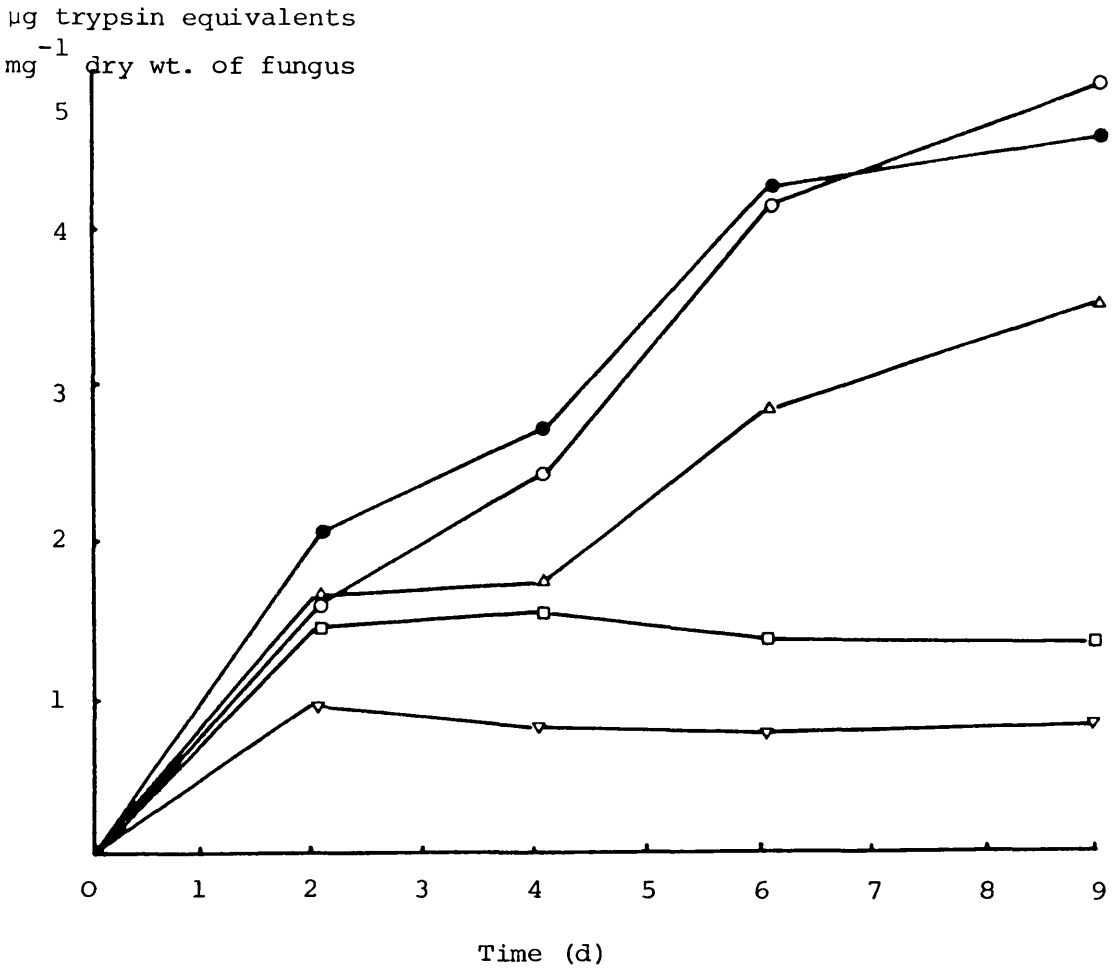


Fig. 23. Total protease production in batch cultures containing 1% BSA (●—●), 1% BSA + 0.01 M alanine (○—○), + 0.05M alanine (△—△) + 0.1 M alanine (□—□) and 1% BSA + 0.1 M NAG (▽—▽).

Each result represents the mean of 3 replicates. The results are representative of 2 similar experiments.

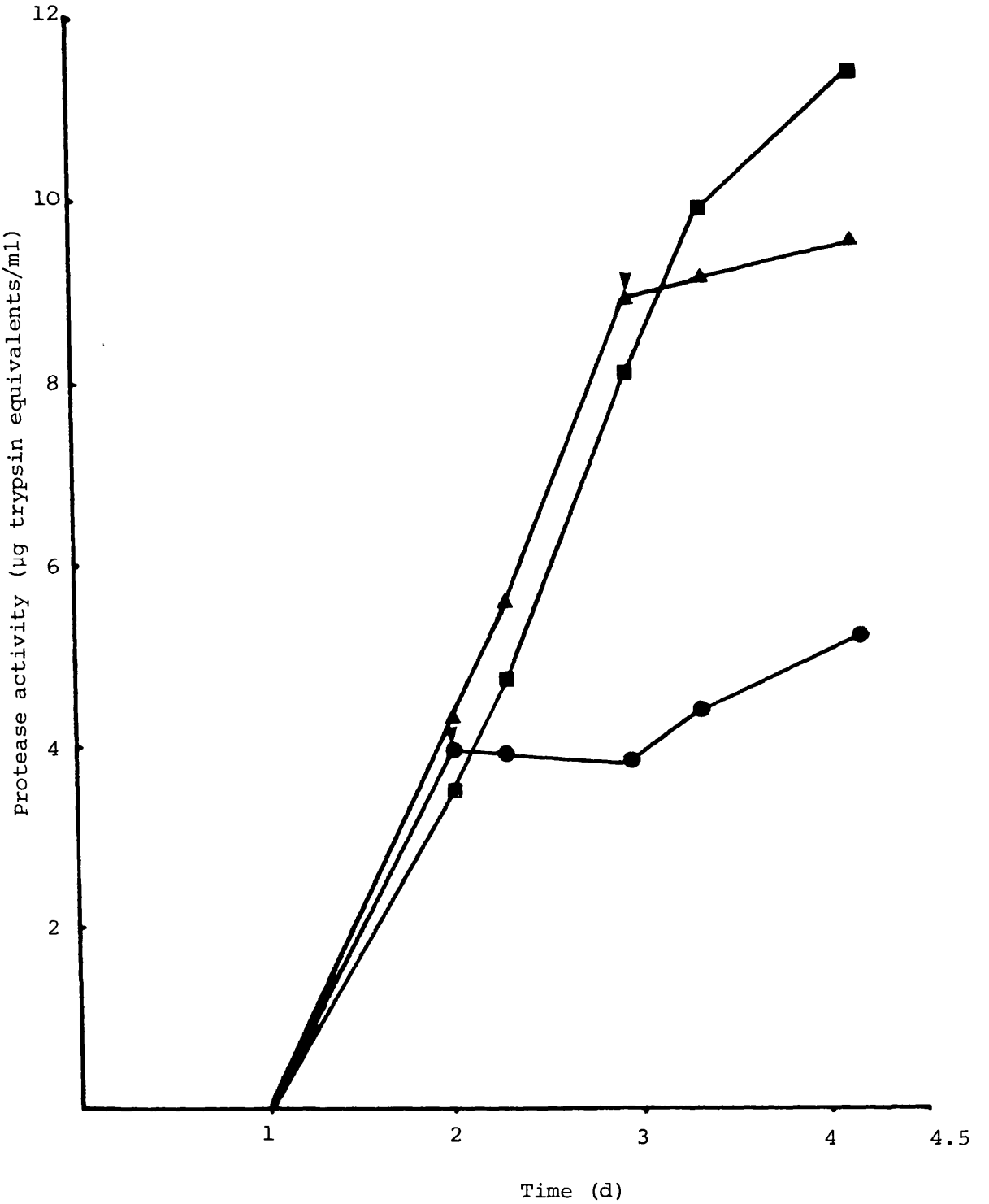


Fig. 24. Effect of cyclohexamide on the production of extracellular protease (assayed at pH 8.2) by *M. anisopliae* (ME1) grown on 1% chitin culture. Cyclohexamide (10 µg/ml) was added 48 h (●—●) or 70 h (▲—▲) after inoculation. Controls (■—■). The media were buffered with 0.2 M MES, pH 5.0. Each result is the mean of 3 replicates. The results are representative of 2 similar experiments.

Table 13. The effect of various carbon and nitrogen sources on growth and protease production in MEl.

1. Compounds which increased the rate of growth but repressed the production of protease

Casamino acids	1%
L-Alanine	0.05 - 0.1 M
L-Glycine	0.1 M
Glycyl-glycine	0.1 M
L-Tyrosine	0.1 M
L-Cysteine	0.1 M
Glucose	0.055 M
N-acetylglucosamine	0.055 M

2. Compounds which had only slight effect on the growth but repressed protease production

Urea	0.1 M
NH_4Cl	0.05 M

3. Compounds which had little effect on either growth or protease production

Lactose	0.055 M
Xylose	0.055 M

Carbon sources were fed from diffusion capsules at linear rates to buffered (pH 6.2) shake cultures of *M. anisopliae* (MEl) containing standardised mycelial inocula (Materials and Methods). The results obtained were compared with those from mycelium inoculated into unrestricted cultures containing carbon sources at 1% levels (Figs. 25 to 27).

Production of alkaline protease, amino peptidase and carboxy-peptidase occurred in cultures with restricted supplies of glucose or amino acids as would be expected if CR is the main or only form of control. Production of enzymes in batch cultures was delayed presumably until the carbon source was reduced to a non repressing level. The low levels of aminopeptidase in batch cultures indicate that this enzyme is subject to comparatively severe CR.

To accurately determine variations in acid protease levels in culture fluids, activities were measured at pH 5.5, 6.0 and 8.0. The alkaline protease has activity at all these values, whereas the acid protease has a far narrower pH profile (see Fig. 22). The results shown in Table 14 indicate that the acid protease is produced in relatively small amounts in non-repressing conditions, suggesting that its production is at least partially constitutive. However, as synthesis was far less than that of the alkaline enzyme maximum production may require the presence of proteins or other compounds which enhance its synthesis.

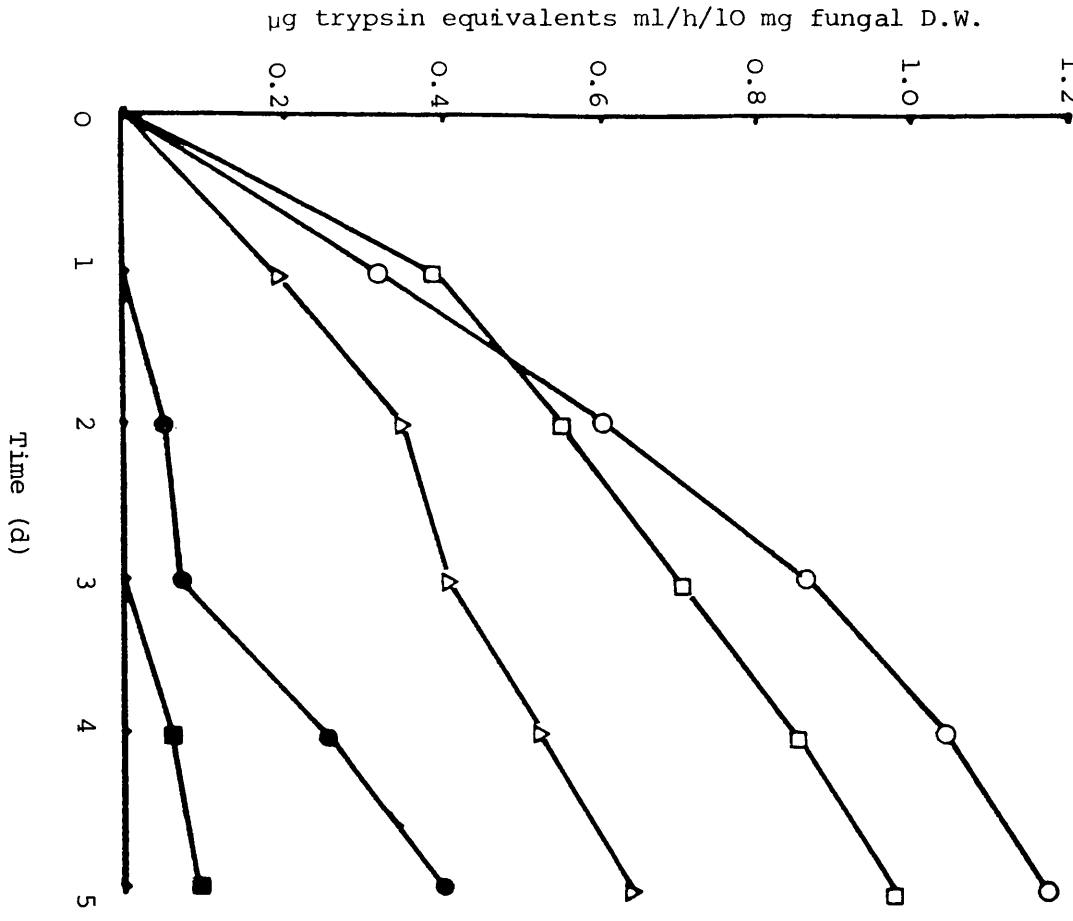


Fig. 25. Protease

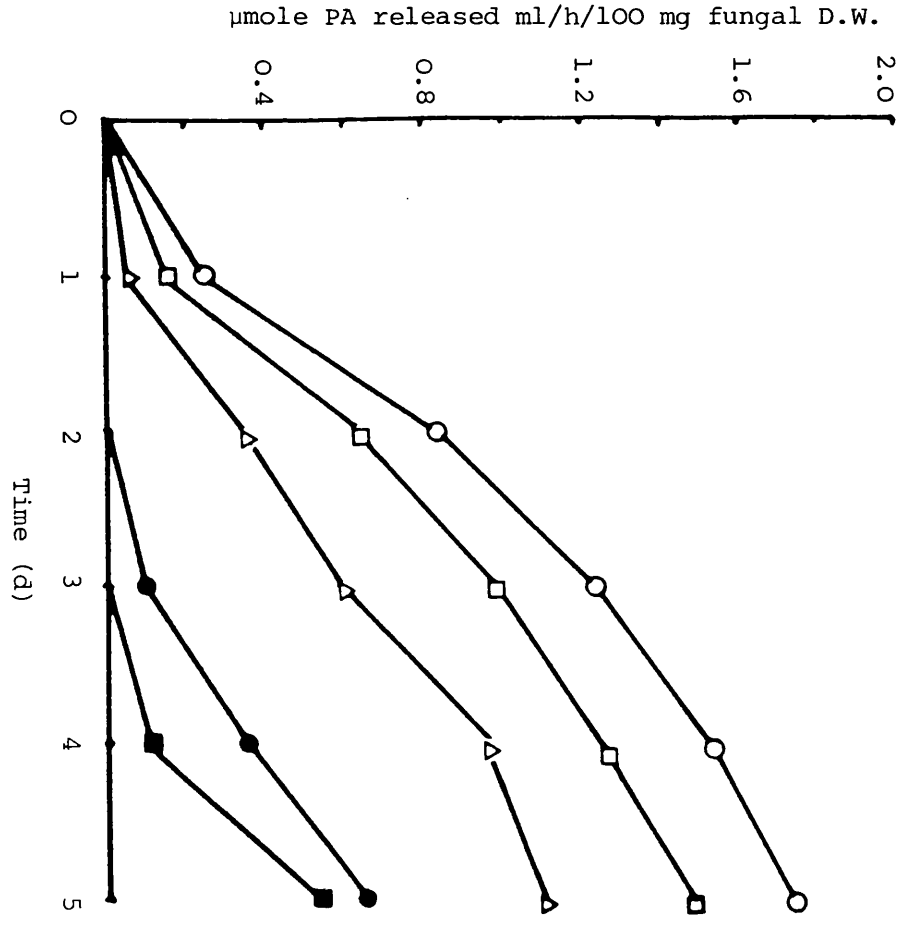


Fig. 26. Carboxypeptidase

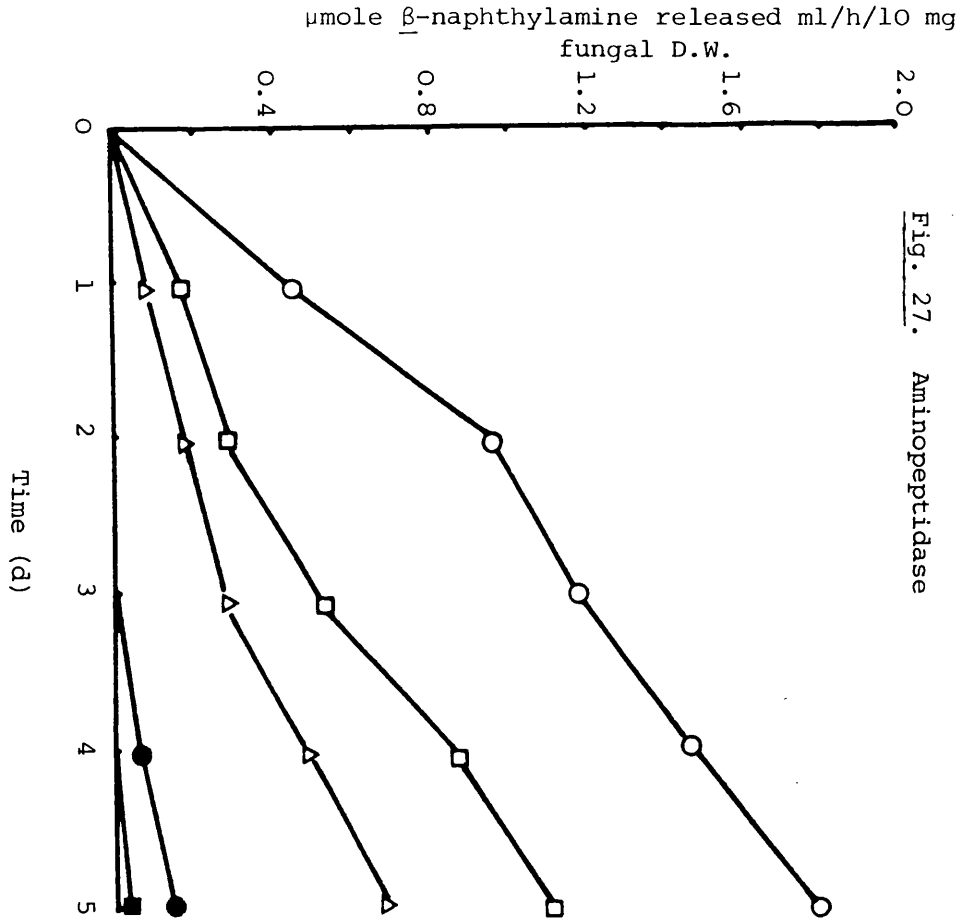


Fig. 27. Aminopeptidase

Figs. 25 to 27. Production of proteolytic enzymes by *M. anisopliae* (MEl) with carbon sources in unrestricted cultures (1% levels) (closed symbols) or in restricted supply (open symbols).

Carbon source: (O) Na glutamate; (□) alanine; (Δ) glucose

Each result is the mean of at least 5 replicates.

Table 14. Protease production in restricted supply cultures of *M. anisopliae* (ME1). Activity after 4 d was measured at pH 5.5, 6.0 and 8.0 and expressed in terms of µg trypsin equivalents $ml^{-1}h^{-1}$.

Carbon source	Assay pH 5.5	6.0	8.0	activity pH 5.5 / activity pH 8.0
glucose	0.09	0.09	0.64	0.14
alanine	0.16	0.11	0.89	0.18
Na glutamate	0.19	0.13	1.04	0.18

E. Production of proteolytic enzymes in cultures deficient in essential catabolites

All proteins contain carbon and nitrogen, most contain sulphur and a few contain phosphorus. A series of transfer experiments were performed to determine which, if any, of these essential catabolites regulates the production of protease in *M. anisopliae* (MEl).

Standardised fungal inocula (Materials and Methods) were incubated for up to 48 h in media lacking either carbon (-C), nitrogen (-N), carbon and nitrogen (-CN), sulphur (-S) or phosphate (-P), and the results compared with those from complete media (CM) that contained: 1% sucrose, 0.2% NH_4Cl, 0.1% KH_2PO_4 and 0.05% $MgSO_4$. The results illustrated in Fig. 28 indicate that no alkaline protease was produced in CM, -P or -S media. However limitation of carbon or nitrogen derepressed extracellular protease as reflected by its rapid production. This required *de novo* protein synthesis as cyclohexamide (lO µg/ml) prevented the appearance of protease in -CN media. Modifying the concentration of C or N demonstrated that both were independently capable of partially repressing protease production, even in the absence of the alternative repressing catabolite (Fig. 28).

Addition of 1% bovine serum albumin (+BSA) to media enhanced production of protease over the first 8 h, subsequently however protease levels were higher in -CN flasks.

Regulation of the acid protease was compared with that of the alkaline enzyme by measuring activities at 5.5, 6.0 and 8.0

(Table 15). Some acid protease was produced in -CN media confirming that it is partially constitutive; however, addition of BSA enhanced its synthesis to a greater extent than occurred with the alkaline enzyme which suggests that production is partly adaptive. Addition of glucose or NH_4Cl to BSA medium repressed both proteases; the effect of glucose was especially severe with the acid enzyme. Evidently therefore repression overrides the enhancing effect of BSA in enzyme production. In all media enzyme production substantially increased after 24 h, presumably due to exhaustion of one or more of the catabolites.

Regulation of Lipase Production

In order to determine whether extracellular lipase could be induced by lipids as carbon source, *M. anisopliae* (MEl) was grown on beeswax, olive oil myristin and caproin. Although profuse growth occurred the extracellular fluids from these cultures showed only slightly greater lipase activity than was obtained from cultures containing cuticle (Fig. 8) or glucose. For example the 5 d old olive oil culture had an extracellular lipase activity of 0.78 μmoles $ml^{-1}h^{-1}$ whereas the comparable values for cuticle and glucose cultures were 0.63 and 0.42 μmoles $ml^{-1}h^{-1}$ respectively. Nevertheless, the rapid growth of *M. anisopliae* in lipid media implies that lipase production occurs but that activity remains largely cell-bound or the extracellular form(s) is unstable.

Table 15. Protease production by *M. anisopliae* (ME1) upon transfer of growing mycelium to cultures containing BSA and additional catabolites. Activity after 8 h was measured at pH 5.5, 6.0 and 8.0 and expressed in terms of µg trypsin equivalents $ml^{-1}h^{-1}$

Growth medium	Assay pH 5.5	6.0	8.0	r $\frac{\text{activity pH 5.5}}{\text{activity pH 8.0}}$
-CN	0.51	0.29	2.2o	0.23
+ 1% BSA	1.11	0.46	3.46	0.32
+ 1% glucose + 1% BSA	0.11	0.15	0.82	0.13
+ 0.2% NH_4Cl + 1% BSA	0.03	0.03	0.07	0.43

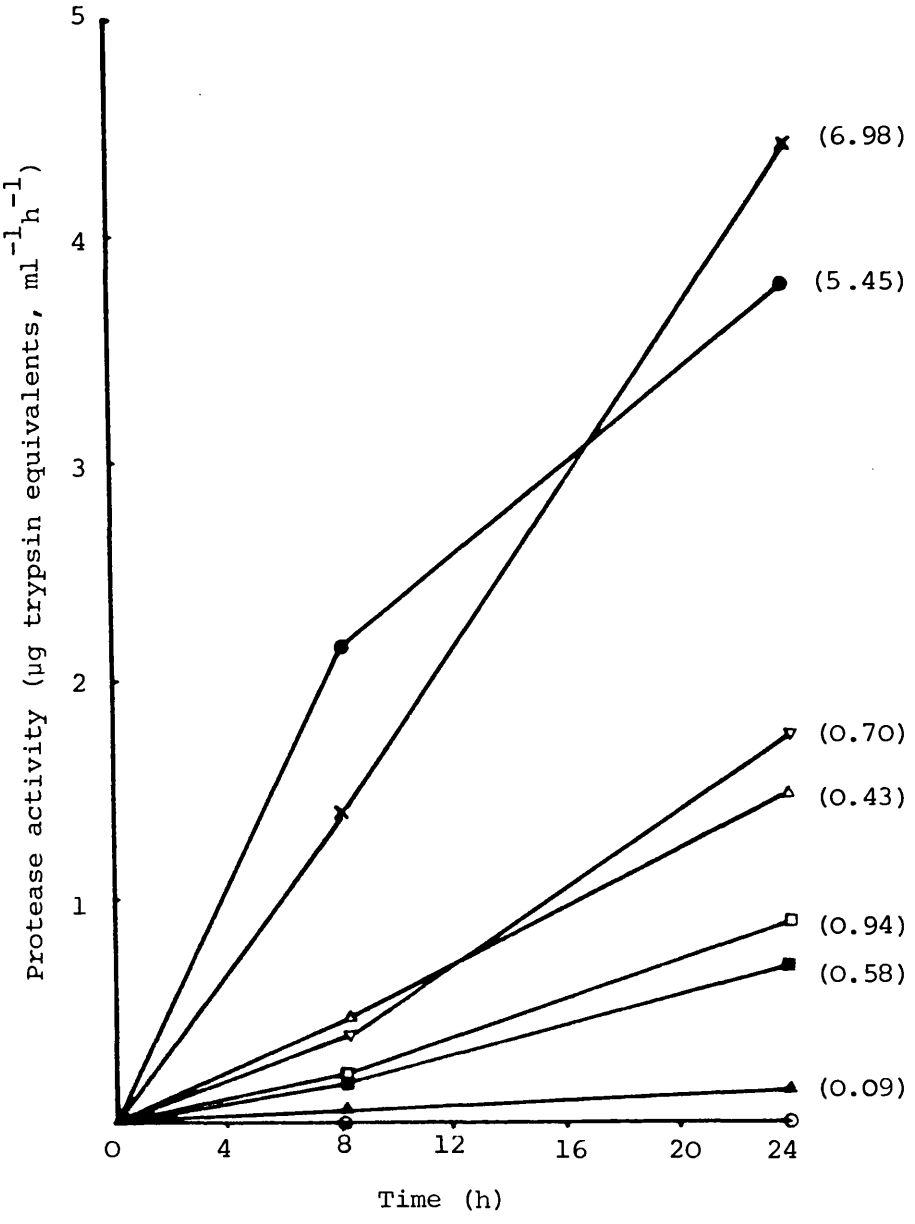


Fig. 28. Protease (pH 8.2) production by *M. anisopliae* (ME1) upon transfer to BSA medium (●—●) or nutrient-limiting conditions: (✕—✕) -CN; (▽—▽) 1% C, -N; (△—△) 3% C, -N; (□—□) -C, 0.05% N; (■—■) -C, 0.1% N; (▲—▲) -C, 0.3% N; (○—○) CM or -P or -S. Letter designations (CM, C, N, P or S) given in text. Figures in parenthesis indicate protease production per 100 mg fungal dry wt. after 24 h. Each point represents the mean of 3 replicates. The results are representative of 2 similar experiments.

Table 14. Regulation of production of CDE by *M. anisopliae* ME1. Summary.

Enzyme	Regulation[a)]	Comments
Chitinase	I/CR	Induced by NAG and glucosamine
NAG'ase	C/CR	Synthesis enhanced by amino-sugars. The effect of CR was relatively weak.
Alkaline protease	C/CR	Production can be initiated by C and N derepression alone
Acid protease	C/CR	Production can be initiated by C and N derepression alone. Synthesis considerably enhanced by protein.
Aminopepticase	C/CR	Subject to severe 'CR' (cf. alkaline protease and carboxy-peptidase)
Carboxypeptidase	C/CR	
Lipase	C	Possibly wall bound.

a) I = induced, C = constitutive; CR = catabolic repression.

DISCUSSION

An initial study on the general growth characteristics of *M. anisopliae* (MEl) as a representative isolate of entomopathogenic spp. showed that it could grow on a wide variety of carbohydrates, lipids and amino acids. Utilization of various polysaccharides not represented in insect hosts such as cellulose and xylan presumably reflects their alternative saprotrophic existence. Excepting utilizing of endogenous reserves, nutrition during penetration of host cuticle will also be saprotrophic, thus ability to utilize available components of cuticle may be of potential importance to pathogenicity. Growth on and from the host surface is a precarious and critical phase of infection. In this context long chain fatty acids, beeswax oils and several of the amino acids found in cuticle supported growth of *M. anisopliae* (MEl). The efficient utilization of NAG and alanine is of particular interest considering their predominance in procuticle.

To gain an indication of how host cuticle influences production of cuticle-degrading enzymes, pathogenic strains of *M. anisopliae*, *B. bassiana* and *V. lecanii* were grown on insoluble locust cuticle as sole carbon and nitrogen source. Under these conditions the fungi produced a wide range of extracellular enzymes corresponding to the major components of insect cuticle. To the author's knowledge, this is the first time the potential range of cuticle degrading enzymes (CDE) has been determined for entomopathogens and the first report of production of peptide exohydrolases, NAGase (chitobiase), catalase, deoxyribonuclease, ribonuclease and phosphoamidase. Furthermore this is the first study of

entomopathogenic fungi where lipase has been assayed with an authentic lipase substrate i.e. a lipid emulsion. Other workers have used Tweens (e.g. Pekrul *et al.*, 1979) or tributyrin (e.g. Samsinakova *et al.*, 1971) which can be hydrolysed by a range of serine hydrolases (e.g. Benoehr *et al.*, 1966; Brokerhoff and Jenson, 1974).

If it is assumed that a similar combination of enzymes is produced *in vivo* then *prima facie* the most important in host penetration would be the endo-acting proteases lipase and chitinase which could potentially destroy the integuments integrity by solubilising cuticle polymers. For plant pathogenic fungi where the range and role of enzymes is better understood, exo-acting enzymes do not effect extensive wall degradation (Cooper, 1983), and this will probably also be true of degradation of insect cuticle. The major function of the peptide-exohydrolases presumably is to further reduce the size of peptide fragments released by proteases and make them available for nutrition. The function of the NAG'ase is more ambiguous as the chitinase is apparently capable of releasing monomer from chitin directly (Sect. II).

The regulation of synthesis of cuticle-degrading enzymes was examined by using carefully controlled culture conditions which allowed selective examination of potential inducers and repressors. The evidence suggests that chitinase synthesis is regulated by products of chitinolytic degradation through an inducer-repressor mechanism. Slow feeding of *M. anisopliae* (MEl)

with sugars and alanine in a carbon-deficient medium demonstrated that the most effective inducer of chitinase was NAG, the principal monomeric constituent of chitin. However, although *M. anisopliae* responded to the inducer within 1 d, the accumulation of enzyme over several days remained less than that obtained after growth on chitin. The two most likely explanations for this phenomenon are that the conditions used to achieve carbon limitation hinder enzyme synthesis, or, that other degradation products of chitin have a synergistic effect with NAG inducing greater chitinase production than NAG alone. Induction of chitinase synthesis is apparently not specific to NAG as glucosamine also allowed production of the enzyme. This may be an adaption by the fungi to the fact that chitin from natural sources appears to be partially deacetylated (Hackman and Goldberg, 1965; 1974; Rudall and Kenchington, 1973), while chitosan also contains 10 - 28% acetylated residues (Monaghan, 1973). Therefore when interpreting data from this and other reports it should be considered that apparent chitinase activity might, at least to a limited extent, be due to chitosanase activity on deacetylated parts of the chitin. The converse however is unlikely to be true as most reports suggest that chitosan is not attacked by chitinase (e.g. Karrer and White, 1930; Otakara, 1964; Monreal and Reese, 1969; Nord and Wadstrom, 1972).

Besides the monomer other candidates for inducers of chitinase are soluble oligomers of chitin (Monreal and Reese, 1969). By analogy with cellulases which are induced by cellobiose (Cooper and Wood, 1975; Cooper, 1977; 1983), chitobiose would be expected to function as an inducer of chitinase. It is possibly of relevance

therefore that the disaccharide is the stereochemical repeating unit of α- chitin (Carlström, 1962) and at least one well characterised chitinase, that of a *Streptomyces* sp. releases chitobiose from colloidal chitin as the major degradation product (Berger and Reynolds, 1958; Skujins *et al.*, 1970). Unfortunately, chitobiose was not available in sufficient quantities to allow its use in diffusion capsules but in any event it seems unlikely for two reasons that it could function as a major inducer of chitinase activity in *M. anisopliae* (MEl). Firstly, extracellular and cell bound activities of the constitutive NAG'ase (chitobiase) would probably degrade chitobiose to NAG before it enters the cell. In culture fluids chitinase was never found in the absence of NAG'ase, thus either a selective inhibitor of the glycosidase or NAG'ase-deficient mutants would have to be used to show unequivocally that chitobiose rather than its degradation product (NAG) was inducing chitinase. Secondly, the major end product of not only NAG'ase but chitinase is NAG (Sect. II), which suggests that enzymes of the chitinase complex would be incapable of generating sufficient chitibiose from chitin to effect induction. The disparity between modes of induction of chitinase and cellulase is probably due to the abundance of glucose (the monomeric unit of cellulose) both in the free state and as various polymers (e.g. starch, callose, glycogen, dextrins) (Cooper, 1983), compared with NAG which although a component of the intercellular region of animal cells and bacterial cell walls, is found predominantly in chitin and perhaps chitosan.

Observations with batch cultures were basically expressions

of the same induction-repression mechanism. Thus there was a negative correlation between growth rate and enzyme production, e.g. in 3% chitin cultures growth was greater but enzyme production lower than cultures containing 1% or 2% chitin. Also, the addition of readily utilised catabolites such as NAG, amino acids and olive oil exerted a non-specific repression of chitinase synthesis. In addition maximum levels attained in cultures were inversely related to the accessibility of the substrate i.e. the least accessible form (an unmodified crystalline chitin) induced higher levels of chitinase than the more readily degraded colloidal chitin (see also Sect. II). A similar situation has been described for cellulases and the results have also been interpreted in terms of CR (e.g. Horton and Keen, 1966; Yamane *et al.*, 1970). Analysis of culture fluids from batch cultures confirmed that inducing sugars had accumulated to repressive levels in cultures where a degree of repression had apparently occurred (e.g. on 3% chitin, or with 1% colloidal chitin). These results with batch cultures suggest that whether the fungus is living saprotrophically in debris containing chitin, or penetrating insect cuticle, the accessibility and amount of chitin relative to other available nutrients may determine levels of chitinase synthesised through the inducer: repressor system.

An interesting contrast to mode of regulation of chitinase was provided by NAG'ase, which was produced in all media and was little affected by CR. The reasons for this relatively loose control over synthesis are not known but may reflect a function

of this enzyme in cell wall changes during growth. Although unlikely to be an adequate explanation for its constitutive production, extracellular NAG'ase will probably function along with basal levels of chitinase to release inducers of chitinase from chitin. In this context it is noteworthy that NAG'ase but not chitinase effectively degrades the dimer and trimer of NAG (Sect. II, 1J).

The complexities of chitinase attack on chitin have not been studied in the same detail as the substrate specificities and mode of action of cellulases, but there are indications that chitinases may also exist in at least two forms distinguished by their activities towards crystalline and colloidal chitin (Monreal and Reese, 1969; Tuinova *et al.*, 1976a,b). However, in *M. anisopliae* (ME1) culture filtrates there appeared to be a close correlation in patterns of activity against these two substrates indicating that a single enzyme is probably responsible. There was however evidence from restricted feed cultures of an enzyme that depolymerised chitosan and was induced principally by glucosamine. The presence of such an enzyme might be predicted considering the heterogeneous nature of amino sugar polymers. A chitosanase produced by *Streptomyces* sp. No. 6 is also induced by glucosamine (Price and Storck, 1974).

The results discussed above particularly with reference to the fine control of chitinase by induction and CR allows certain data obtained by other workers to be re-interpreted. As for *M. anisopliae* (ME1) in this study Monreal and Reese (1969) using

unrestricted batch cultures of *Serratia marcescens* found production of far greater amounts of chitinase on chitin than on NAG and they suggested induction was probably by soluble oligomers.However as more chitinase was produced on low concentrations of NAG (0.2%) than at higher concentrations, induction and CR by the monomer is a probability.

Young and Carroad (1981) demonstrated in the same strain of *S. marcescens* that chitinase was produced in restricted feed cultures with NAG as limiting substrate. Unfortunately, they did not test any alternative carbon sources in limiting amounts so control by CR alone cannot be ruled out. However, taking their work in conjunction with that of Monreal and Reese (1969) it seems clear that chitinase in *S. marcescens* is regulated by NAG induction: CR in a rather similar way to *M. anisopliae* (MEl).

In unrestricted batch cultures of *B. bassiana* amino sugars allowed production of small quantities of chitinase (36 µg NAG released over 4 h from 0.5 ml of concentrated 1% NAG medium) whereas no chitinase was detected in glucose or amino acid cultures (Smith and Grula, 1983). It is possible therefore that even under conditions where CR is presumably operating, there is still some specificity of induction. Monaghan (1973) found that a strain of *B. bassiana* produced a chitosanase when grown on *Rhizopus* hyphae whereas growth on colloidal chitin or colloidal chitosan did not induce the enzyme. Chitosan in *Rhizopus* hyphae is presumably relatively inaccessible compared to the colloidal preparations and it seems probable therefore that these results can be explained in terms of CR. A strain of *M. anisopliae* studied

by Monaghan (1973) did not apparently produce a chitosanase suggesting there may be considerable variation between isolates of this species in specificity of chitinolytic enzymes.

On the basis of pH activity profiles, *M. anisopliae* (MEl) produces at least two extracellular proteases. Production of both enzymes appeared to be constitutive as production could be initiated by carbon and/or nitrogen derepression alone. Cyclohexamide prevented the production of proteases when growing mycelia were transferred to derepressing conditions, indicating that repression was probably exerted at the level of enzyme synthesis rather than the secretion process. Similar results with regard to derepression were found in *Aspergillus* spp. (Cohen, 1981), *Neurospora crassa* (Cohen and Drucker, 1977) and *Candida lipolytica* (Ogrydziak *et al.*, 1977), although with the exception of some *Aspergillus* spp. (Cohen, 1981) protease production was also derepressed upon limitation of sulphur - a feature not evident with *M. anisopliae* (MEl). It is noteworthy that sulphur-containing amino acids appear to be lacking from cuticle (Table 3). Since all proteins contain carbon and nitrogen these findings are consistent with the idea that a major function of the extracellular protease is to make nutrients available.

Protein culture media supplemented with additional catabolites also demonstrated a repression mechanism. Several compounds including amino acids and readily utilisable carbohydrates such as NAG increased the growth rate markedly while simultaneously causing a non-specific repression of enzyme formation.

As with several species of *Aspergillus* (Cohen, 1981) but unlike *N. crassa* (Cohen and Drucker, 1977), *M. anisopliae* (MEl) did not require a protein in the medium to induce protease production. However, protein added to -CN medium initially enhanced production, especially of the acidic enzyme. The large amounts of protease produced on media containing proteins in batch cultures and on plates also indicates that protein may stimulate enzyme synthesis. Several previous studies have shown that protease production by micro-organisms is higher when grown on proteins than on other nutrients (O'Sullivan and Mathison, 1971; Ichikawa, 1954; Sakamoto and Shuzuyl, 1957; Ogrydziak *et al.*, 1977) and this may also be true of *B. bassiana* and *M. anisopliae* (Kucera, 1971; 1981). However, with the exception of the studies by Ogrydziak *et al.*,(1977) and Cohen and his co-workers the possibility of regulation by CR has not been considered. The present study suggests that CR could potentially have influenced enzyme synthesis on amino acids, inorganic nitrogen sources and the other readily utilised nutrients with which proteins were usually compared (e.g. O'Sullivan and Mathison, 1971)

Overall, these results show that synthesis of protease is regulated by the composition of media with respect to protein levels and other metabolites. Thus protein in the media will enhanced enzyme production but easily metabolisable compounds (i.e. NAG or amino acids) will repress it. However although production of both proteases was enhanced by proteins and inhibited by CR, differences in their mode of regulation are such that very

little acidic protease is produced on glucose/$NaNO_3$ media whereas it predominates over the alkaline enzyme when growth is on a locust homogenate. This was probably due to the acidic protease being more adaptive and subject to comparatively severe CR. It is also possible that production of the acidic protease may be linked to the presence of specific proteins or other compounds which enhance its synthesis. There is evidence for such a phenomenon with another strain of *M. anisopliae* (Kucera, 1981) though again failure to consider the effects of CR handicap interpretation of this work. As the alkaline protease appears stable (activity declines slowly at 30^{o}C and is constant over a wide range of pH values, Sect II.J),relatively non-specific (Sect. II 2H)and has a wide pH optimum there is no obvious role for the acidic enzyme. The high production on locust homogenate may be significant as *Metarhizium* proteases are toxic to at least one insect, *Galleria mellonella* (Kucera, 1980).

Regulation of production of the exo-hydrolases was examined with diffusion capsules. The results were consistent with CR as the main or only form of control. Most other studies on regulation of peptide exohydrolases also suggest control by CR. Thus growth of a yeast in minimal media results in increased levels of endocellular carboxypeptidase Y (Saheki and Holzer, 1975) and aminopeptidase (Ramos *et al.*, 1983); *cf* activities in rich media. To the author's knowledge the only report of an adaptive exopeptidase concerns a yeast carboxypeptidase which is produced in far higher amounts on the "inducer" CBZ glygyl leucine than on ammonium or a mixture of amino acids (Wolf and Ehmann, 1978). However the relative enzyme levels they describe can be explained

by CR alone, especially as they also report that the peptide-induced increase is completely repressed by amino acids.

The ability of sugars and amino acids to repress production of chitinolytic and proteolytic enzymes suggests that rapid enzyme synthesis is only likely in those host tissues where the concentration of readily metabolisable compounds is low. This is probably the case in insect cuticles as the components are largely insoluble until released by CDE. However, repression could operate if ever the release from cuticle of degradation products exceeded fungal requirements.

In vitro a sequential appearance of enzymes occurred when *M. anisopliae, B. bassiana* and *V. lecanii* were grown on locust cuticle. These results can be explained in terms of the known regulation of CDE and the structure of insect cuticle in which chitin is embedded in a protein matrix (Neville, 1975). The first enzymes produced were the proteases and exopeptidases. Their rapidity of production (< 24 h) would be of obvious advantage in pathogenesis providing nutrients and enabling the onset of cuticle penetration before host defence mechanisms became effective. Chitinase appeared substantially later than enzymes of the proteolytic complex. This order of appearance is supported by the sequence of cuticle constituents solubilised into the culture medium, when an eventual rapid increase in amino sugars followed early release of amino acids.

The late appearance of chitinase is presumably a result of

induction as chitin eventually becomes available after degradation of encasing cuticle proteins. Chitin thus exposed could provide inducers after slight degradation by the small amounts of basal chitinase produced on most media (Table 8). Analogous systems have been described for a β-1,3-glucanase by Lilley and Bull (cited in Bull, 1972) and cell wall-degrading enzymes of plant pathogens (Cooper and Wood, 1975). Alternatively, inducers could be provided by the action of the constitutively produced NAG'ase, although no evidence was found for this in later studies in which the effect of purified NAG'ase on insect cuticle was examined (Sect. II, 5B).

The late appearance of cell-free lipase in cuticle cultures could possibly be accounted for by the predominance of the chitin-protein complex. *In vivo,* lipolytic substances of the epicuticle will predominate in the environments of germinating conidia. Although the presence of lipids in media allowed rapid growth of *M. anisopliae* (MEl), lipase production was not increased thus it seems likely that the lipase(s) was either wall-bound or unstable when extracellular. Wall-bound forms should be considered in future studies of sequential production.

Analogously, sequential production of cell wall-degrading enzymes of several plant pathogens such as *Fusarium oxysporum* (Jones *et al.*, 1972),*Colletotrichum lindemuthianum* (English *et al.*, 1971) and *V. albo-atrium* (Cooper and Wood, 1975) occurs during growth in plant-cell walls. Appearance of the various enzymes reflects the physiochemical susceptibility of the

respective wall polymers (Cooper, 1977).

As substantial degradation of the chitin component of cuticle requires prior removal of the protein (Sec. II 5B) it seems probable that the pattern of synthesis of cuticle degrading enzymes in cultures containing host cuticle may also occur when the pathogens are infecting insects, at least in the absence of CR.

Production of enzymes by fungi *in vivo* will be described later (Sect. III).

SECTION II

SECTION II

Properties of enzymes produced by *M. anisopliae* (ME1)

1. Properties of chitinolytic enzymes

The properties of extracellular chitinase from *M. anisopliae* (ME1) were investigated in order to provide optimum conditions for assay and purification, and as a prerequisite to investigations on the mode of action of chitinase and its effects on insect cuticle.

Unless otherwise stated, experiments were conducted with dialysed filtrates from 5 or 10 d cultures grown on 1% ground (< 0.2 mm) chitin.

A. Nature of chitin substrate

Particle size of chitin is likely to affect accessibility to enzymic degradation. The influence of size and agitation are shown in Table 16. Hydrolysis was related to particle size with finely divided preparations being more susceptible to degradation e.g. colloidal chitin was degraded at > 7 times the rate of crystalline chitin (> 0.5 mm diameter). Agitation increased NAG release from particulate crystalline chitin but had little influence on degradation of colloidal or flake chitin. Thus in subsequent assays involving colloidal or ground crystalline chitin reaction mixtures were agitated under identical conditions to enable direct comparison of activities.

Table 16. Effect of agitation and chitin particle size on activity of *M. anisopliae* (MEl) chitinase

Substrate	Still		Agitated (190 rev/min)		r $\frac{\text{still}}{\text{agitated}}$
	µmole NAG $ml^{-1}h^{-1}$	% maximum activity	µmole NAG $ml^{-1}h^{-1}$	% maximum activity	
1. Colloidal chitin	1.05 (1.21-0.98)	100	1.12 (1.18-1.08)	100	0.939
2. Crystalline chitin (< 0.2 mm)	0.33 (0.35-0.32)	31	0.55 (0.58-0.52)	49	0.598
3. Crystalline chitin (>0.5 mm)	0.14 (0.15-0.13)	13	0.29 (0.32-0.27)	26	0.477
4. Flakes of chitin	0.09 (0.10-0.08)	8.6	0.10 (0.10-0.10)	8.9	0.91

Reaction mixtures consisted of 1 ml Cit-PO_4 buffer (0.1 M, pH 5.1) containing 5 mg of chitin, and 1 ml of culture filtrate. After incubation for 1 h at 35°C reaction products were estimated by the Nelson-Somogyi procedure (1944). Each result is the mean of 3 replicates. Ranges are given in parenthesis. The results are representative of 2 similar experiments.

B. Substrate Concentration

The effect of colloidal chitin concentration is shown in Fig. 29. It is clear that under standard conditions (35°C, 1 h incubation) chitin concentrations of 3-5 mg ml^{-1} used in assays (Nelson-Somogyi and Morgan-Elson) are not limiting or inhibitory.

C. Effect of pH

Chitinase. The effect of pH on chitinase activity is shown in Fig. 30. The rate of hydrolysis increased almost linearly between pH 3.5-5.0. A fairly broad pH optimum occurred with maximal activity at 5.3 beyond which activity slowly declined. Substantial activity (> 15%) was detected at either extreme of the pH range tested (3.5-7.5).

N-acetylglucosaminidase. The effect of pH on N-acetylglucosaminidase (chitobiase) measured with *p*NP-β-N-acetylglucosaminide, was about 5.0 (Fig. 30) with significant activity extending over the pH range tested. As with chitinase the pH profile was assymetrical with a more rapid decrease in activity on the acid side of the optimum.

D. Effects of buffer type on activity

Buffer constituents affect the activity of some chitinases (Waterhouse *et al.*, 1961; Cohen, 1974) although apparently not others (Hackman and Goldberg, 1964). Determination of chitinase activity in citrate, citrate-phosphate (Cit-PO_4), acetate, maleate and succinate buffers was made. Buffer constituents had a considerable effect on apparent enzyme activity (Table 17) with acetate buffer resulting in highest activity.

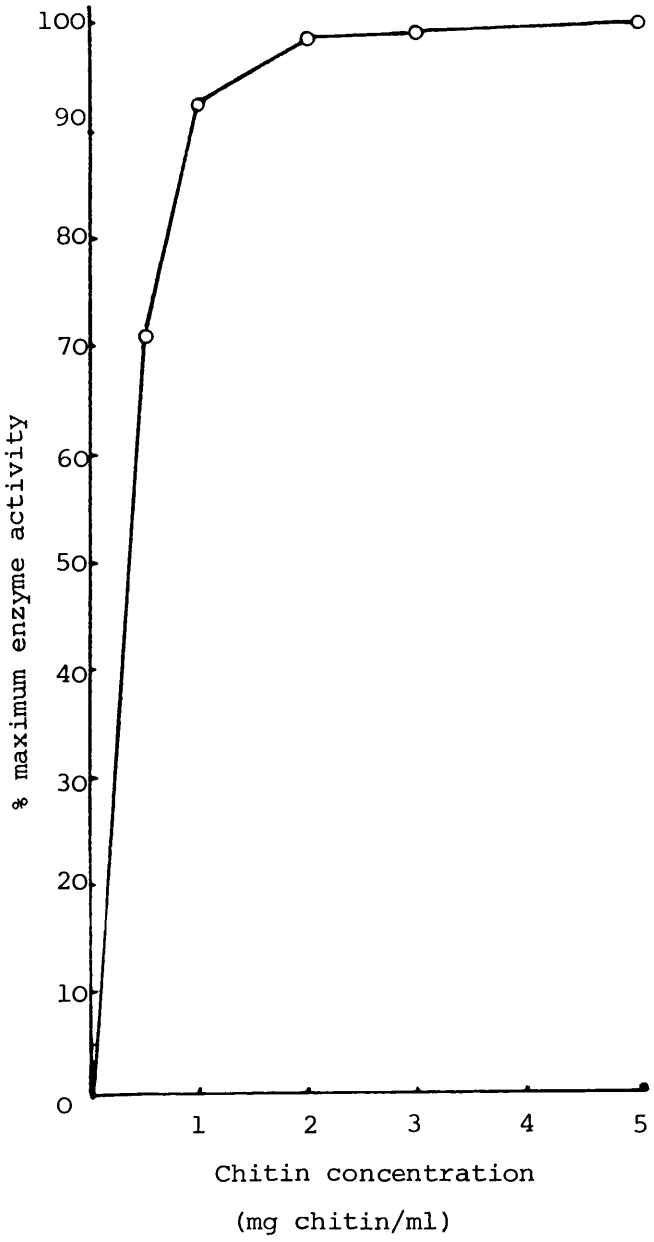


Fig. 29. Effect of colloidal chitin concentration on chitinase activity. Each point is the mean of 3 replicates. The results are representative of 2 similar experiments using different enzyme preparations.

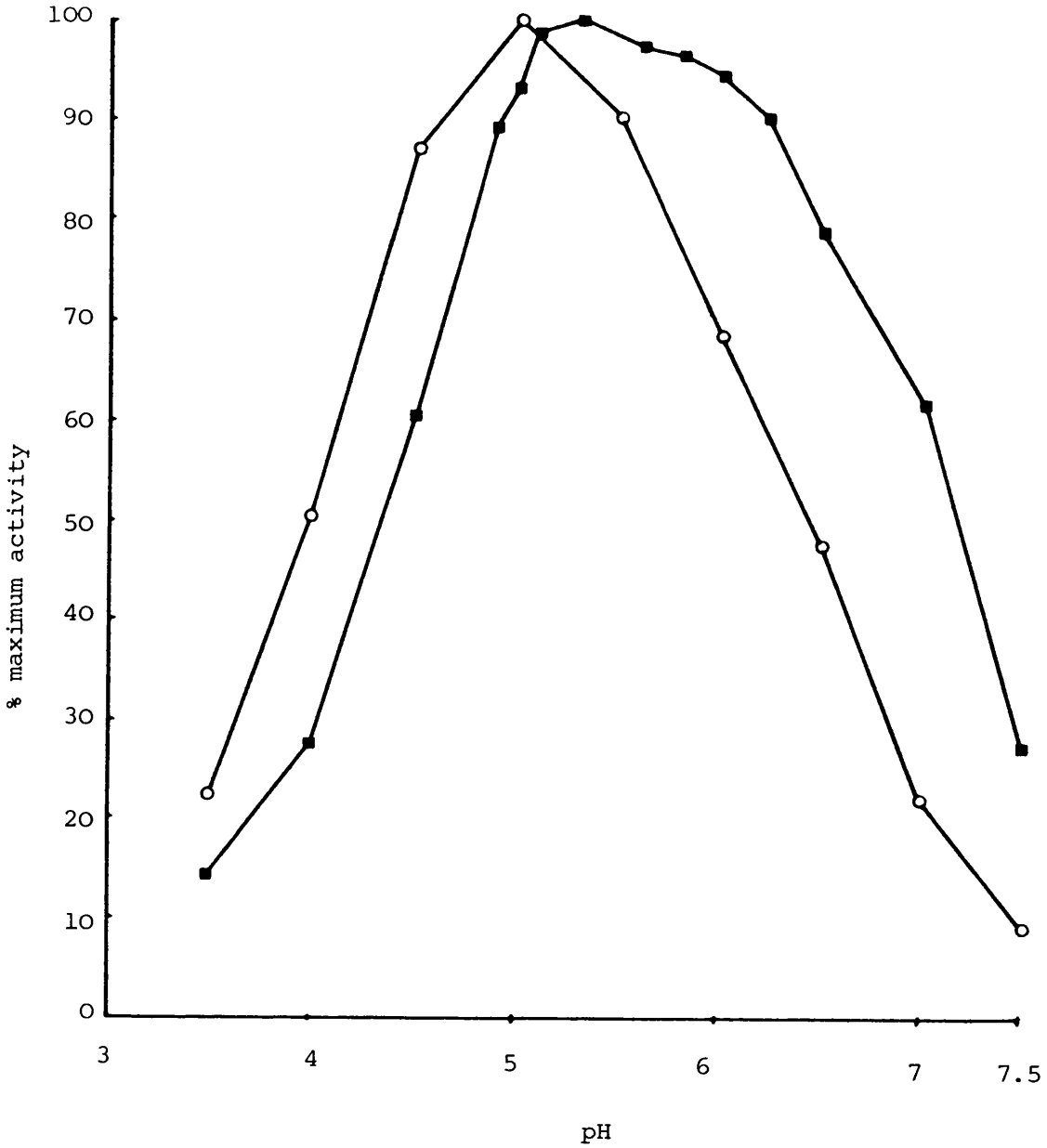


Fig. 30. pH response curves for chitinase (■—■) and NAG'ase (O—O) activities in culture filtrates of *M. anisopliae* (ME1).
Chitinase: the reaction mixture consisted of 1 ml buffer (Britton Robinson, Universal buffer at pH values ranging from 3.5 to 7.5) containing 5 mg colloidal chitin plus 1 ml of culture filtrate. After 30 min incubation at 35°C on a shaking water bath (190 rev/min) reaction products were estimated by the Nelson-Somogyi procedure (Nelson, 1944).
NAG'ase: the substrate solutions were prepared as described in Materials and Methods using Britton Robinson Universal buffer of the required pH.
Each point is the mean of 3 replicates. The results are representative of 2 similar experiments using different enzyme preparations.

In spite of this Cit-PO_4 buffer was used in future determinations in view of a report that acetate may act as an activator or catalyst of chitinase,particularly in non-optimal pH conditions (Cohen, 1974).

Table 17. Effect of buffer constituents on chitinase activity.

Buffer (pH 5.1)	Chitinase activity[a] (µmole NAG $ml^{-1}h^{-1}$)	% maximum activity
0.1 M acetate	2.2 (2.25-2.18)	100
0.1 M Cit-PO_4	1.72 (1.76-1.66)	78
0.1 M succinate	1.66 (1.70-1.63)	75
0.1 M citrate	1.34 (1.36-1.31)	65
0.1 M maleate	1.32 (1.35-1.30)	64

a) 5 mg colloidal chitin was suspended in 1.8 ml of each of the various buffer solutions at pH 5.1 and 0.2 ml of filtrate added. The reaction mixture was incubated for 1 h at 35°C. Results are the mean of 3 replicates. Ranges are given in parenthesis.

E. Effect of pH on stability of chitinase

Samples of chitinase partially purified by ammonium sulphate precipitation and column chromatography were adjusted to pH values ranging from 3.5 to 7.0 with Britton-Robinson Universal buffer. After incubating for 2 h at 35°C the solutions were assayed for activity on colloidal chitin at pH 5.1 by the Nelson-Somogyi (1944) method. As illustrated in Fig. 31 there was a

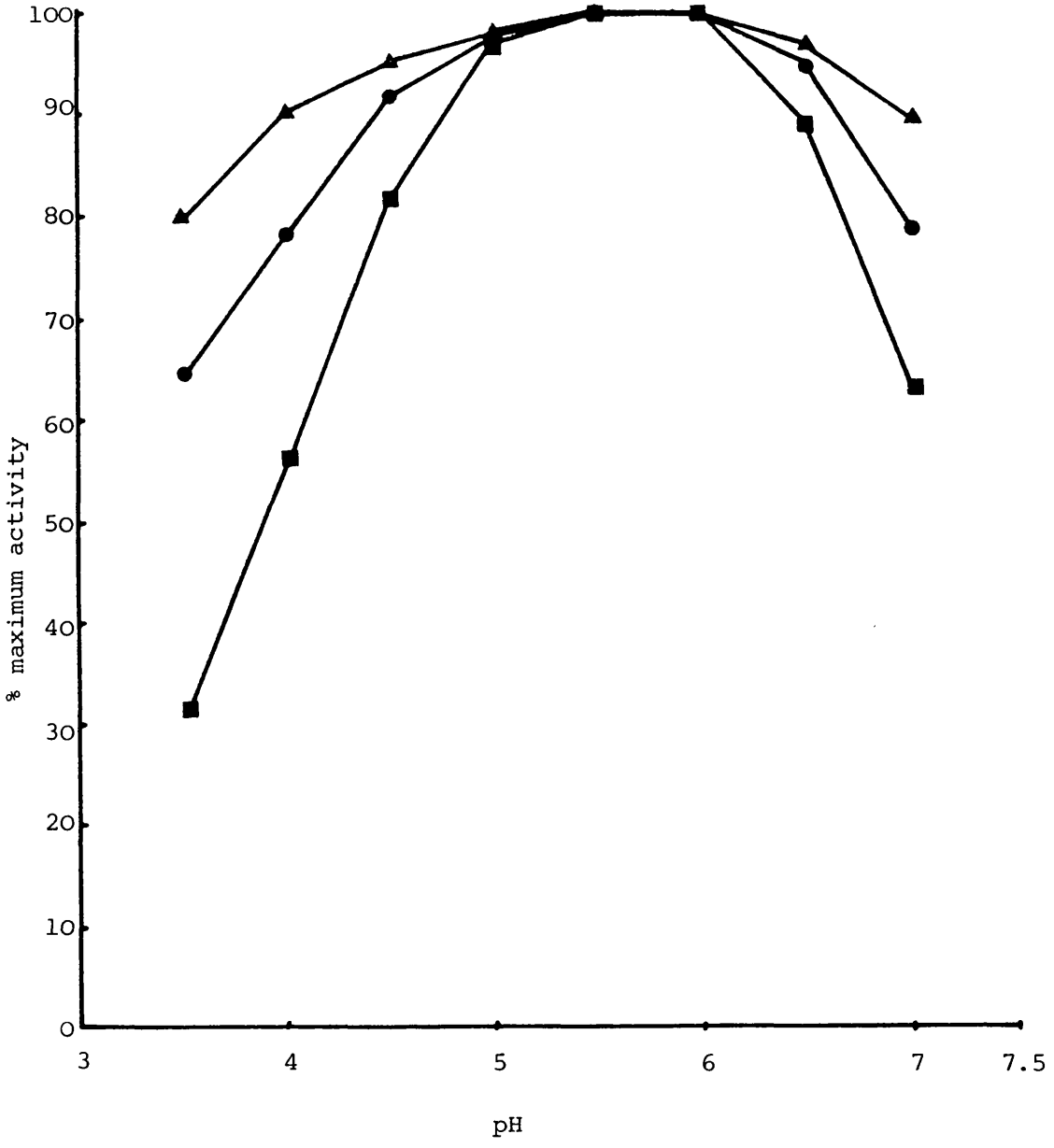


Fig. 31. Effect of pH on stability of partially purified chitinase (■—■) incubated for 2 h at 35°C; (●—●) incubated for 24 h at 4°C; (▲—▲) incubated for 24 h at 4°C with finely dispersed locust chitin.

Each point is the mean of three replicates. The results are representative of two similar experiments using different enzyme preparations.

considerable loss of activity at pH values below 5 and above 6.

Unestam (1968) has reported that the chitinase of *A. astaci* is stabilised at extremes of pH by its substrate. To investigate whether this applies to the chitinase of *M. anisopliae* (MEl), enzyme was incubated at 4°C with or without finely dispersed locust chitin (5 mg ml^{-1}). After 24 h at 4°C virtually no activity had occurred against the locust chitin (as determined by estimation of reducing sugars), but the chitin had partly stabilised chitinase, especially at values below 4.5 and above 6.5.

F. Effect of temperature on chitinase activity

From the results shown in Fig. 32 it is possible to derive the optimum temperature of chitinase activity over different periods of incubation and these are shown in Fig. 33. As the optimum temperature reflects a balance between the effect of increased temperature on activity and the denaturation of enzyme protein, it is predictable that the shorter the incubation period the higher will be the apparent optimum temperature.

To determine whether the chitinase had been protected from heat inactivation by substrate, enzyme was incubated in controls lacking chitin at 37°C and 45°C. At both temperatures the enzyme was almost completely denaturated after 3 h exposure. Similar stabilisation by substrate has been noted by Unestam (1968) for *Aphanomyces* chitinase.

Fig. 32. Effect of temperature and time on the release of NAG from colloidal chitin. (O—O) 25°C; (×—×) 37°C; (Δ—Δ) 45°C; (□—□) 62°C.

Fig. 33. The optimum temperature of chitinase activity, with measurement of NAG released after 2 h (□—□), 4 h (O—O) and 8 h (Δ—Δ).

The reaction mixture consisted of 5 mg colloidal chitin suspended in 3 ml of Cit-PO_4 buffer (pH 5.1, 0.1 M) to which 2 ml of diluted (2x) filtrate and a drop of toluene were added. At 0, 2, 4, and 8 h the tubes were shaken and 2 0.5 ml aliquots removed. Each aliquot was diluted to 2 ml with buffer and assayed for reducing groups by the Nelson-Somogyi test.

Figs. 32 and 33. Each point is the mean of 4 replicates. A second experiment (3 replicates) produced very similar results.

Fig 32.

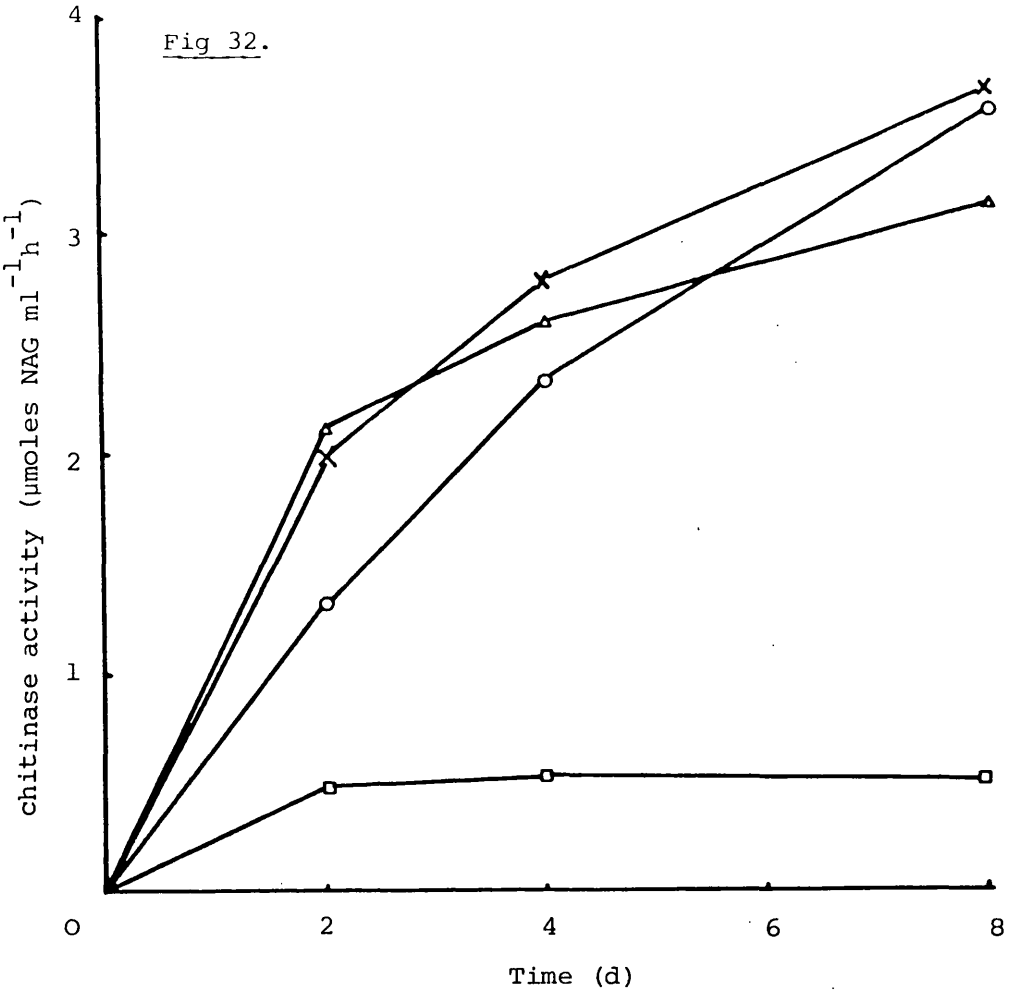
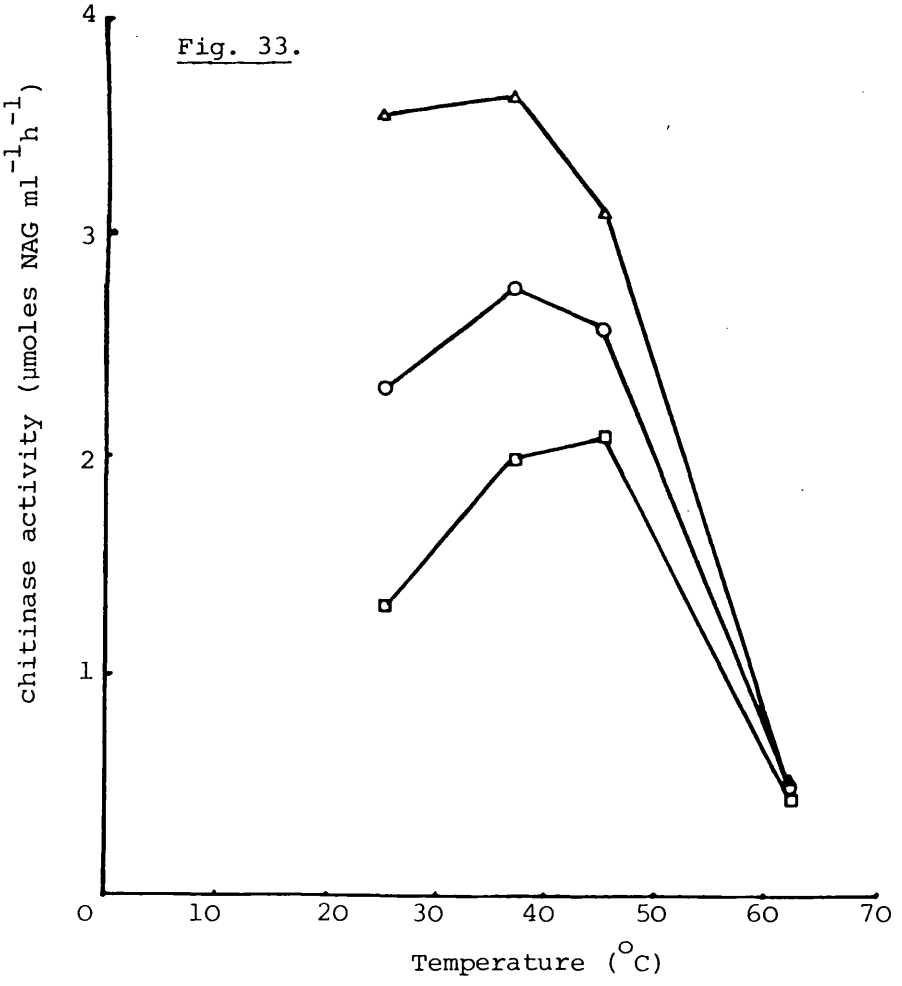

chitinase activity (µmoles NAG ml^{-1}h^{-1})
4
3
2
1
0
2
4
6
8
Time (d)

Fig. 33.


chitinase activity (µmoles NAG ml^{-1}h^{-1})
4
3
2
1
0
10
20
30
40
50
60
70
Temperature (°C)

G. Influence of dialysis, monovalent ions and chitin breakdown products on enzyme activity.

Dialysis (2 x 12 h, 600 vol dist. water, pH 6.0) of culture filtrates increased the specific activity of chitinase by 122% and NAG'ase by 136%. However the divalent metal chelating agent Na_2EDTA (5 mM) in the dialysing medium did not further influence activity. These results indicate that small molecules and ions are not required as cofactors, NAG and other breakdown products of chitin are presumably also removed by dialysis and this may account for the increased activity of the two enzymes. This possibility was tested and the results are shown in Table 18. NAG significantly (*c.* 80% at 0.2 M) inhibited NAG'ase (Fig. 34) but showed no inhibition of chitinase. Chitinase was however inhibited by both the dimer and trimer which also had a smaller inhibitory effect on NAG'ase. However, this may have been at least partly due to NAG released from the saccharides by the NAG'ase.

The influence of monovalent ions was investigated to provide information for subsequent extraction of cell-bound enzymes. The effect of K^+ was minimal but Li^+ and Na^+ ions were inhibitory above 50 mM (Fig. 35). Similarly KCl had no effect on NAG'ase activity at 400 nm.

H. Fractional precipitation with ammonium sulphate

Filtrates from 5 d MEl cultures grown on 1% chitin/basal salts were subjected to increasing levels of $(NH_4)_2SO_4$ as described in Materials and Methods, then dialysed before assay for chitinase and chitobiase (NAG'ase) activities (Table 19). Chitinase was absent below 40% (w/v) saturation. The highest

Table 18. Effect of NAG, chitobiose and chitotriose on activity of chitinolytic enzymes.

Inhibitor	Chitinase activity[a], (ΔOD_{595} ml h^{-1})	% Max. activity	NAG'ase activity[b], (µmole PNP $ml^{-1}h^{-1}$)	% Max. activity
Unamended control	39.0 ± 1.3[d]	100	1.32 ± 0.05	100
NAG 30 mM	38.8 ± 1.8	99.5	0.68 ± 0.03	51.5
200 mM	37.8 ± 2.4	97.0	0.26 ± 0.02	19.7
Chitobiose 2 mM[c]	32.1 ± 2.2	82.0	1.20 ± 0.07	90.9
Chitotriose 2 mM[c]	29.8 ± 1.6	76.5	1.14 ± 0.06	86.4

a) A partially purified chitinase preparation (0.5 ml) was pre-incubated with NAG, chitobiose and chitotriose in 1 ml of Cit-PO_4 buffer (0.1 M, pH 5.3). After 30 min 10 mg of ground chitin azure was added and reaction mixtures were incubated for 1 h at 30°C before determining activity.

b) The effects of NAG and saccharides were tested without pre-incubation. The reaction mixture contained 0.5 ml enzyme and 1 ml of Cit-PO_4 buffer (0.1 M, pH 5.0) containing 1 mg PNP-β-N-acetylglucosaminide and the potential inhibitor.

c) Lack of available material precluded use of larger quantities of saccharides.

d) Mean values ± SE, n = 5. The experiment was repeated twice with similar results. A different enzyme preparation was used on each occasion.

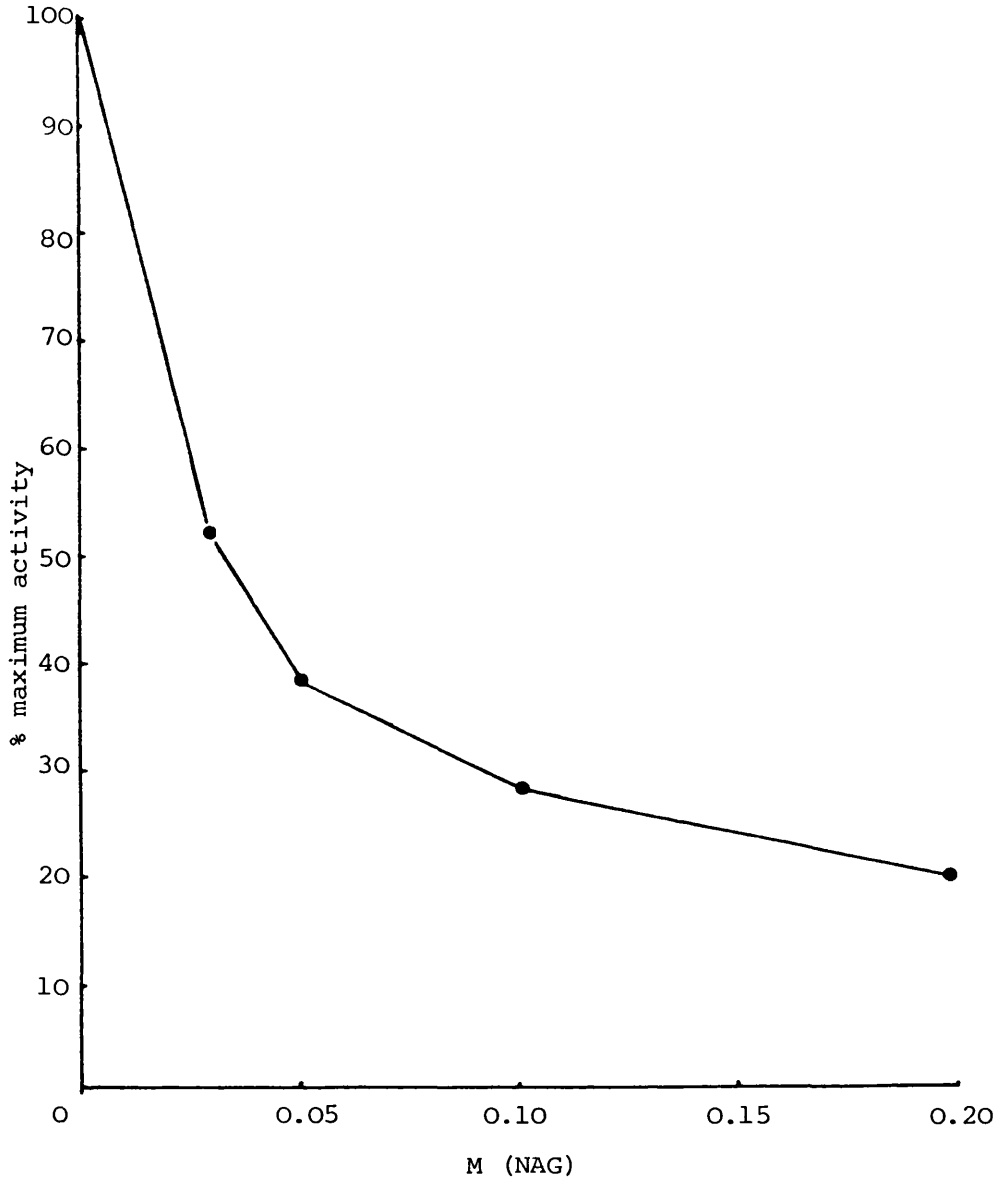


Fig. 34. Influence of NAG on NAG'ase activity. Culture filtrates were exhaustively dialysed against several changes of distilled water and used directly as sources of enzyme. NAG was added to reaction mixtures at the following concentrations: 0, 0.03 M, 0.05 M, 0.1 M and 0.2 M.

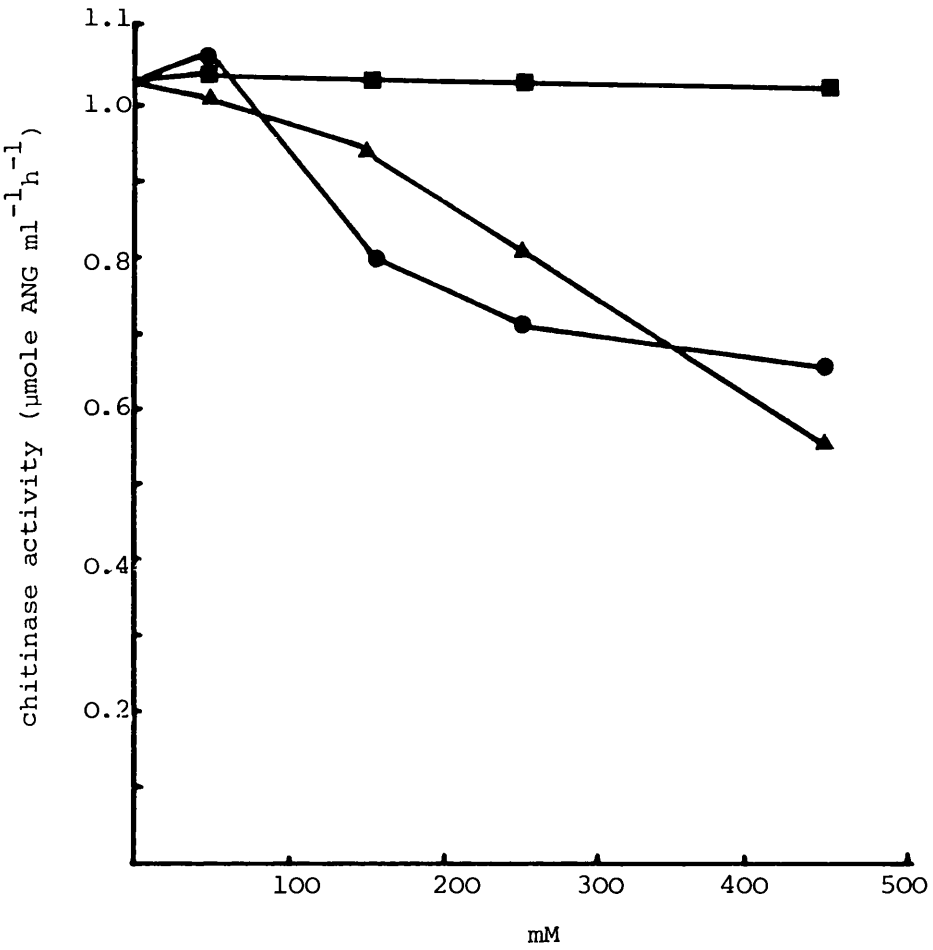


Fig. 35. Influence of monovalent ions on chitinase activity. Exhaustively dialysed culture filtrates were used directly as sources of enzyme. Na^+ (●—●), Li^+ (▲—▲) or K^+ (■—■) chloride were added to reaction mxitures at O (EDTA, O.OO5M), 5O mM, 15O mM, 25O mM, 45O mM and incubated for 1 h at 35°C.

amount of activity and of protein precipitated was obtained in the 60-80% fraction, but the greatest degree of purification (x3.32) was achieved at 40-60%. The total chitinase recovery combining all fractions was 63.16%.

Most chitobiase activity occurred in the 20-40% and 40-60% fractions in which specific activity was increased *c.* two-fold. At higher saturations there was no overall purification of the enzyme.

Since it was intended to use purified chitinase in studies of cuticle degradation it was important to determine whether partially purified chitinase contained other hydrolytic enzymes. The fractions 60% to 100% (% w/v) saturation were combined and the hydrolytic activity tested with several polysaccharides as substrates (pH 5.0, 2 h, 35^{o}C, Nelson Somogyi determination). Of the polysaccharides tested only chitin was digested; very low or no hydrolysis occurred with the glucans starch, cellulose and dextran, or on xylan.

I. Molecular weight determination

The protein obtained from ammonium sulphate precipitation was fractionated on a Sephadex G-100 column (Fig. 36). The chitinase eluted as a broad zone with a peak estimated as molecular weight *c.* 33,000 daltons (Stokes radius (Rs) 24.5 Å), and a minor peak of 66,000 daltons. The numerical relationship between these molecular weights suggests that the minor peak is a result of dimerisation. Glycosidase activity (*vs* *p*NP-N-acetyl-β-D-acetylglucosaminide) eluted near the void volume

Table 19. Fractional precipitation of chitinase and chitobiase of *M. anisopliae* (MEl) with ammonium sulphate.

Ammonium sulphate saturation (% w/v)	Enzyme activity (% of maximum)	Specific activity (units/mg protein)	Purification	Enzyme activity (% of maximum, w/v)	Specific activity (units/mg protein)	Purification
culture filtrate	100	358.00	-	100	567.85	-
20	0	0	0	0.33	107.29	0
40	7.6	667.44	1.86	14.5	1,075.67	1.89
60	25.0	1,188.77	3.32	36.3	1,456.05	2.56
80	39.9	660.12	1.84	29.5	411.44	0
100	27.5	702.38	1.96	19.3	415.71	0

as a relatively narrow peak with a molecular weight of 110,000-120,000 daltons, R_s 38.5 Å.

J. Specificities of NAG'ase and chitinase

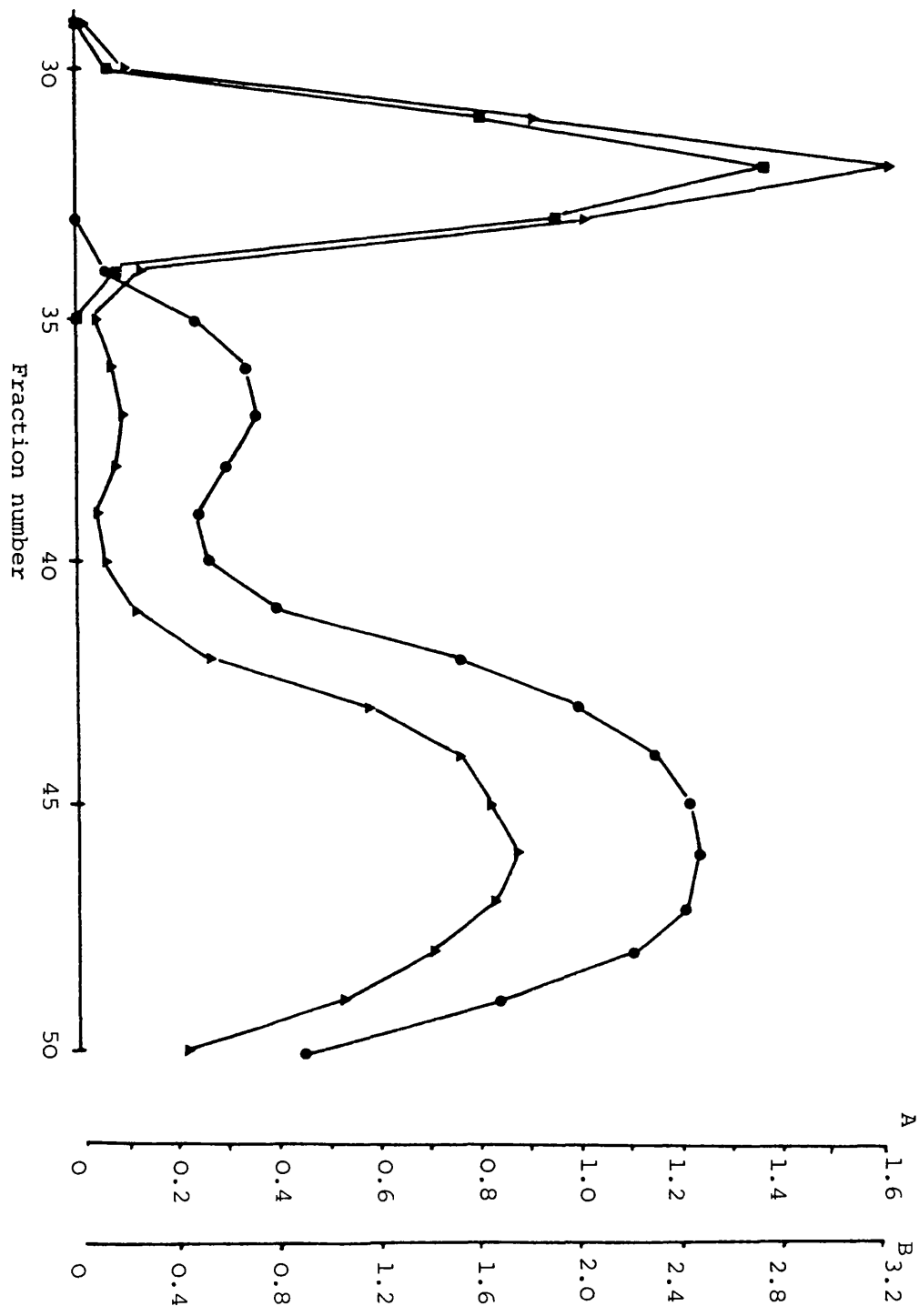
Activity against chitobiose, chitotriose, chitotetraose and the PNP-derivative of N-acetylgalactosamine closely followed the NAG'ase profile obtained from Sephadex G-100 columns (Fig. 36, Table 20). For determining activity against chitin, NAG'ase fractions (31-32) were pooled and concentrated (*c.* x 2.5) in a dialysis bag surrounded by polyethylene glycol (MWT 20,000). Slight activity against colloidal chitin and locust chitin was apparent, but the possibility of trace levels of residual chitinase cannot be ruled out. No activity was detected against crystalline chitin or chitosan.

The chitinase fractions (42-46) were further purified by adsorption onto colloidal chitin (see Materials and Methods). The purified chitinase failed to hydrolyse chitobiose (Table 20) and displayed no glycosidase activity against any of the aryl glycosides. The enzyme showed only trace activity against the trimer of NAG but rapidly attacked the tetramer. Thus *in vivo* oligosaccharase activity may result from the joint participation of NAG'ase and chitinase activities (see Fig. 36).

Chitinase rapidly degraded colloidal chitin(1 ml enzyme solubilised 7 mg (d/w) of chitin in buffer (4 ml, 0.02 M Cit-PO_4, pH 5.1) within 24 h). Colloidal chitosan and crystalline chitin were less amenable to degradation but activity on them was still substantial.

Fig. 36. Sephadex G-100 gel filtration of chitinolytic enzymes from the protein fraction of *M. anisopliae* (ME1) cuticle culture filtrate precipitated at 20-60% NH_4SO_4 saturation.

A 2.5 x 40 cm column of Sephadex G-100 was equilibrated and eluted with 0.2 M phosphate buffer (pH 6.5). Activities against colloidal chitin (●—●), chitotetraose (▲—▲) and *P*NP-β-N-acetylglucosaminide (■—■) were measured as described in Materials and Methods.



(A) = Activity against PNP-β-N-acetylglucosaminide (measured in µmole PNP released $ml^{-1}h^{-1}$)

(B) = Activities against colloidal chitin and chitotetraose (measured in µmoles NAG released $ml^{-1}h^{-1}$).

Table 20. The action of *M. anisopliae* (ME1) chitinase and NAG'ase on chitin, chitin oligosaccharides and arylglycosides.

Substrate	NAG'ase fractions	Chitinase fractions
*p*NP-β-glucuronide[a]	0	0
*p*NP-cellobioside[a]	0	0
*p*NP-β-glucoside[a]	0.002	0
*p*NP-β-N-acetylgalactosaminide[a]	0.14	0
*p*NP-β-N-acetylglucosaminide[a]	1.21	0
Chitobiose[b]	2.65	0
Chitotriose[b]	3.15	0.006
Chitotetraose[b]	2.89	1.65
Colloidal chitin[b]	0.002	1.32
Locust chitin[b]	0.002	0.93
Crystalline chitin[b]	0	0.56
Chitin azure[c]	0	6.32
Colloidal chitosan[b]	0	0.78

a) enzyme units, μmole NP released $ml^{-1}h^{-1}$

b) enzyme units; μmole NAG released $ml^{-1}h^{-1}$

c) enzyme units, $OD_{595}\ h^{-1}$

Assay conditions are described in Materials and Methods. Results are the means of 3 replicates. The experiment was repeated twice using different enzyme preparations.

K. Mode of action of chitinase

Information on the products of enzymolysis is necessary for a complete understanding of enzyme induction and nutrition of the pathogen. It may also assist in assessing the relative role of different enzymes in cuticle penetration.

As a first step, the mean degree of polymerisation (DP) of degradation products was estimated by measuring release of amino sugars; (including those contained in higher saccharides) by the method of Smith *et al.* (1979) and comparing values with those obtained for monomeric N-acetylglucosamine (Reissig *et al.*, 1955).

For the first 4 h incubation of colloidal chitin with a glycosidase-free chitinase preparation (purified by column chromatography and adsorption onto colloidal chitin), total amino sugar residues slightly exceeded the amount of free monomers present (Fig. 37); e.g. after 2 h the mean DP was estimated as 1.46. After 4 h the monomer accounted for almost all the amino sugar residues released. These results suggest that either chitobiose is produced mainly in the initial but not later stages of chitin degradation or alternatively that chitinase has a limited ability to release higher oligosaccharides whichare subsequently further degraded by the enzyme. As chitinase is unable to degrade to any significant extent either the dimer or trimer, oligomers released initially must have been at least the level of chito-tetraose.

With NAG'ase, total amino sugar residues equalled production of NAG throughout indicating that the monomer was the sole reaction product.

Further information on the composition of reaction mixtures was obtained by descending paper chromatography as described in Materials and Methods. A diagrammatic representation of chromatograms is shown in Fig. 38. Hydrolysis of crystalline chitin by chitinase for 2 to 24 h produced only one spot which corresponded to the NAG standard. Hydrolysis of colloidal chitin gave two products, the fastest corresponded to NAG and was clearly the major component. The minor product had an identical R_{NAG} to chitobiose. The size and intensity of the NAG spot increased considerably in relation to that of chitobiose during the (2-24 h) induction. Hydrolysis of chitotetraose gave three spots, the furthest from the origin was faint and corresponded to NAG, the second spot which was the most prominent occupied the position expected of chitobiose while the third spot, also faint, corresponded to the trimer standard. As expected, the chitinase had no activity on chitobiose.

The absence of intermediary oligomers among chitin breakdown products probably means that NAG is released directly from insoluble chitin. Either the chitinase has an exo-acting component or alternatively the reaction proceeds by multiple attack viz. random cleaving of bonds followed by release of mono- or dimers from exposed ends (Cooper *et al.*, 1978). That chitinase may function as an endo-acting enzyme was suggested by the breakdown

products of chitotetraose. The relatively high amounts of chitobiose produced indicates that the central link may be the preferred point of cleavage.

Hydrolysis of the chitotetraose by NAG'ase gave three spots in the same positions as those produced by chitinase, but in contrast the major component was NAG. Hydrolysis of chitobiose also yielded NAG.

2. Characterisation of proteolytic enzymes

The properties of proteolytic enzymes produced in cultures of *M. anisopliae* (MEl) grown on locust cuticle medium were examined. The results of these experiments were used to provide optimum conditions for assay and purification, and as a prerequisite to investigations on their modes of action and effects on insect cuticle.

Unless otherwise stated these experiments were conducted with clarified dialysed filtrates from 5 d cultures grown on 1% ground locust cuticle.

A. Nature of active site

Proteolytic activity can be classified into categories (serine, metal and sulphydryl) based on their active site catalytic mechanism as determined using specific inhibitors (Hartley, 1960; Ryan, 1979).

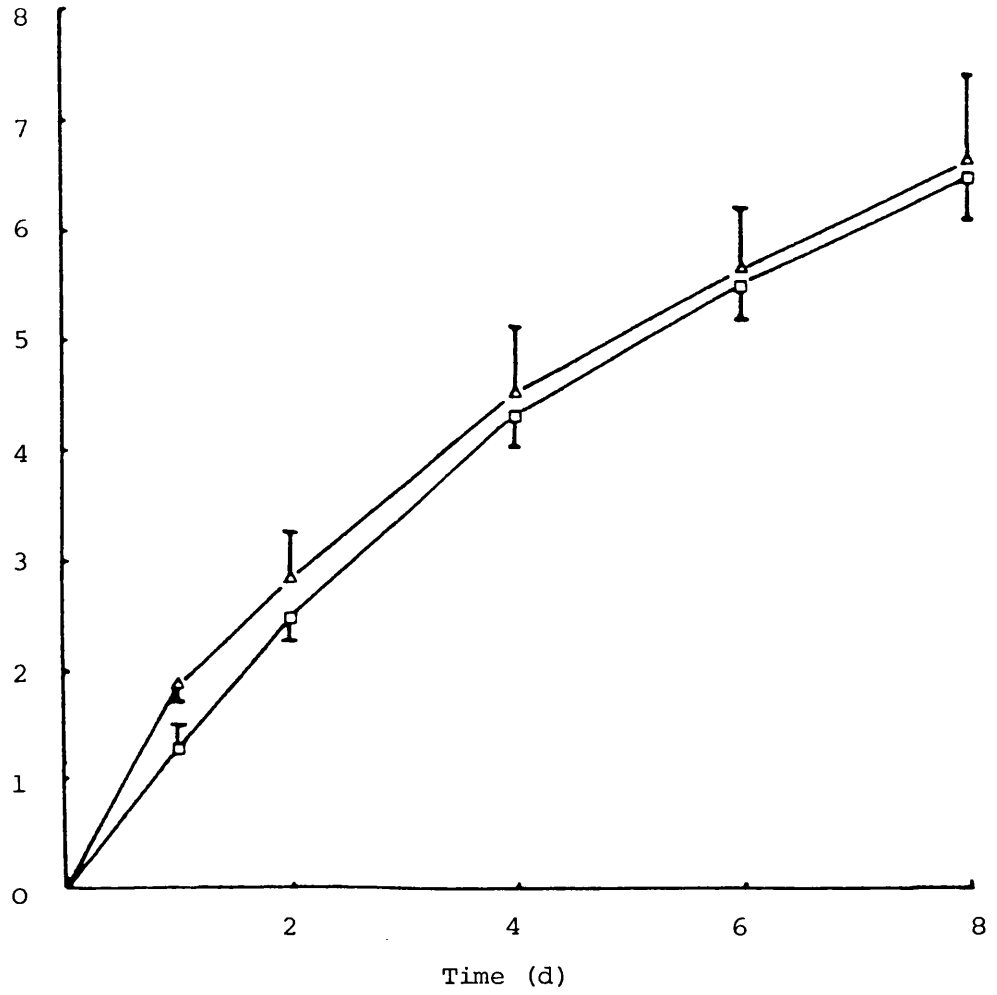
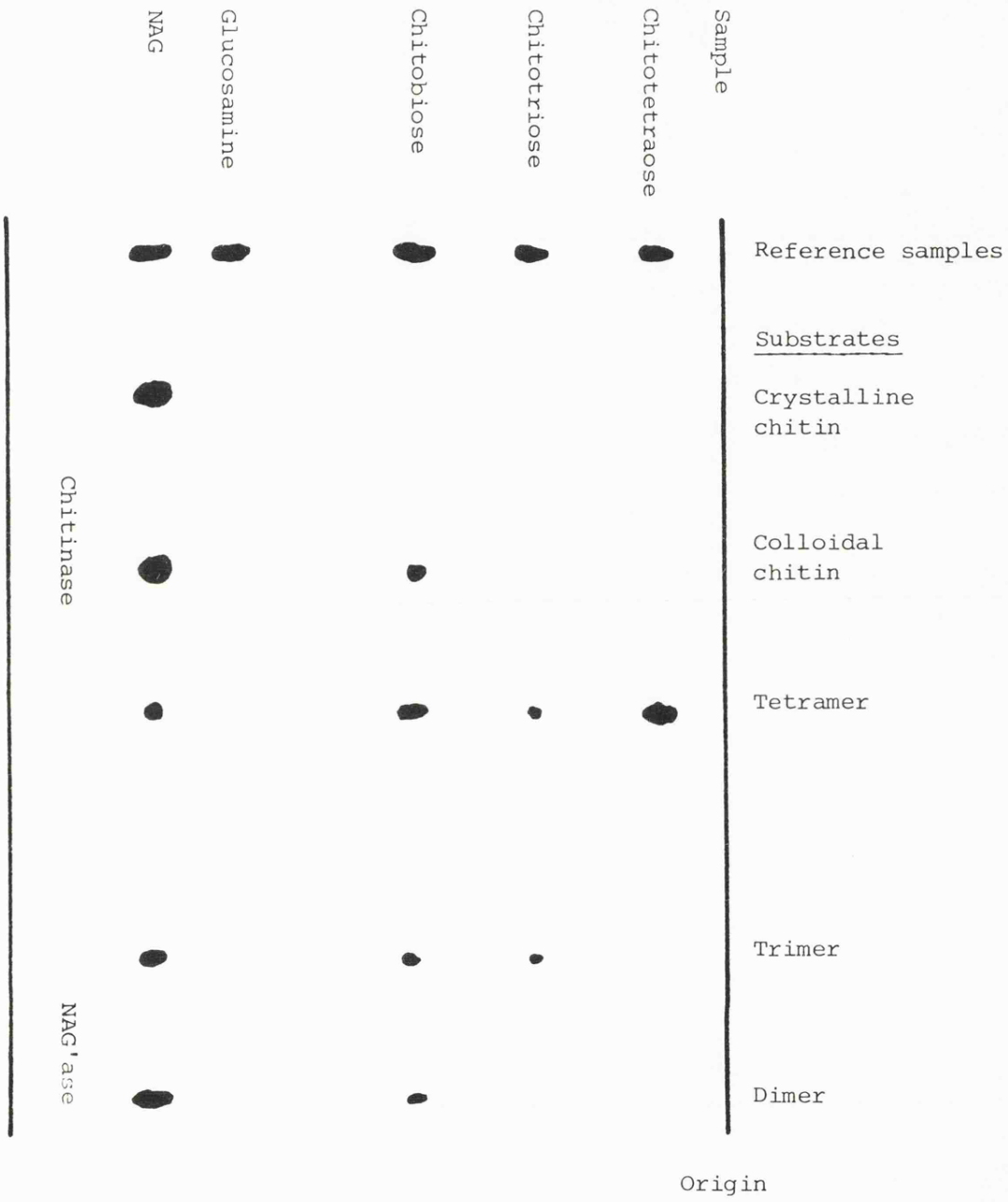


Fig. 37. Total amino sugars (Δ—Δ) and monomers (□-□) released during hydrolysis of chitin by *M. anisopliae* (MEl) chitinase. Each symbol is the mean of 6 replicates ± SE. The results are representative of two similar experiments.

Fig. 38. Products of degradation of chitin and oligosaccharide substrates by purified chitinase and partially purified NAG'ase of *M. anisopliae* (ME1). The figure is drawn to scale. Paper chromatograms were developed with butanol-ethanol-water-NH_3 (40:10:49:1). Spots were located with the spray reagent of Partridge (1949). Colours of spots in daylight: NAG (Brown); glucosamine (Brown/Green); chitobiose (Brown/Orange); chitotriose (Orange); Chitotetraose (Orange).


Sample
Chitotetraose
Chitotriose
Chitobiose
Glucosamine
NAG
Reference samples
Substrates
Crystalline chitin
Colloidal chitin
Tetramer
Trimer
Dimer
Chitinase
NAG'ase
Origin

Although probably not inclusive of all enzymes the classification does include most known proteinases and is valuable in their characterisation (Ryan, 1979).

Dialysis of crude filtrate against Tris buffer (Table 21) had no effect on protease but increased substantially both actual activity and specific activity of the aminopeptidase and carboxypeptidase. This suggests that any low molecular weight co-factors or activators must be bound to the enzyme molecule. Amino acids and other products of the hydrolysis of cuticle are presumably also removed by dialysis and this may account for the increased activity of the two exo-peptide hydrolases. This explanation is supported by the observation that preincubation with alanine (10^{-2} M) resulted in a substantial inhibition of amino peptidase (residual activity 54%). The method used to assay carboxypeptidase (release of an amino acid from a CBZ-peptide) precluded a similar investigation.

The effect of NaCl and Co^{2+} on activity was investigated as metallic ions are known to have a considerable influence on peptidase activity (Ryan, 1973). NaCl and Co^{2+} (as $CoCl_2.6H_2O$) strongly activated the carboxypeptidase (Table 21). Co^{2+} is a specific activator of some carboxypeptidases (Coleman and Vallee, 1961); NaCl may have increased the overall rate of hydrolysis by increasing ionic strength as reported by Lumry, Smith and Glantz (1951).

Both carboxypeptidases and aminopeptidases have been reported

to form active complexes with metals such as Co^{2+} or Zn^{2+} (Coleman *et al.*, 1961; Ray and Wagner, 1972), and it follows that inhibition may be effected by chelating agents which remove the metal ions from the active complex (Plumbley and Pitt, 1979; Nakadai *et al.*, 1973). Such inhibition occurred for both exohydrolases in this study but only when filtrates were exhaustively dialysed against EDTA, and even then complete inactivation was not achieved (Table 21). This suggests that the metal ions involved in activity are very strongly bound to the active site. The slight inactivation of the protease shows a possible dependence on divalent cations but this conclusion must remain tentative.

Sulfonyl fluorides inhibit enzymes which have a serine residue in the active site. Examples of these are β-esterases which include certain esterolytic proteases (e.g. subtilisin, trypsin and chymotrypsin; Aldridge and Reiner, 1972). As the alkaline protease possesses esterase activity (Table 24) the effect of phenyl methyl sulfonyl fluoride (PMSF) on activity was examined. Like trypsin and chymotrypsin (Fahrney and Gold, 1963) the protease was strongly inhibited by sulfonyl fluoride indicating that it is a serine enzyme and classifiable as a β-esterase (carboxyesterase). The results with the two exo-peptidases reveal that neither of these is dependent on a serine residue for catalysis. The specific sulphydryl reagent ethylmaleimide showed a partial inhibitory effect on carboxypeptidase indicating the possession of sulfhydryl group(s) in the active site but it had no effect on protease or aminopeptidase. A natural protease inhibitor from soybean had relatively little effect whether

preincubated or simultaneously incubated with each enzyme. It is reported however to inhibit crystalline trypsin and the proteases of *T. rubum* (Meevootisom and Niederpruem, 1979).

These results indicate that the protease is a serine hydrolase while the aminopeptidase is a metallo-enzyme. The carboxypeptidase apparently contains a metal-sulphydryl complex.

B. Effect of pH on substrate hydrolysis

Fungal proteolytic enzymes are often classified as acid, neutral or alkaline based upon the analysis of pH activity curves with several protein substrates and the behaviour of the enzymes towards inhibitors.

The effect of pH on proteolytic activity was examined with haemoglobin, casein, hide protein azure and ground locust cuticle (Figs. 39 and 40). Results with casein were influenced by its decreased solubility from pH 3.0 - 6.0. Consequently meaningful pH-activity curves could only be obtained outside this range.

As reported in Section (I) the extracellular proteolytic enzyme system of *M. anisopliae* (MEl) appears to comprise at least two types of protease with separate activity peaks at *c.* pH 5.0-5.5 and 8.0-9.0. The alkaline enzyme was predominant with maximal activity *vs.* haemoglobin at pH 8.2, whereas pH 9.0 was optimal for either casein or locust cuticle.

The relative activity of the minor protease compared to the

Table 20. The effect of various chemical agents on the activity of proteolytic enzymes produced in cuticle culture by *M. anisopliae* (ME1).

Activator/inhibitor	Relative activity % [a] protease	aminopeptidase	carboxy-peptidase
Dialysis[b]	112	206	183
EDTA[c] (Na salt)	89	36	8
PMSF (2×10^{-4} M)[d]	12	97	95
Ethylmaleimide (10^{-3} M)[d]	97	98	57
Soybean inhibitor (0.2 mg ml^{-1})[d]	87	98	98
Alanine (10^{-2} M)[d]	102	54	-
Na^+ (0.1 M)[d]	104	102	154
Co^{2+} (6.6 mM)[d]	109	109	167
Na^+ (0.1 M + Co^{2+} (6.6 mM)[d]	106	110	235

a) Enzyme activities are given as the % of the activity exhibited in the absence of chemicals. In all instances variation between replicates (n=4) of a treatment was less than 7%. The results are representative of 2 similar experiments using different enzyme preparations.

b) Culture filtrate dialysed (2x12h) against Tris buffer(0.02M,pH 7.1).

c) Culture filtrated dialysed (2x12h) against Tris buffer (0.02M, pH 7.1) containing 5 mM EDTA

d) To study the effect of inhibitors and activators 1 volume of purified alkaline protease or culture filtrate was incubated for 30 min at 23°C with an equal volume of the reagent and the residual protease, aminopeptidase and carboxypeptidase activity assayed as described in Materials and Methods.

Fig. 39. pH optima of proteolytic enzymes from *M. anisopliae* (ME1) grown on liquid cultures containing 1% ground locust cuticle. The substrates used for assay are: (▲—▲) Haemoglobin; (●—●) casein; (■—■) Hide protein azure and were prepared in Britton-Robinson Universal buffer.

Fig. 40. pH optima of pure alkaline protease (●—●) and proteases in crude cuticle culture filtrate (■—■) with ground locust cuticle as substrate.

Figs 39 and 40. Each point is the mean of 3 replicates. Experiments were repeated twice with similar results. Different culture filtrates were used on each occasion.

Fig. 39.

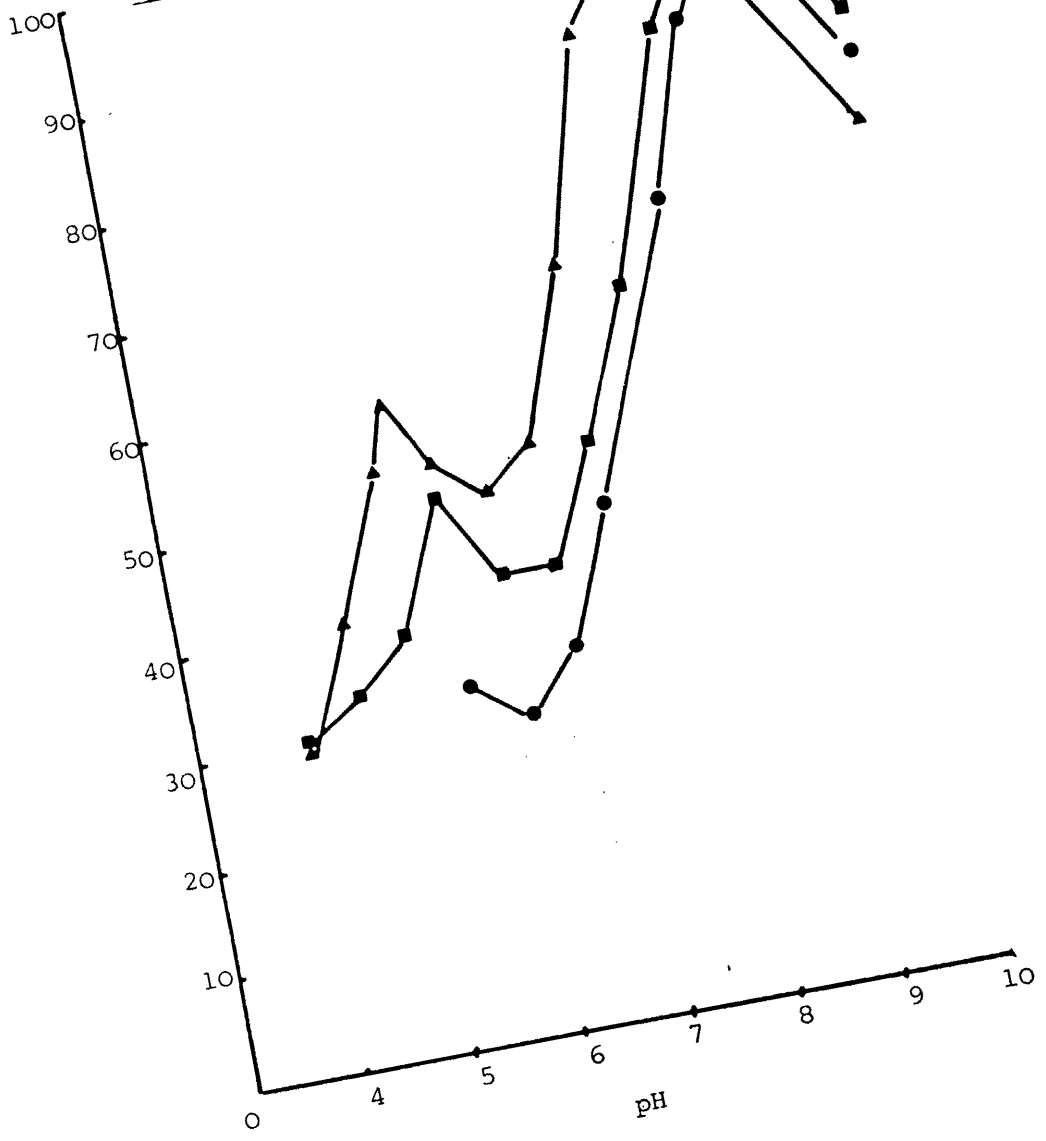
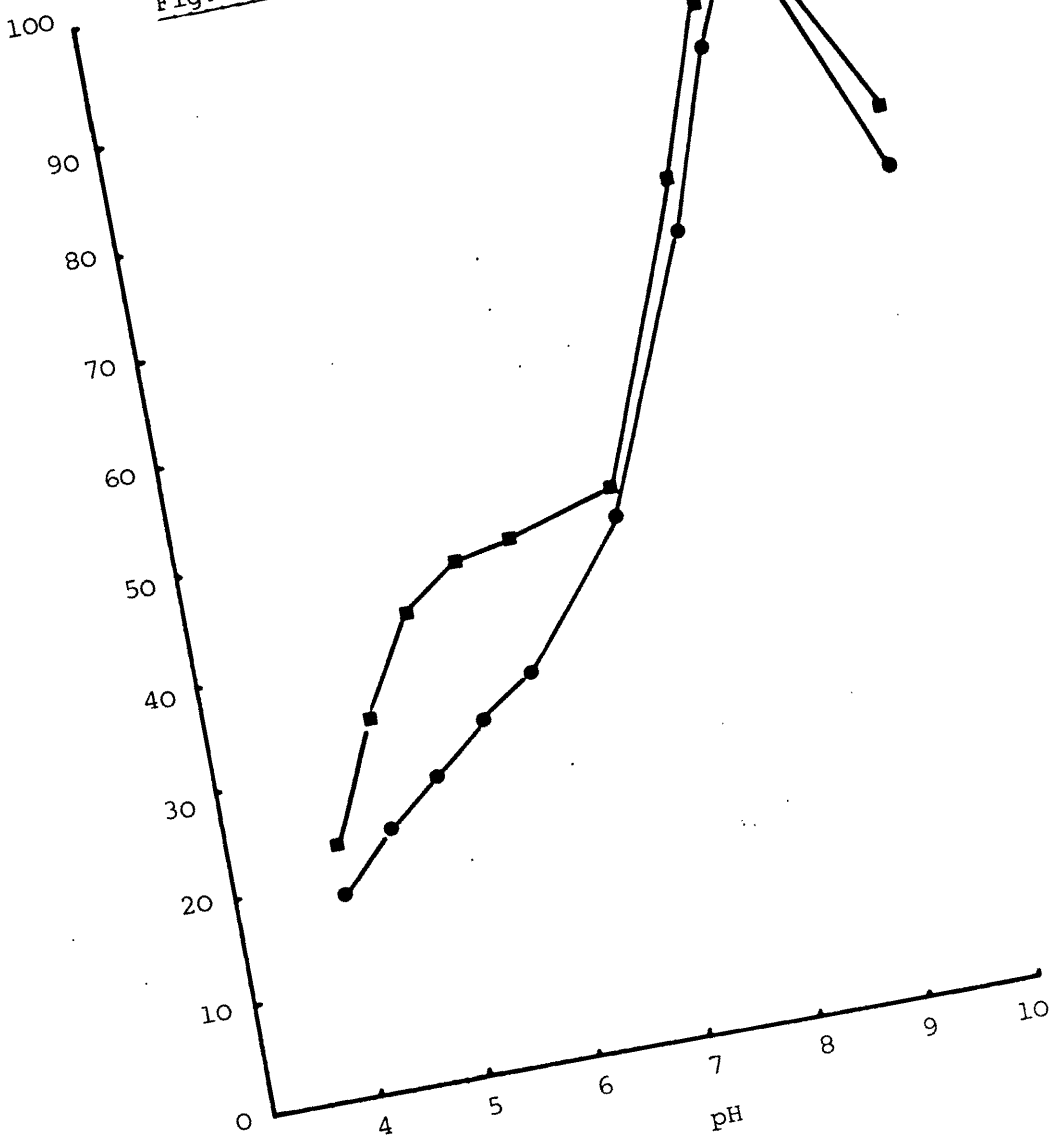

100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

Fig. 40


100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

major alkaline form appeared to vary with the substrate. Activity at pH 5.5 comprised 60.5% and 48% of maximum activity with haemoglobin and cuticle respectively. Figs. 39 and 40 show the hydrolysis of cuticle by proteases in crude culture filtrate compared with that effected by purified alkaline protease. Presuming that the disparity in activity at the lower pH range is due to the minor protease than at its greatest contribution (pH 5.0) it comprises 36% of the total activity.

C. Effect of pH on protease stability

Samples of purified alkaline protease were adjusted to pH values ranging from 4.0 - 10.0 with Britton-Robinson buffer. After incubating for 2 h at 35°C or for 24 h at 4°C the solutions were assayed (at pH 8.0, 30°C) for activity using hide protein azure. As illustrated in Fig. 41 the enzyme was stable at all pH values at 4°C. At 35°C there was some loss of activity particularly at pH values below 6.0 and above 9.0. The protease was stable to 24 h dialysis at 4°C against Tris buffer (0.02 M, pH 7.1). Overnight dialysis against Tris, pH 9.5 resulted in the loss of *c.* 35% of the protease activity.

D. Effect of temperature and protease stability

Samples of purified alkaline protease were adjusted to pH 8.0 with Britton-Robinson buffer, and subjected to heating for 10 min at various temperatures within the range of 30°C to 70°C. The residual proteolytic activities obtained were assayed at 30°C and compared to the enzymatic activity of unheated controls. The protease was stable within the temperature range of 30 to

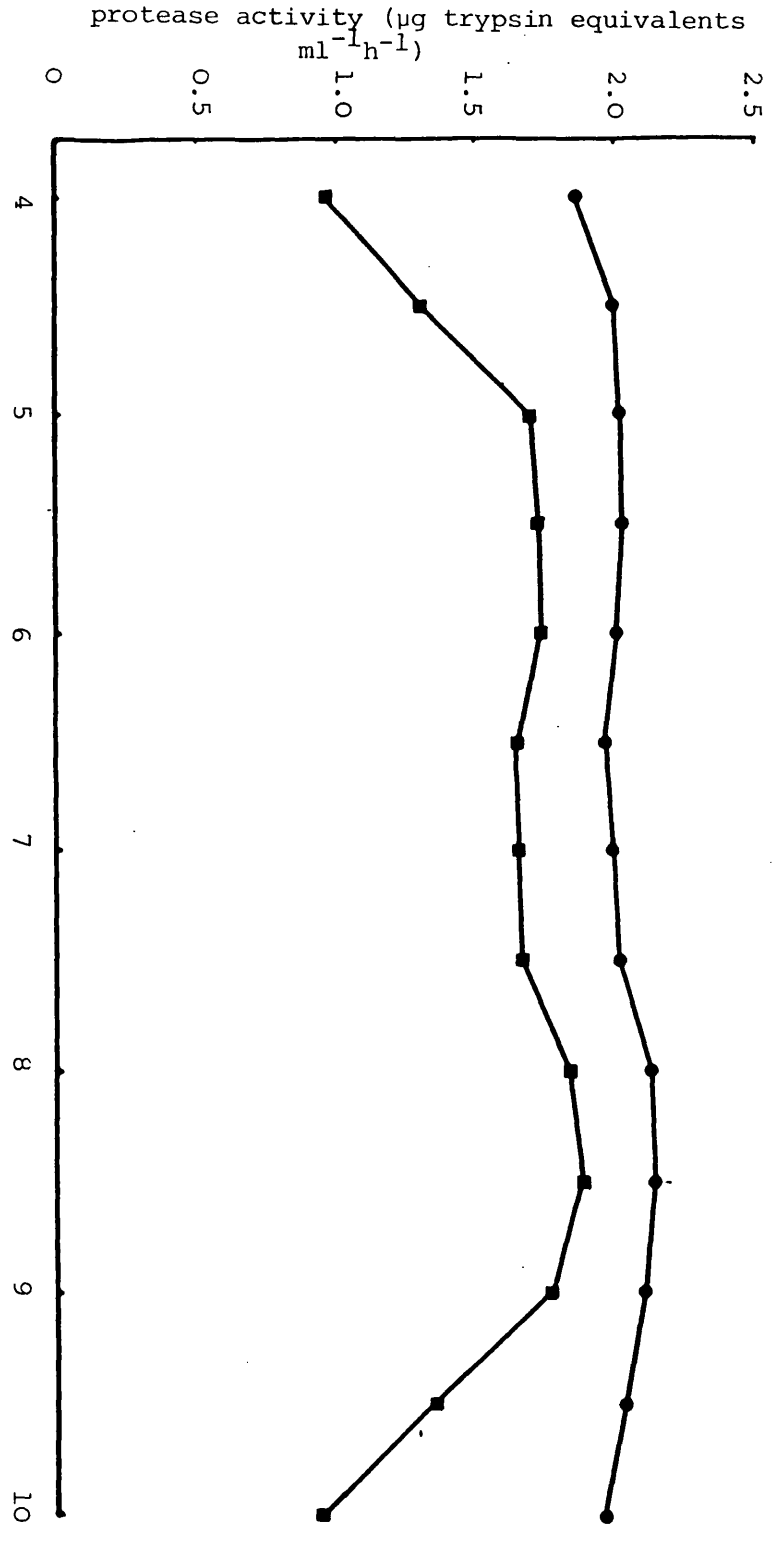


Fig. 41. Effect of pH on stability of *M. anisopliae* (MEl) alkaline protease at 4°C for 24 h (●—●) and 35°C for 2 h (■—■). Each point is the mean of 4 replicates. A second experiment (3 replicates) produced very similar results.

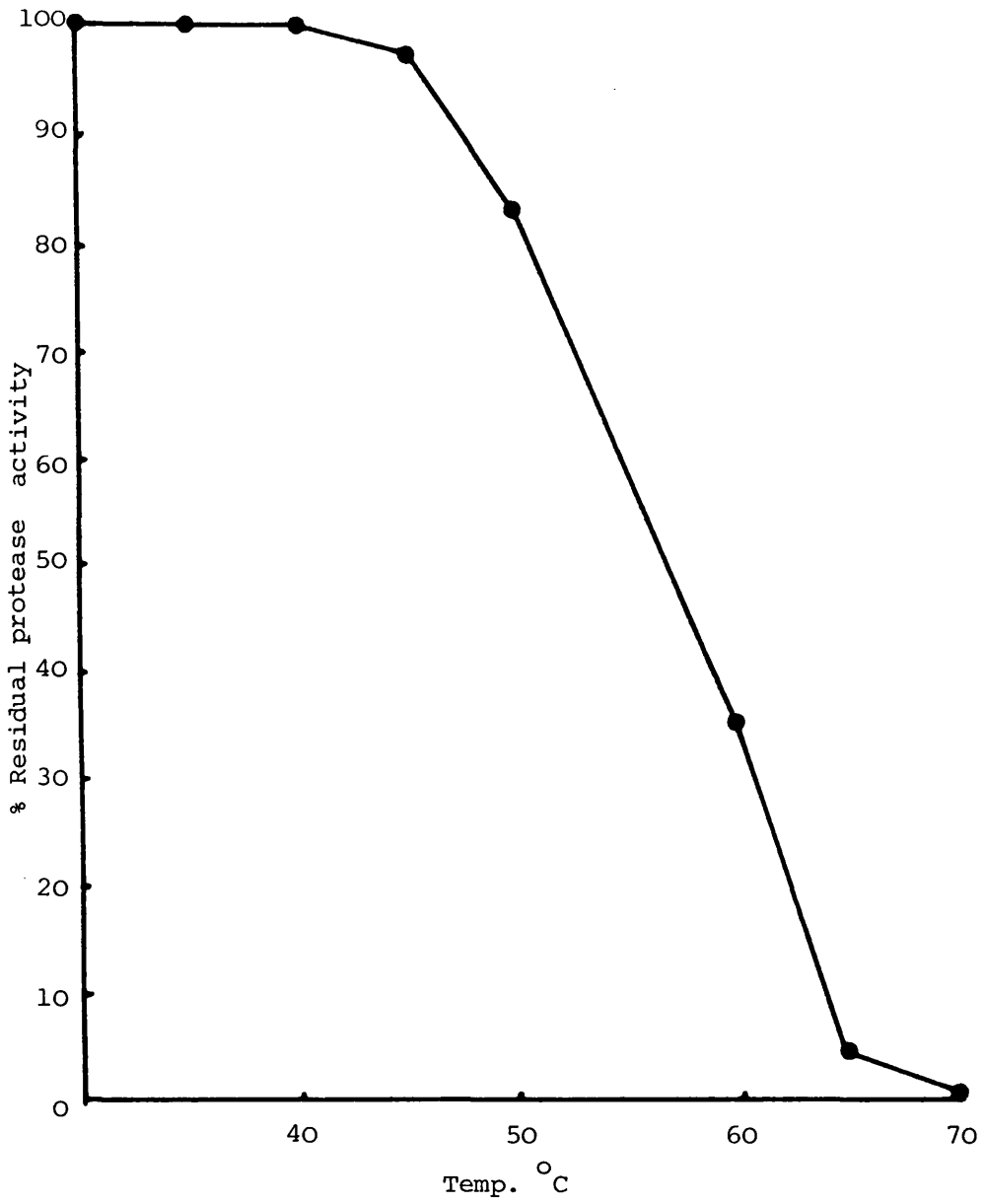


Fig. 42. Heat inactivation of alkaline protease from *M. anisopliae* (MEl). Pure protease, adjusted to pH 8.0, was heated for 10 min at temperatures indicated prior to assay with casein. Each point is the mean of 4 replicates. A second experiment (3 replicates) produced very similar results.

Table 21. Fractional precipitation of alkaline protease of *M. anisopliae* (MEl) with ammonium sulphate.

Ammonium sulphate saturation (%)	Enzyme activity (% of maximum)	Specific activity (units/mg protein)	Purification
Filtrate	-	15.4	-
20	0.4	3.9	0
40	6.5	14.6	0
60	21.1	25.61	1.660
80	47.4	19.85	1.289
100	24.6	16.93	1.100

40°C (Fig. 42). Above 45°C enzymatic activity decreases sharply, with total inactivation occurring at 70°C.

E. Fractional precipitation with ammonium sulphate

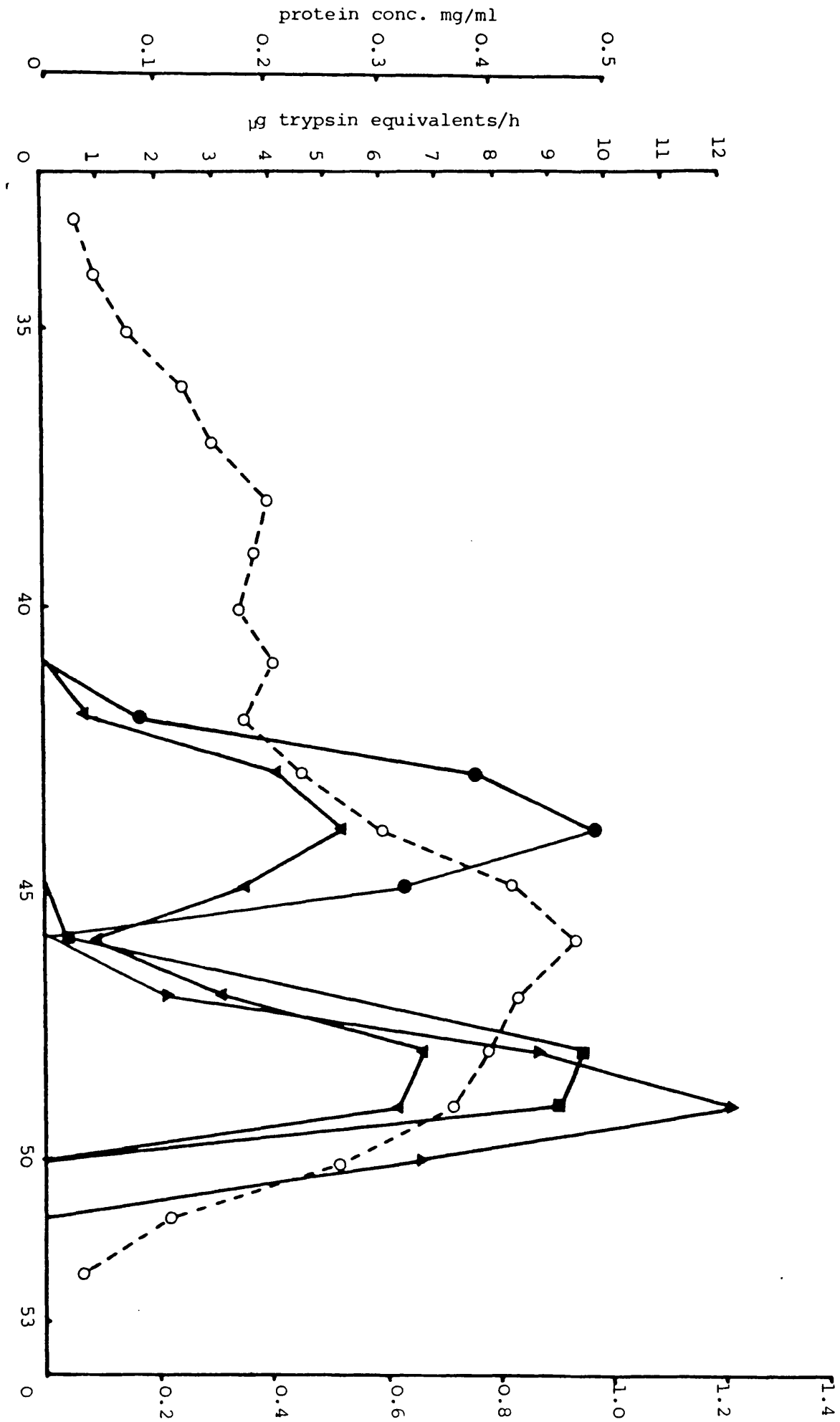
Filtrates from 5 d *M. anisopliae* (ME1) cultures grown on 1% chitin or cuticle/basal salts were subjected to increasing levels of ammonium sulphate saturation as described in Materials and Methods, then dialysed and assayed for protease. The largest amount of protein precipitated was in the 60 - 80% (w/v) fraction and this contained 47% of the total protease (Table 21). 25% of the remaining protease was in the 80 - 100% (w/v) saturation fraction.

For most practical purposes the 40 - 100% ammonium sulphate saturation was used as a means of concentration only since the technique did not result in a very significant purification nor did it resolve the protease from enzymes of the chitinolytic complex (Table 19).

F. Molecular weight determination

The protein obtained from the ammonium sulphate precipitation step was fractionated on a Sephadex G-100 column (Fig. 43). A large protein peak occurred near the end of the fractionation range which shows that the bulk of the proteins in chitin culture filtrate have molecular weights between 23,000 - 30,000. The alkaline protease eluted as a single peak with a molecular weight of *c.* 25,000 daltons (R_s 20.9 Å). The protease was well separated from the aminopeptidase peak (M.Wt. 35,000 daltons, R_s 27.5 Å) but overlapped with carboxypeptidase activity (M.Wt. 26,500,

Fig. 43. Sephadex G-100 gel filtration of the protein fraction precipitated at 40-100% NH_4SO_4 saturation of *M. anisopliae* (MEl) cuticle culture filtrate. A 2.5 x 47 cm column of Sephadex G-100 was equilibriated and eluted with 0.15 M phosphate buffer (pH 6.5). Protein concentration (O-O); protease activity, pH opt. 8.2 (▲-▲); carboxypeptidase (■-■); aminopeptidase (●-●); peptidase activity against G-G-P (▼-▼).


protein conc. mg/ml
0
0.1
0.2
0.3
0.4
0.5
µg trypsin equivalents/h
0
1
2
3
4
5
6
7
8
9
10
11
12
Fraction number
0
35
40
45
50
53
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Carboxypeptidase (µmole PA/h/ml)
Aminopeptidase (µmole naphthylamide 10 min^{-1}); peptidase (µmole amino acids h^{-1} ml^{-1})

R_s 21.5 Å). Activity against the tripeptide L-glycylglycyl-phenylalanine closely followed the elution profiles of the two peptide exo-hydrolases.

G. Iso-electric focussing of proteolytic enzymes

The protein obtained from the ammonium sulphate precipitation step was applied to an LKB 8101 column and resolved in a pH 3 to 10 gradient. The alkaline protease separated into two distinct zones with a major peak pI 9.5 - 10.0 and a minor peak pI 4.5. The acid protease occurred in a fairly narrow zone, pI 5.6 (Fig. 44).

These results suggest that the alkaline protease is composed of two separate molecular species.

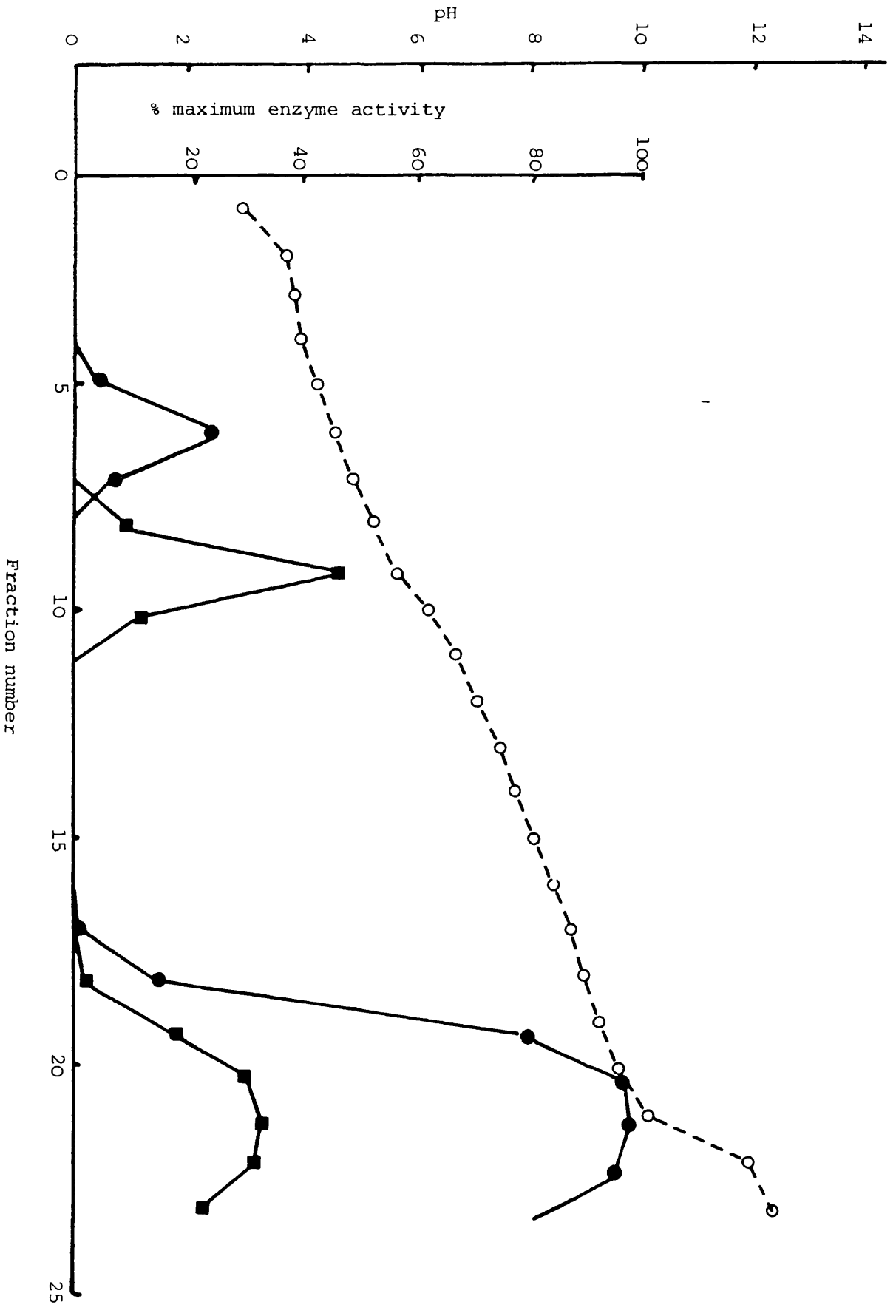
H. Substrate specificity of proteases

Fungal proteases appear to be relatively non-specific as evidenced by activity against a wide variety of proteinaceous substrates (e.g. Tominaga and Tsujisaka, 1976; Singh and Vezina, 1971), The range of activity of the alkaline protease, pI 9.5, purified by NH_4SO_4 precipitation and iso-electric focussing (Fig. 44) was determined against several widely differing proteins (Table 22). Moderate to high activity was evident on casein, urea-denatured haemoglobin (Materials and Methods), gelatin, bovine fibrinogen and locust cuticle but little degradation of the highly ordered collagen occurred. The enzyme also degraded three commercial preparations of insoluble proteins conjugated with dyes.

The protease did not hydrolyse any of the following peptides -

Fig. 44. Iso-electric focussing (pH 3 - 10) of proteases in 40 - 100% NH_4SO_4 saturation fraction of *M. anisopliae* (ME1) cuticle culture filtrate.

(■–■) protease activity assayed at pH 5.5; (●–●) protease activity assayed at pH 8.2; (○– –○) pH.


pH
0
2
4
6
8
10
12
14
% maximum enzyme activity
20
40
60
80
100
0
5
10
15
20
25
Fraction number

dipeptides : L-alanyl-L-alanine, L-alanylglycine, glycyl-L-alanine, glycylglycine L-valyl-L-alanine, glycyl-L-proline; tripeptides: alanylalanylalanine, alanylglycyl, glycine, glycylalanylalanine, glycylglycylalanine, valyl glycylglycine, prolylglycylglycine, tetrapeptides: alanylalanylalanyl alanine, glycylglycyl glycylglycine and pentapeptides: alanylalanylalanyl-alanylalanine. In contrast, culture filtrates of *M. anisopliae* (MEl) as well as those from *B. bassiana* and *V. lecanii* hydrolysed di and tri alanyl peptides (Table 25), presumably through the action of peptide exo-hydrolases (see Fig. 43).

The protease failed to hydrolyse L-alanylnaphthylamide indicating that it does not possess amino peptidase activity. It did however have trace activity against CBZ-glycylphenylalanine. This activity was inhibited by PMSF (1×10^{-4} M) but not by EDTA or ethylmaleimide (1×10^{-3} M) thereby clearly distinguishing it from the major carboxypeptidase activity present in culture filtrates (Table 21).

The *M. anisopliae* (MEl) protease like trypsin or chymotrypsin is an esterolytic protease as confirmed by its activity against *p*NP proprionate (1.24 µmole $ml^{-1}h^{-1}$). The rates of hydrolysis of esters and chromogenic substrates by *M. anisopliae* (MEl) protease were determined by specific spectrophotometric procedures (Materials and Methods) and compared with the rates shown by trypsin (Sigma) and culture filtrates from strains of *M. anisopliae, B. bassiana* and *V. lecanii* (Table 24). The enzyme preparations from four strains of *M. anisopliae* preferentially hydrolysed the trypsin substrates p-toluene sulfonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-arginine-p-nitroanilide (BAPNA).

Table 22. Hydrolysis of various protease substrates by *M. anisopliae* (MEl) alkaline protease.

Substrate	Activity
Hide protein azure[a)]	135 ± 2.7 OD_{595}/h
Keratin azure[a)]	57 ± 1.4 OD_{595}/h
Elastin congo[a)]	48 ± 1.7 OD_{450}/h
	OD_{280}/h
Gelatin[b)]	1.27 ± 0.033
Casein[b)]	0.82 ± 0.022
Haemoglobin[b)]	0.63 ± 0.018
Bovine fibrinogen[b)]	0.55 ± 0.026
Ground cuticle[b)]	0.29 ± 0.017
Collagen[b)]	0.036 ± 0.005

a) Assay and units are as described in Materials and Methods

b) Assay and units are as described for activity against collagen in Materials and Methods

Each result is the mean of 5 replicates ± S.E.

Table 23. Hydrolysis of peptides by proteolytic enzymes of *M. anisopliae*, *B. bassiana* and *V. lecanii*

	M. anisopliae		*B. bassiana*	*V. lecanii*
	Purified enzyme	Culture filtrate	Culture filtrates	
L-alanyl alanine	0	1.89[a]	0.96	1.62
L-alanyl alanyl-alanine	0	2.01	1.20	1.72
L-alanyl naphthyl-amide	0	1.04	0.75	0.01
CBZ-glycyl phenyl-alanine	0.02	1.27	0.24	2.8

Note: The amount of protease in each preparation (assayed against hide protein azure, pH 8.0) was diluted to 5 µg trypsin equivalents. Peptidase activities were assayed as described in Materials and Methods. Activities are expressed as µmoles $ml^{-1}h^{-1}$.

a) Mean values N = 4. The results are representative of 3 experiments using different culture filtrates on each occasion.

Table 24. Hydrolysis of trypsin substrates (BAPNA and TAME) and chymotrypsin substrates (BTPNA and BTEE) by enzyme preparations from *M. anisopliae* (MEl, RS23, RS455 and RS549), *B. bassiana* and *V. lecanii*.

Enzyme source	Amount of enzyme (µg trypsin equivalents)[a]	Substrates BAPNA[b]	BTPNA[b]	TAME[b]	BTEE[b]
Purified enzymes					
Trypsin	2	NT	NT	12	0.001
M. anisopliae (MEl)	5	39.41[c]	1.61	3.31	1.3
Culture filtrates					
M. anisopliae (MEl)	5	49.49	1.75	3.73	1.53
" " (RS23)	5	38.14	0.24	5.04	0.03
" " (RS455)	5	24.83	0.06	3.86	0.03
" " (RS549)	5	18.87	0.06	3.67	0.03
B. bassiana	5	3.01	63.12	0.83	6.12
V. lecanii	5	1.10	47.28	0.29	5.02

a) Culture filtrates were diluted to give equal activities against hide protein azure.

b) Assay and units are as described in Materials and Methods.

c) Mean values; n = 3. The results are representative of 3 experiments using different culture filtrates on each occasion.

However the *M. anisopliae* proteases differed from trypsin in exhibiting comparatively low esterase activity. Thus the TAME esterase activity of *M. anisopliae* (MEl) protease was *c.* 9% of that exhibited by trypsin. In addition the fungal proteases were less specific - especially that from *M. anisopliae* (MEl) which had considerable activity against the chymotrypsin substrate N-benzoyl-L-tyrosine ethyl ester (BTEE).

Filtrates of *B bassiana* and *V. lecanii* preferentially hydrolysed the chymotrypsin substrates BTEE and N-benzoyl-L-tyrosine-P-nitroanilide (BTPNA) which suggests some specificity for proteins containing aromatic acids. However in contrast to chymotrypsin (Walsh and Wilcox, 1970), protease from both fungi also showed a limited activity against TAME.

3. Partial characterisation of *M. anisopliae* (MEl) aminopeptidase

A. Effect of pH on activity

The effect of pH on aminopeptidase activity from filtrates of MEl grown on cuticle was studied using L-alanyl-β-naphthylamide in Britton Robinson buffer (pH 5.0 to 9.5). Aminopeptidase was most active at pH 7.0 with the pH profile showing a rather steeper slope on the acid side of the optimum (Fig. 45).

B. Iso-electric focussing

The iso-electric point was determined on iso-electric focussing plates. The application, running and staining techniques are described in Materials and Methods. With either L-alanyl-4-methoxy-β-naphthylamide or L-leucyl-4-methoxy-β-naphthylamide

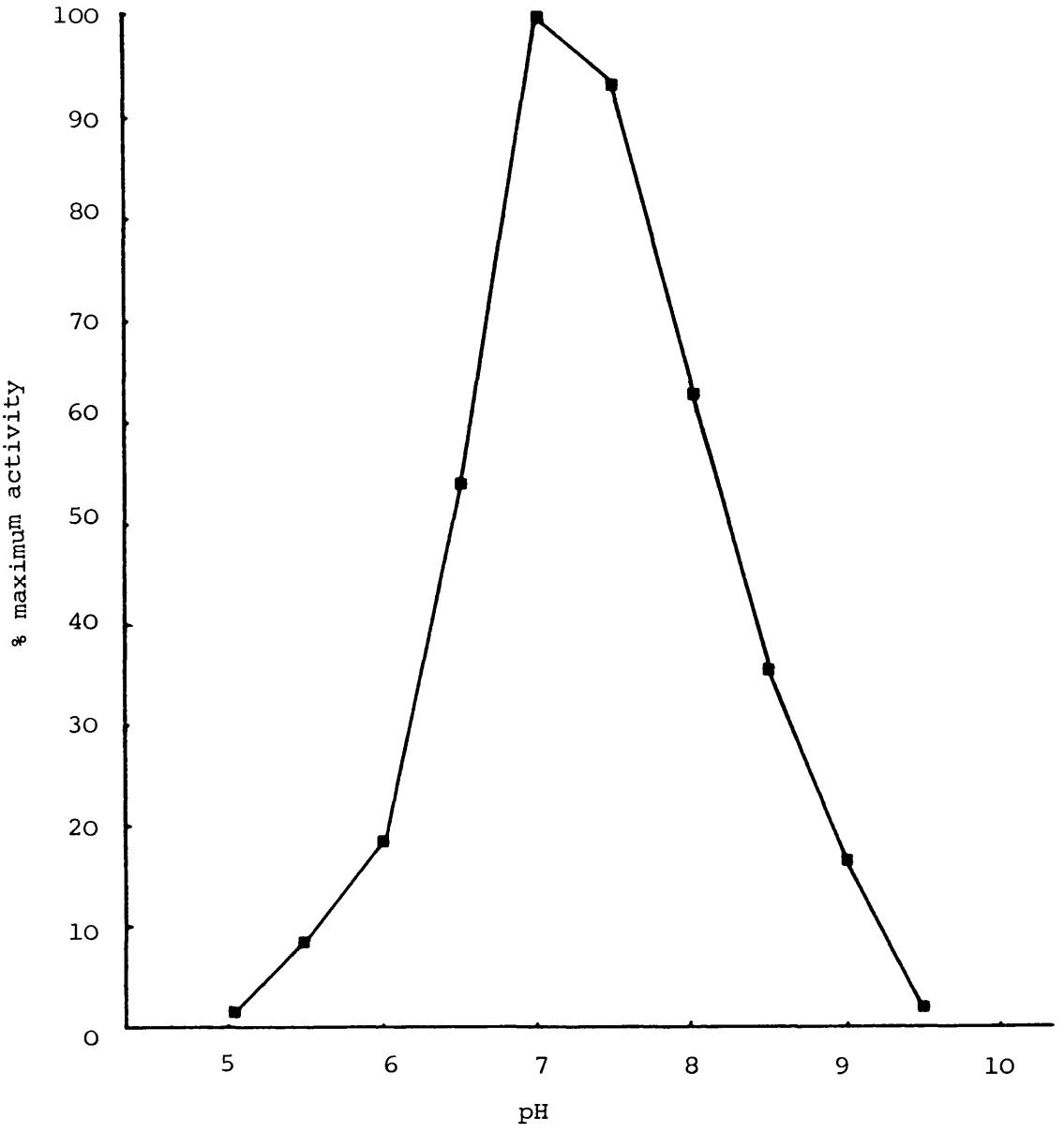


Fig. 45. pH optima of aminopeptidase from *M. anisopliae* (MEl) grown on 1% ground locust cuticle. L-alanyl naphthylamide used as substrate. Each point is the mean of three replicates. The results are representative of two similar experiments using different enzyme preparations.

as substrate a single band of activity was detected with an estimated pI of 4.7 (Plate 6). Incubation of plates with both substrates simultaneously still only resulted in one band indicating that a single protein is probably responsible for both activities.

C. Action on L-alanine β-naphthylamide and L-leucine β-naphthylamide

The ability of culture filtrates from 6 strains of *M.anisopliae* to degrade L-leucine naphthylamide and L-alanine naphthylamide was compared. The results (Table 25) show that all strains preferentially hydrolysed L-alanyl naphthylamide indicating that activity against peptides will depend on the nature of the N-terminal amino acid.

4. Partial characterisation of esterase activity

A. The effect of pH on esterase and lipase activity

The effect of pH on the rate of enzymatic hydrolysis of *p*NP-caprylate is shown in Fig. 46. As the pH increased from 6.5 to 7.5 the rate of hydrolysis increased linearly. A fairly broad pH optimum was observed between 8.0 and 8.5 beyond which the rate of non-enzymatic hydrolysis of the substrate was too high to measure enzymolysis accurately.

The lipase pH profile (Fig. 46) was almost symmetrical, with an optimum *c.* pH 7.0. Substantial activity extended from 5.5 to 8.5 (> 10%).

Plate 6. Acrylamide gel zymograms of proteins (blue bands) and aminopeptidase (pink/red bands) in cuticle culture filtrates. Detection of proteins and amino peptidase activity against L-alanine-4-methoxy-β-naphthylamide was as described in Materials and Methods.

Table 25. Activity of aminopeptidase in culture filtrates of *M. anisopliae* against L-alanine naphthylamide and L-leucine naphthylamide.

Strain	Substrate L-alanine naphthylamide[a) b)]	L-leucine naphthylamide[b)]
M. anisopliae		
ME1	1.00	0.171[c)]
RS23	1.00	0.105
RS298	1.00	0.255
RS549	1.00	0.122
RS543	1.00	0.136
RS455	1.00	0.166

a) Culture filtrates were diluted to give equal activities against L-alanine-β-naphthylamide.

b) Assay and units are as described in Materials and Methods

c) Mean values, N = 3. The results are representative of three experiments using different enzyme preparations on each occasion.

B. Inhibition studies on esterase

Most esterases can be classified into arylesterases (Esterase A) that are inhibited by N-ethylmaleimide and other thiol alkylating agents, and carboxyesterases (Esterase B) which are serine hydrolases and therefore inhibited by PMSF, Paroxan and other organophosphorus compounds (Aldridge, 1953; Vrisch, 1971). It is reported that quinone HCl has a more pronounced effect on lipase than vs esterase activity and might possibly be used to distinguish between them (Nachlas and Seligman, 1949 a,b). Using PNP caprylate as substrate only PMSF was an effective inhibitor indicating that most or all esterase activity is the result of carboxyesterase(s).

Table 26. Effect of inhibitors on hydrolysis of PNP caprylate by cuticle culture filtrate (5 d) of *M. anisopliae* (ME1).

Inhibitor[a)]	Activity (%)
Control	100
N-ethylmaleimide (10^{-3} M)	106
PMSf (1 x 10^{-4} M	20
Quinone HCl[b)]	92

a) Filtrate was incubated for 20 min with inhibitors at concentrations indicated before assaying for activity. In all instances variation between replicates (n=4) of a treatment was less than 6%.

b) Tris buffer (0.1 M, pH 8.0) used in place of Britton Robinson buffer.

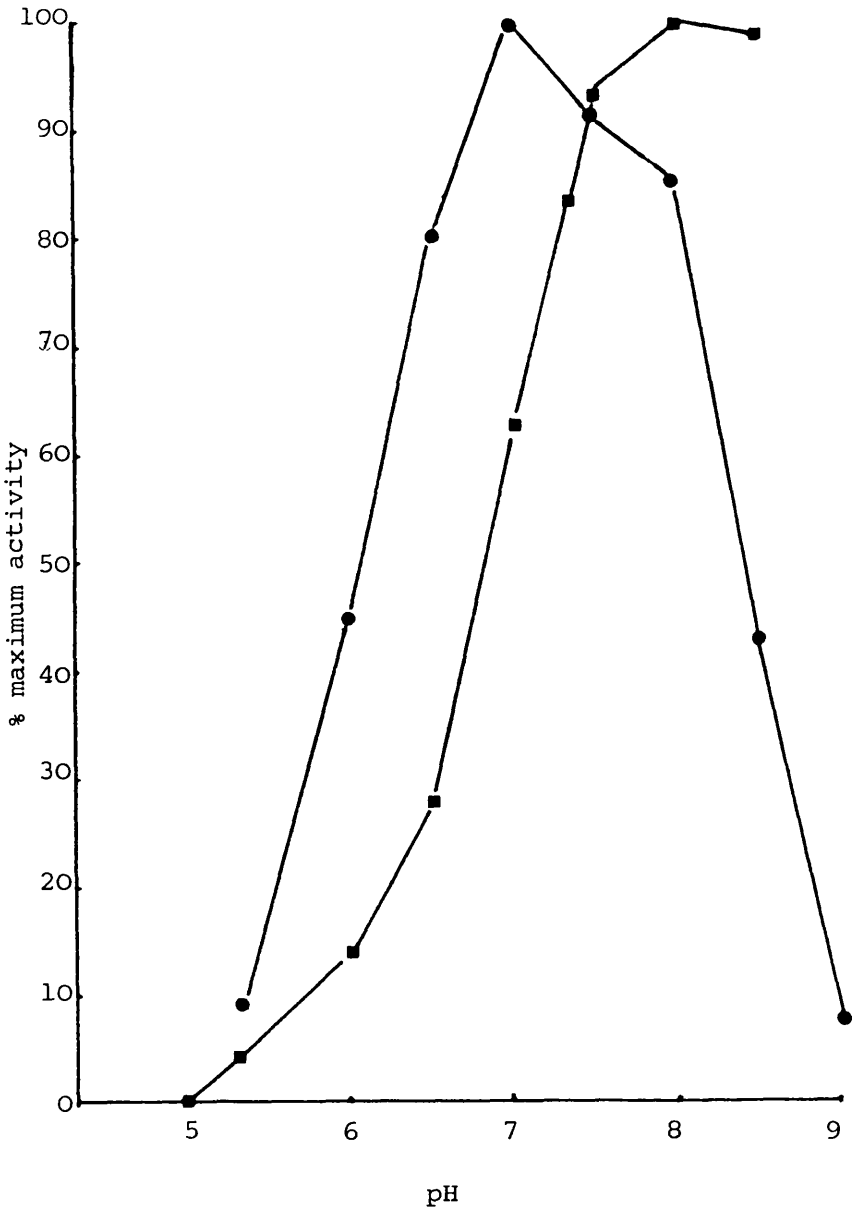


Fig. 46. pH response curve for lipase (●—●) (from 10 d culture filtrate) and esterase (■—■) (from 3 d culture filtrate). Lipase was assayed with olive oil, esterase with PNP caprylate (Materials and Methods). Each point is the mean of three replicates. The results are representative of two experiments using different culture filtrates.

C. Esterase specificity

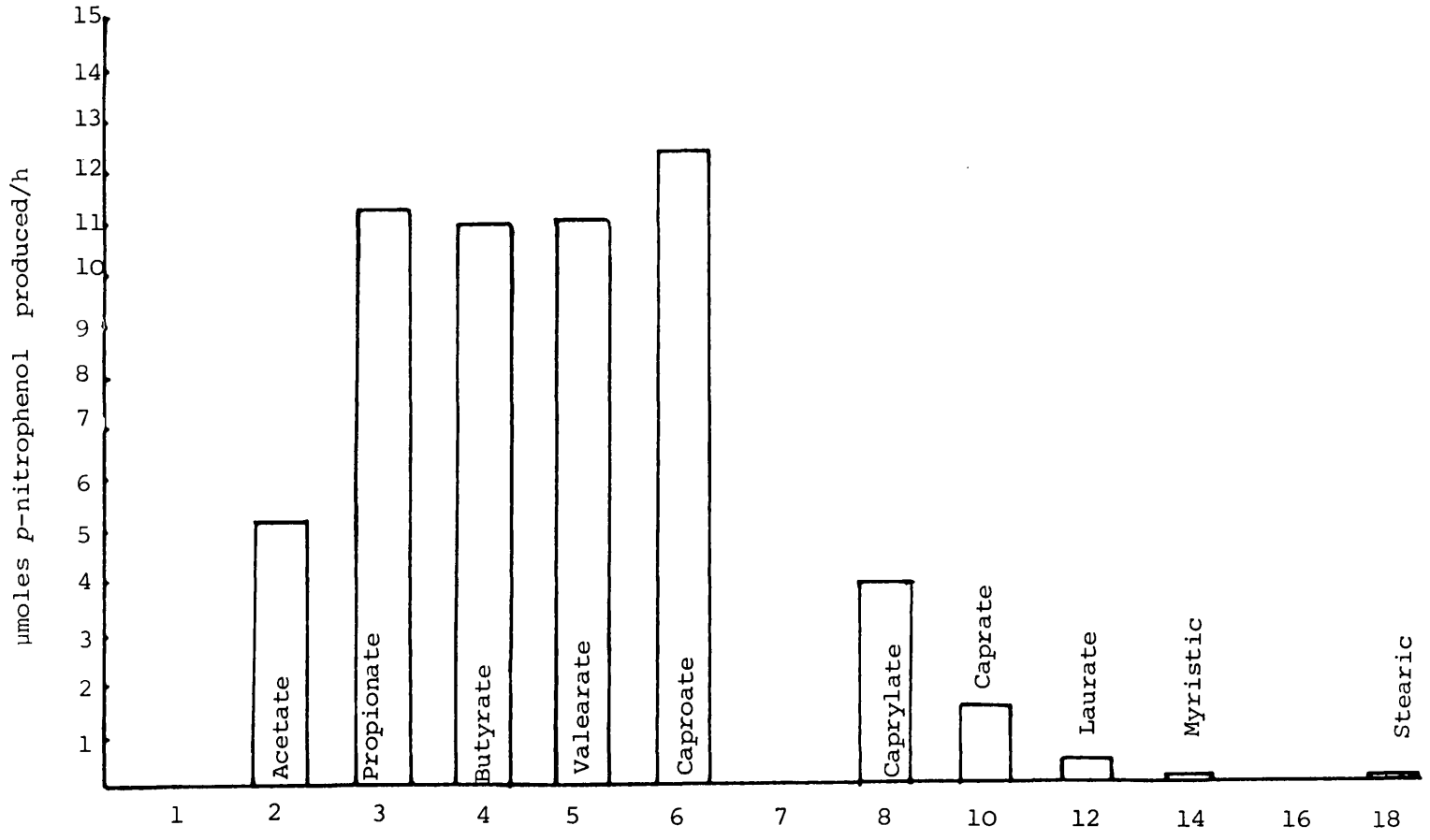
A common dogma is that lipases only hydrolyse fatty acid esters at a lipid-water interphase (i.e. in an emulsion) where as esterases hydrolyse substrates in solution (Hofstee, 1960; Brokerhoff, 1973). In practice, however the differences between the two activities are relative rather than absolute, such that generally esters of short chained fatty acids (C_2-C_4) are preferentially hydrolysed by esterases and long chained esters (> C_8) are preferentially split by lipases (Shnitka, 1974). The specificity of esterase activity produced by *M. anisopliae* (MEl) in cuticle cultures (3 d) was characterised with several *p*-nitrophenyl esters possessing acyl moieties varying from C_2 to C_{18} (Fig. 47). Activity was greatest against short and intermediate length fatty acids with only trace activity occurring above C_{10}. This confirms the earlier results (Sec. 1) that lipase is not produced extracellularly by young mycelium. Activity against myristic acid (C_{14}) rose in older cultures (7 - 14 d) but remained low at less than 1 μmole $ml^{-1}h^{-1}$.

The specificity of esterases is probably due to many phenomena but steric and hydration effects can be especially important with this group (Brokerhoff and Jensen, 1974).

D. Gel-filtration.

The protein obtained from the ammonium sulphate precipitation step was fractionated on a Sephadex G-100 column (Fig. 48). The major esterase peak eluted with a profile very similar to that obtained for alkaline protease. As this protease also degrades PNP esters (Sect. II, 2H), and both activities behave similarly with PMSF it seems probable that the alkaline protease is a major if not

Fig. 47. Hydrolysis of *p*-nitrophenyl esters by MEl cuticle culture filtrate.



Each result is the mean of 3 replicates. The results are representative of 2 similar experiments using different culture filtrates.

sole contributor to total esterase activity. However unlike protease, esterase activity extended into fractions 40 - 46 indicating that other esterases are present.

E. Iso-electric focusing of esterases

Esterases frequently exist in multiple molecular forms presumably isoenzymes (Masters and Holmes, 1972) and this has led to their use in phylogenic studies (Shnitka, 1974). De Conti *et al.* (1980) electrophoretically tested strains of *M. anisopliae* grown on minimal media and detected four or five separate esterase activities. However their method,which involved sectioning gels before staining , is unlikely to have given good resolution. In addition complex growth media may be necessary to allow production of the full range of esterases. This study was undertaken to determine whether iso-electrophoretic zymogram techniques could provide a sensitive assay of filtrate esterases, to compare zymograms between *M. anisopliae* (ME1),*B bassiana* and *V. lecanii*, and to develop techniques for the separation of these esterases.

F. Comparison of iso-electrophoretic zymograms of esterase activity in *M. anisopliae, B. bassiana* and *V. lecanii*.

The iso-enzyme distributions found when focussing in gels and esterase located directly with naphthyl esters in the range pH 3-9 are illustrated in Fig. 49. Although the esterase patterns differed markedly between species, each pathogen produced a single major peak of activity which developed within seconds of applying the staining solution (indicated by an arrow in Fig. 49). Over the course of an hour other bands appeared so that the esterase activities produced

by *M. anisopliae*, *B. bassiana* and *V. lecanii* were represented by at least 25, 18 and 17 iso-enzymes respectively. In addition to sharply demarcated and regularly spaced bands there were several which were faint, broad or indistinct. Staining of proteins from ME1 cultures with Coomassie blue revealed about 30 bands distributed in pI regions 3 to 5 and 7.5 to 9.5. Occasionally protein bands coincided with esterase bands (e.g. pH 9.5) but in many cases the amount of esterase protein was too small to be located with Coomassie blue.

G. Characterisation of esterases

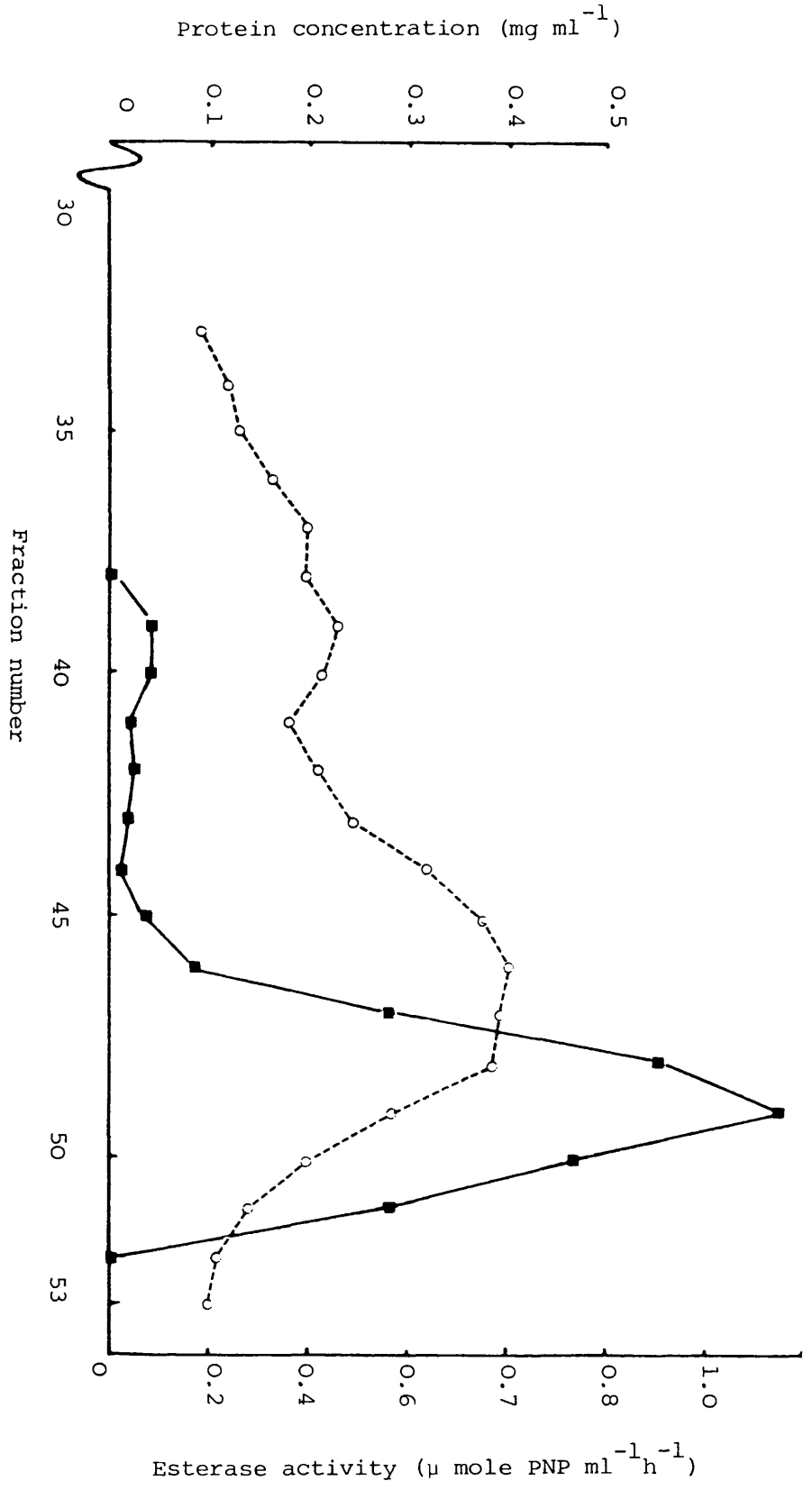
It was possible by means of flat bed iso-electric focussing to recognise *c.* 25 distinct esterases in *M. anisopliae* (ME1). A first step towards understanding the functional and biological significance of these different enzyme forms was attempted by dividing them into functionally distinct groups. Esterases which have similar substrate specificities or are similarly inactivated may have common biochemical and physiological attributes. Two methods were tried in this study:

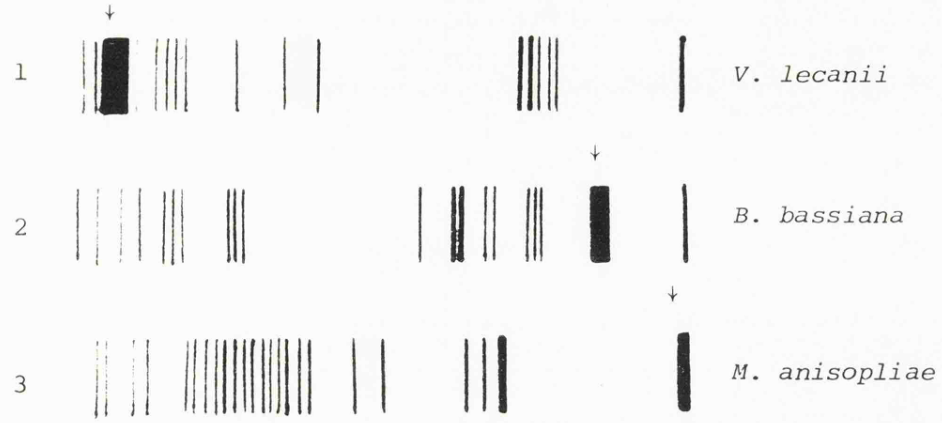
1. A comparison of iso-electrophoretic zymograms obtained with different esterase substrates.
2. Testing the effect of the serine hydrolase inhibitor PMSF.

The esterases from *M. anisopliae* (ME1) showed three major groups of activities: one group (15 bands) had a pI of approximately 5-6, another group (3 bands) were of pI *c.* 7.3 and there was a single major activity of *c.* pI 9.5. Staining the gels with different naphthyl esters (Materials and Methods) gave no additional bands but

Fig. 48. Sephadex G-100 gel filtration of the protein fraction of *M. anisopliae* (ME1) cuticle culture filtrate precipitated at 40 - 100% NH_4SO_4 saturation.

A 2.5 x 40 cm column of Sephadex G-100 was equilibrated and eluted with 0.2 M phosphate buffer (pH 6.5). Protein concentration (O—O); p-nitrophenyl palmitate hydrolysis (■—■) were measured as described in Materials and Methods .


Protein concentration (mg ml^{-1})
0
0.1
0.2
0.3
0.4
0.5
Fraction number
30
35
40
45
50
53
0
0.2
0.4
0.6
0.7
0.8
1.0
Esterase activity (μ mole PNP ml^{-1}h^{-1})



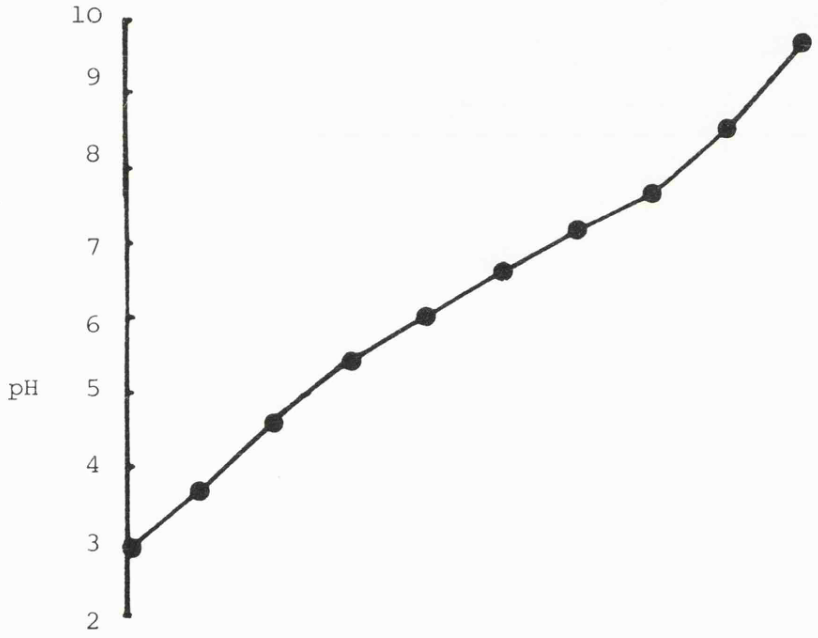


Fig. 49. Gel isoelectric focussing of ammonium sulphate precipitated filtrate from 1) *V. lecanii*, 2) *B. bassiana* and 3) *M. anisopliae* (MEl) grown on cuticle media. Staining α-naphthylacetate + α-naphthyl-propionate ↓ = major activity.

Table 27. Detection of esterase iso-enzymes on iso-electric focussing plates using different naphthyl esters.

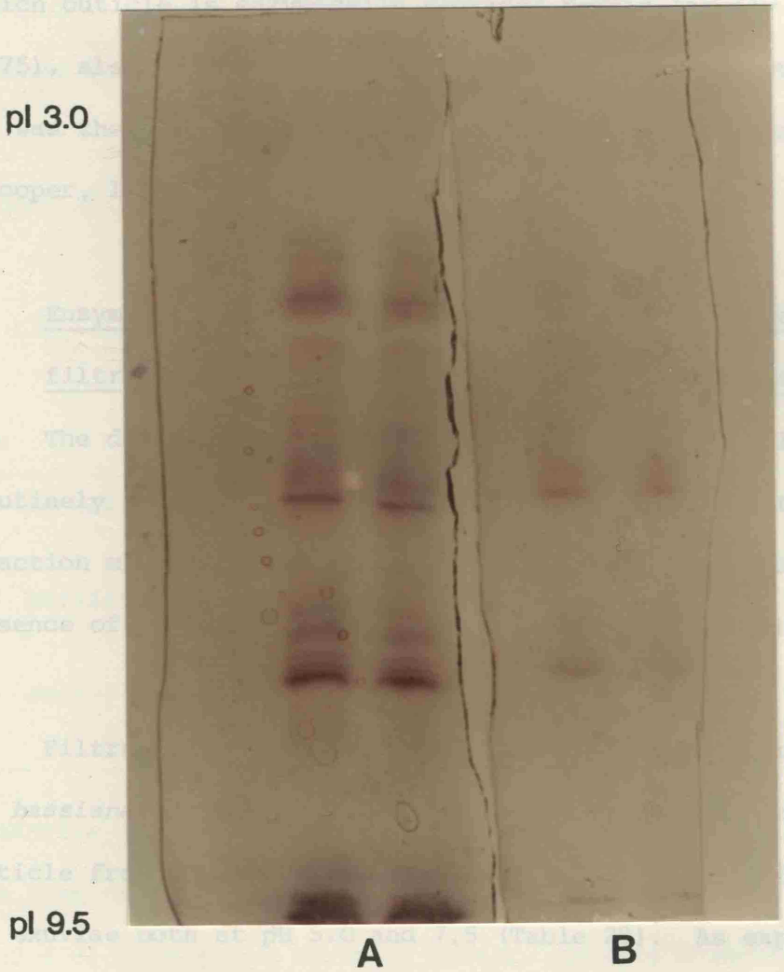
Substrate	Activities in pI region 5-6	7.1-7.4	9.5
α-naphthyl acetate	+++	++++	++
β-naphthyl acetate	++	++	+
α-naphthyl propionate	++++	+	++++
α-naphthyl caprylate	O	O	O
α-naphthyl myristate	O	O	O

O, no staining; + → ++++, various degrees of staining.

the relative activities of the groups varied considerably (Table 27). The strongest staining in pI regions 5-6 and 9.5 was achieved with α-naphthyl propionate but α-naphthyl acetate gave better staining in the pI region 7.3. β-naphthyl acetate produced rather diffuse bands with poor localisation, while no staining was detected when α-naphthyl caprylate or α-naphthyl myristate were used.

The effect of preincubating gels for 20 min in a solution of PMSF (10^{-4} M) is shown in Plate 7 . All of the stronger activities were partially inhibited while minor activities could no longer be detected. The esterases can therefore be classified as serine hydrolases (esterase B). As the alkaline protease is also a serine hydrolase and has a pI of *c.* 9.5 (Fig. 44) it probably accounts for the major esterase activity on the gel.

Plate 7. Acrylamide gel zymograms of esterases in 5 d cuticle culture filtrate. A) control, preincubated for 30 min with water. B) pre-incubated for 30 min with PMSF (1×10^{-4} M). Detection of esterase activity against α-naphthyl propionate was as described in Materials and Methods.



5. Cuticle degradation by enzymes from entomopathogens

To date no studies have been performed to determine the effect of purified fungal enzymes on insect cuticle (Charnley, 1984), and only one study has tested the degradative effects of a culture filtrate (Samsinakova *et al.*, 1971). Yet before it is possible to ascribe any significance *in vivo* to extracellular enzymes it is necessary to demonstrate that activities produced in culture have cuticle-degrading activity either separately, in combination, or in sequence. This is particularly important as the mechanisms by which cuticle is enzymically degraded remain largely unknown (Neville, 1975), also pure enzymes may assist in unravelling cuticle structures as was the case with cell wall-degrading enzymes from plant pathogens (Cooper, 1983).

6. Enzymatic release of soluble products from cuticle by culture filtrates of *M. anispliae*, *B. bassiana* and *V. lecanii*

The digestion of cuticle *in vitro* by culture filtrates was routinely performed as described in Materials and Methods. Control reaction mixtures containing cuticle alone, and filtrate in the absence of substrate were included in each experiment.

Filtrates from 5 d cuticle cultures of *M. anisopliae* (MEl), *B. bassiana* and *V. lecanii* degraded the protein and chitin in ground cuticle from 3 d old adult locusts (0.2 mm - 0.5 mm fragments), and in exuviae both at pH 5.0 and 7.5 (Table 28). As expected from their pH optima (Figs 39 and 30) proteolytic activity was greater and chitinolytic activity lower at alkaline pH. The greater activity of culture filtrates against whole cuticle *cf.* exuviae shows the relative resistance to enzymolysis of sclerotized exocuticle.

Fig. 50 shows the release from cuticle of amino acids and sugars over a 24 h period by *M. anisopliae* culture filtrate. At the end of this time about 16% of the protein (at pH 7.5) and 4% of the total chitin (at pH 5.0) had been solubilised from the cuticle (percentages calculated as described in Materials and Methods).

Essentially similar results (not shown) were obtained with equivalent filtrates of *V. lecanii* and *B. bassiana*. During the early stages there was a rapid release of soluble products from the cuticle. However, after 2 h activities of protease and chitinase became almost linear at their optimal pH.

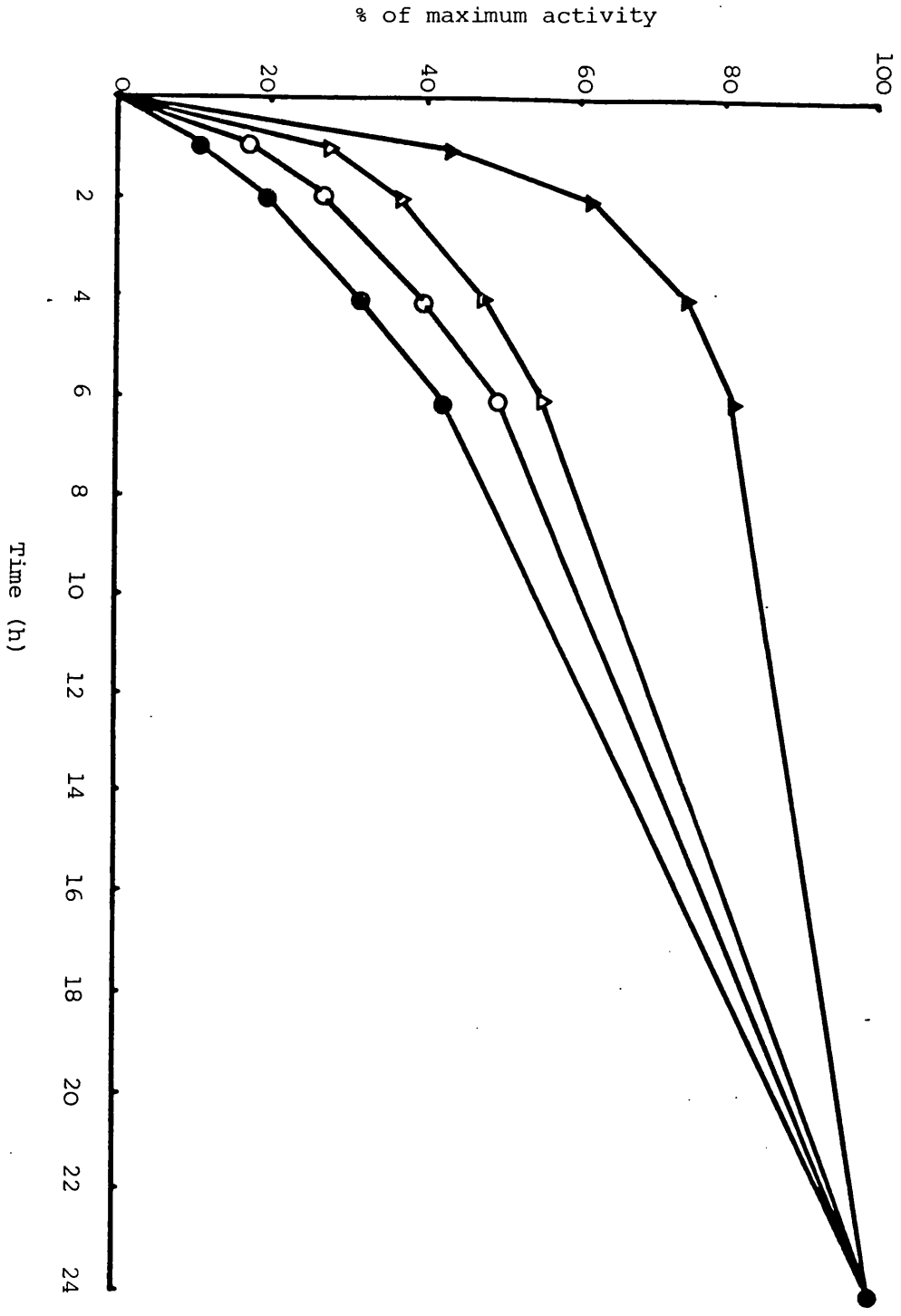
Table 28. Effect of cuticle culture filtrates from 3 entomopathogens on cuticle (1) and exuviae (2)

		Proteolytic activity (µmole amino acids ml^{-1})		Chitinolytic activity (µmole NAG ml^{-1})	
pH		5.0	7.5	5.0	7.5
V. lecanii	(1)	0.24	0.72	0.037	0.018
	(2)	0.13	0.44	0.018	0.006
B. bassiana	(1)	0.16	0.39	0.034	0.013
	(2)	0.10	0.25	0.012	0.003
M. anisopliae	(1)	0.36	0.74	0.027	0.008
	(2)	0.16	0.38	0.009	0.003

Reaction mixtures contained 30 mg ground locust cuticle or exuviae (0.2 mm - 0.5 mm fragments), 2 ml culture filtrate (5 d cultures) and 4 ml of Britton Robinson buffer. After 4 h incubation at 30°C reaction products were assayed as described in Materials and Methods. Each result is the mean of 3 replicates. The results are representative of 3 similar experiments using different enzyme preparations.

Fig. 50. Release of cuticle constituents during enzymolysis by fluid from 5 d cultures of *M. anisopliae* (MEl).

Product, pH of reaction mixture and maximum amount formed are (●—●) amino acids, pH 7.5, 8.97 µmole ml^{-1} (○—○) amino acids, pH 5.0, 3.64 µmole ml^{-1} (▲—▲) amino sugars, pH 7.5, 0.011 µmole ml^{-1} (△—△) amino sugars, pH 5.0, 0.225 µmole ml^{-1}


% of maximum activity
0
20
40
60
80
100
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)

A. Detection of chitin in cuticle fragments by fluorescence staining

Calcofluor M2R New binds to β-glucans including NAG oligomers and has been used in fungi to demonstrate areas of incomplete cell wall formation in which the chitin remains exposed (Gull and Trinci, 1974; Kritzman *et al.*, 1978). Fluorescein isothiocyanate conjugated wheat germ agglutinin (FITC-WGA) has also been employed in studies on fungal cell surfaces as it is specific for oligosaccharides of NAG and binds to chitin (Horisberger and Vonlanthen, 1977). These compounds were exploited in this study to identify and locate chitin in cuticle fragments before and after enzymolysis.

Both calcofluor and FITC-WGA stained only the cut edges of cuticle fragments where NAG polymers are presumably exposed (Plate 8). Very small pieces (< 0.2 mm) of cuticle showed greater overall staining, and hair sockets bound the compounds, albeit weakly. Cuticle particles of various sizes were used as carbon sources in fungal cultures and, after growth, compared with uninoculated controls for their ability to bind calcofluor and FITC-WGA. Intense fluorescence was observed over many particles of both ground and unground cuticle after 4 d growth (Plate 10). A similar degree of staining (Plate 11) was obtained by boiling pieces of cuticle in 30% KOH for 15 min to remove bound protein (Jeuniaux, 1964). Exuviae was far more resistant to staining and the more highly pigmented pieces of sclerotized cuticle showed no increase in staining even after 5 d growth.

These results suggest that fungal proteolytic enzymes can remove bound proteins of unsclerotized cuticle, thus increasing the number

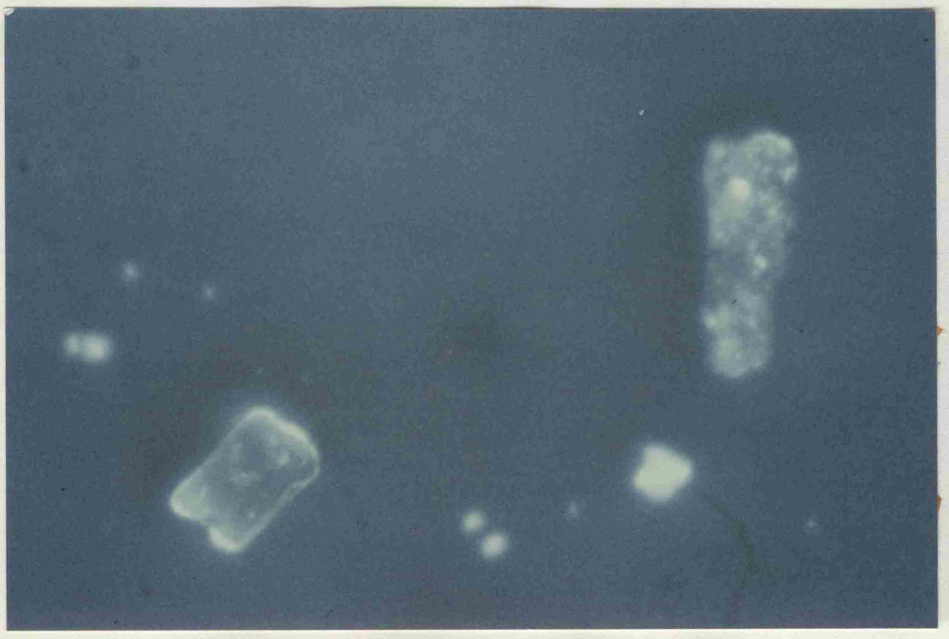
Plates 8 to 11. Detection of chitin in cuticle fragments by fluorescence staining with FITC-WGA.

Plate 8. Staining (mainly) of the broken edges of ground cuticle (< 0.2 mm).

Plate 9. Increased overall staining of a particle of unground cuticle (*c.* 0.5 mm) after 3 d incorporation in growth media.

Plate 10. Intense fluorescence over a particle of unground cuticle after 4 d incorporation in growth media.

Plate 11. Intense fluorescence obtained after boiling pieces of ground cuticle in 30% KOH.



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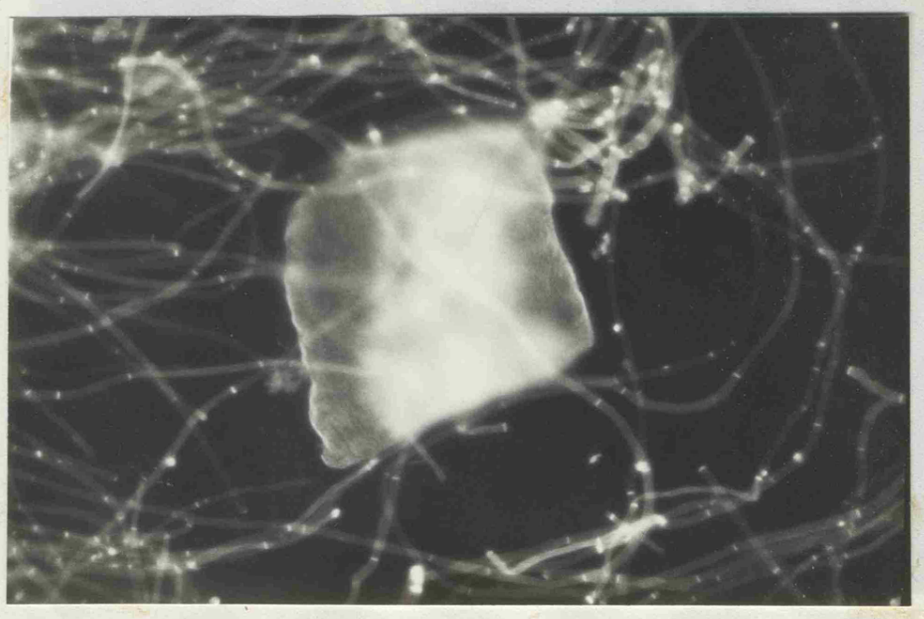

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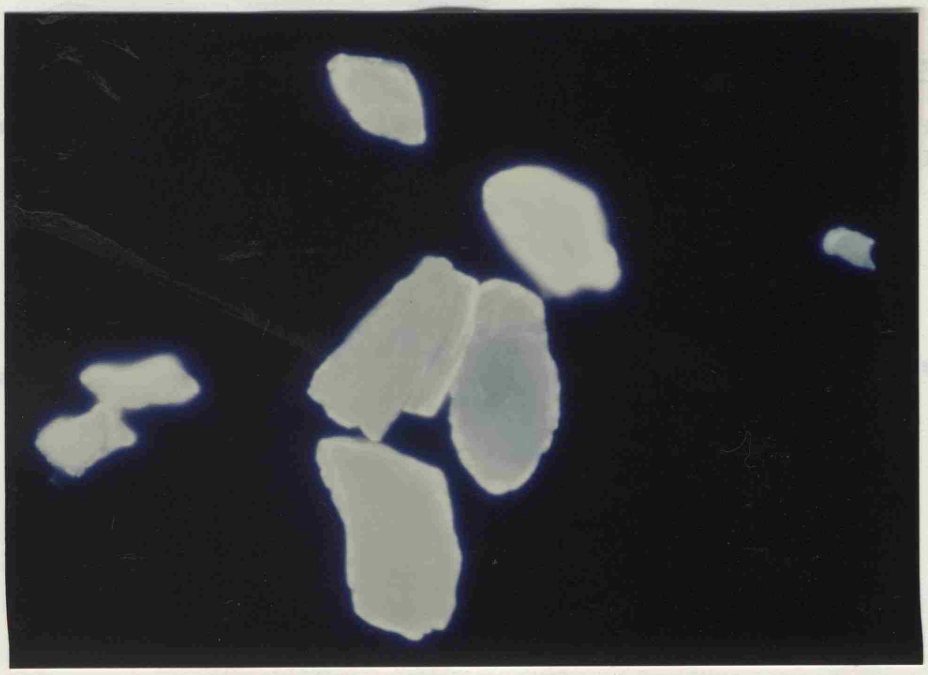


11

of chitin receptor sites available for binding to calcofluor or FITC-WGA. They also provide further evidence of the refractory nature of exuviae.

B. Activity of purified enzymes from *M. anisopliae* (MEl), alone and in combination on cuticle.

The digestion of cuticle *in vitro* by purified enzyme preparations from *M. anisopliae* (MEl)was carried out as described in Materials and Methods. Enzyme preparations were tested singly, in combination, or in sequence. Control reaction mixtures containing cuticle alone and enzyme in the absence of substrate were included in each experiment.

The enzymes used, purification and activities are listed below:

Enzyme	Growth media and purification	Activity
Alkaline protease	Casein (1%) medium. Ammonium sulphate precipitation fraction 60-100% saturation, iso-electric focussing pH 3-10 fractions 19-22 (Fig. 44).	40 µg trypsin equivalents ml^{-1}
Chitinase	Chitin (1%) medium (induction - Sec. I). Ammonium sulphate precipitation fraction 20-60% saturation. Sephadex G-100 column chromatography fractions 42-46 (Fig. 36). Adsorption on colloidal chitin. Dialysis against polyethylene glycol (MWT 20,000)	4 units ml^{-1}
NAGase	NAG (1%) medium. Ammonium sulphate precipitation fraction 20-60% saturation.	4.32 units ml^{-1}

Enzyme	Growth media and purification	Activity
NAGase (contd)	Sephadex G-100 column chromatography fractions 31-33 (Fig. 36). Dialysis against polyethylene glycol (MWT 20,000)	

C. Composition of amino acids solubilised by protease from *M. anisopliae* (ME1)

The major component of locust cuticle is protein (*c.* 75% w/w, see Sect. I. 2). *Prima facie* therefore endo-proteases likely to have a principal role in cuticle degradation. Of those produced by *M. anisopliae* (ME1) the alkaline protease (pI 9.5) appeared to have the greatest cuticle-degrading activity and so was used during this study.

As a first step in determining its mode of action on ground locust cuticle the gross amino acid composition of peptides released over a 24 h period was determined (see Materials and Methods): Table 29. Particular emphasis was placed on the alanine-glycine ratio of solubilised peptides as alanine and glycine are the principal amino acid components of hard (sclerotized) and soft locust cuticles respectively (Andersen, 1973).

At 5 h with *c.* 8.25% of the cuticular proteins solubilized the composition of the released peptides closely resembled that of the substrate cuticle (Table 29). However, in the interval between 5 and 24 h the alanine:glycine ratio changed considerably with soluble glycine increasing by *c.* 275% and alanine by only *c.* 200%

(percentages calculated from the results in Table 29). After 24 h *c.* 20% of the cuticular proteins had been solubilised.The addition of fresh protease hydrolysed a further 5-10% of the cuticle protein after 24 h. The residue that remained was refractory to additional enzymolysis. These results show that protein in fragments of both hard and soft cuticle is available for degradation. Once the available protein in hard cuticle has been removed the decrease in the alanine:glycine ratio suggests that selective enzymolysis of soft cuticle occurs until a residue of refractory exocuticle remains. This interpretation conforms with the results of Andersen *et al.* (1971b, 1973) who found that cuticle deposited on locust sclerites after ecdysis only gradually becomes hardened so that even after several days *c.* 10% remains unsclerotized and presumably susceptible to enzymolysis.

Water alone did not extract any peptides but aqueous buffer solutions in controls removed small quantities of all but one of the amino acid constituents of cuticle. It is well known that buffer solutions can extract ionically bound cuticular proteins (Hackman, 1980) and presumably released the peptides in a similar way. In composition the peptides in control solutions differed from those released by enzymolysis in possessing a high glycine to alanine ratio and an increased proportion of histidine.

The mean number of amino-acid residues in the solubilised peptides was estimated by determining free amino N with ninhydrin before and after hydrolysis with 6 N HCl (Lipke *et al.*, 1971a). Peptides liberated after 5 h with active protease averaged 4.7 residues in length compared

Table 29. Amino-acid composition (in number of residues per 100) of ground cuticle (I); peptides solubilised by *M. anisopliae* protease after (II); 5 hrs and (III) 24 hrs incubation, and(IV) peptides solubilised in controls after 24 h.

	Whole cuticle (I)	Solubilised peptides[a] (II) 5 h	(III) 24 h	Control[a] (IV)
Aspartic acid	4.0	3.6	4.2	3.9
Threonine	2.6	2.3	3.1	2.5
Serine	4.5	4.1	4.6	4.8
Glutamic acid	5.2	4.8	4.9	5.1
Proline	11.5	10.9	11.4	12.9
Glycine	16.5	17.2	17.5	20.2
Alanine	27.1	26.5	20.4	21.9
Valine	8.9	8.1	8.3	5.6
Isoleucine	2.8	2.7	3.2	2.3
Leucine	6.2	6.4	6.4	4.9
Tyrosine	2.8	4.4	4.5	4.0
Phenylalanine	1.2	1.2	2.3	0
Histidine	2.2	3.6	4.4	7.8
Lysine	1.7	1.4	1.9	1.0
Arginine	2.8	2.6	2.7	3.1

a) the concentration of amino acids released into the supernatants (alanine equivalents) in (II), (III) and (IV) were 2.29, 6.16 and 0.22 μmoles ml^{-1} respectively

with 1.87 in controls. The DP did not decrease during further incubation up to 24 h.

D. Effect of pretreating cuticle with different enzymes on subsequent cuticle degradation

Model studies testing the effects of commercial enzyme preparations on soft larval cuticle have indicated that chitinase is only effective in causing disintegration of cuticle if applied after protease (Samsinakova *et al.*, 1971; Smith and Grula, 1981). In addition when *M. anisopliae, V. lecanii* and *B. bassiana* are grown on insect cuticle the production of protease precedes that of induced chitinase (Sect. 1), indicating that chitin in the cuticle becomes available only after degradation of the protein. Evidence for synergism between cuticle-degrading enzymes of *M. anisopliae* (ME1)was therefore investigated as follows:

15 mg samples of cuticle (≃ 0.5 mm fragment size) were vigorously shaken at 30°C with 2 ml of Britton Robinson buffer, 1 ml of enzyme solution and 0.05 ml toluene. After 12 h 1 ml of fresh enzyme was added and incubated for a further 12 h. Controls contained autoclaved enzyme.

Reaction mixtures were centrifuged (2,000 g, 20 min) and the pellets washed in several changes of K phosphate buffer (pH 7.0, 0.2 M) and distilled water to remove enzyme and solubilised peptides. The residue was dried to constant weight at 70°C and stored at 4°C. The cuticle fragments (15 mg) were subsequently suspended in universal buffer and treated with other enzymes. The reaction rates on

enzyme-treated cuticle were compared with controls that had been incubated with autoclaved enzyme.

The sources of substrate were (i) exuviae and (ii) 3-5 d 5th instar larvae. These were used rather than cuticle from adults, as in contrast endocuticle in larval sclerites remains untanned so that only the future exuviae is sclerotized (Andersen, 1973). This allows a direct comparison to be made of enzymatic degradation of tanned (exuviae) and untanned cuticle. Enzyme pretreatments on larval cuticle and the effect on reaction rates of treatments are shown in Table 30 and Fig. 51.

Both protease and chitinase when added separately released significant amounts of degradation products from larval cuticle. NAG'ase alone showed no detectable activity over 24 h. Pretreatment with protease substantially enhanced the activity of chitinase so that chitinase solubilised approximately 10% of the cuticle by 22 h compared to only 2.5% released from cuticle preincubated with autoclaved protease. Release of amino sugars (determined by the method of Smith *et al.*, 1979) equalled production of NAG monomers (Reissig *et al.*, 1955) throughout, indicating that NAG was the sole reaction product. Repeated incubations (4 x 12 h) with fresh chitinase solutions indicated that the available chitin in 0.5 mm fragments amounted to 3-4 % of the cuticle (w/w). Chitinase was ineffective as a preliminary hydrolytic agent for gaining increased yields from the protease.

The exuviae was comparatively resistant to both proteolytic and chitinolytic attack. Pre-treatment with protease had no effect on subsequent chitinase activity.

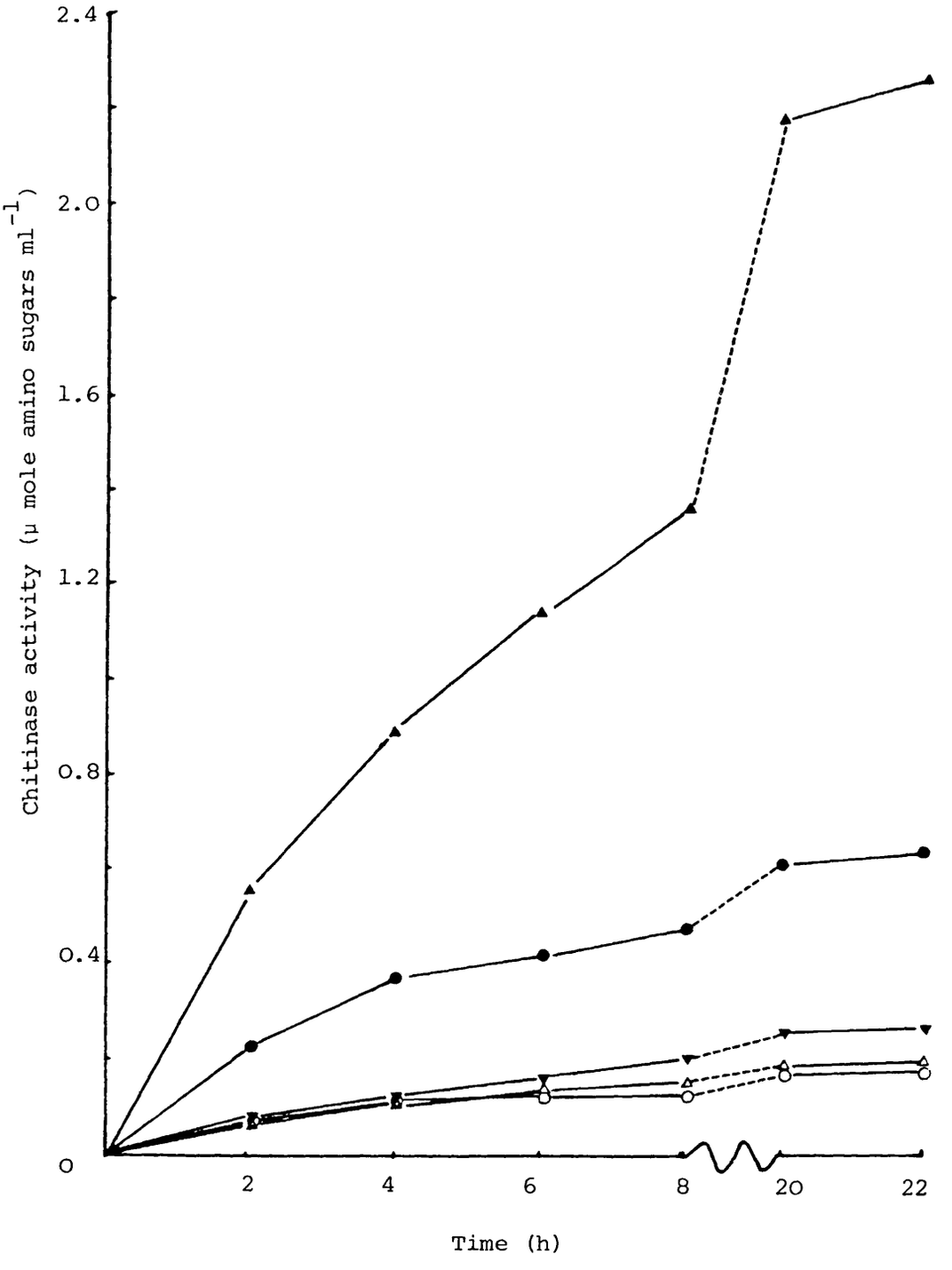


Fig. 51. Effect of pretreating exuviae (open symbols) and cuticle (closed symbols) with active protease (▲-▲); chitinase (▼-▼) or autoclaved protease (●-●) on subsequent degradation by chitinase of *M. anisopliae* (MEl). Each point is the mean of 3 replicates. The results are representative of 3 similar experiments.

Table 30. Comparison of reaction rates of enzymes on untreated cuticle and on cuticle partially degraded by the enzyme shown in Col. 1.

Enzyme pretreatment	Enzyme post-treatment	Effect of pre-treatment on rate of hydrolysis by post-treatment enzyme
Protease	Chitinase	increase
Protease + chitinase	Chitinase	increase
Chitinase	Chitinase	decrease
Chitinase	Protease	none
Protease	NAG'ase	none

Cuticle degradation was measured by release of amino acids (determined after acid hydrolysis (6N HCl) with ninhydrin) or amino-sugars (Smith *et al.*, 1979). Numerical values for some of these treatments are given in Fig. 51.

The effect of NAG'ase on cuticle was too small to be measured with accuracy either before or after treatment with protease. Nor could any synergistic effect be demonstrated between chitinase and NAGase; when tested in combination NAG release did not exceed the amount produced by chitinase alone (Table 31). In contrast when chitinase was combined simultaneously with protease the release of NAG was substantially increased (*c.* x5).

These results suggest that though the action of protease is not a prerequisite for the action of chitinase on *ground* cuticle (with chitin exposed at the cut edges, Sect. II, 5A), it does significantly enhance its activity, presumably by facilitating passage of chitinase to the chitin fibrils within cuticle. Additionally, the ability of chitinase to penetrate the cuticle is likely to be rate limiting as the enzyme binds rapidly to locust chitin (Sect. II, 9A).

The inability of either protease or chitinase to substantially degrade sclerotized cuticle has important implications in pathogenesis as it suggests that penetration of hard cuticle must be largely a result of mechanical pressure.

7. Degradative enzymes in ungerminated conidia

Cuticle-degrading enzymes are produced by germinating conidia in cultures containing host cuticle. These enzymes may have a role in the provision of nutrients during germination and in the early stages of penetration. It was decided to examine whether such enzymes were only produced during and subsequent to germination , or if they were already formed in ungerminated conidia; the latter case would strongly implicate them in the earliest stages of infection.

Table 31. Release of degradation products from cuticle treated with various enzymes separately and in combination for 5 h.

	Amino acids (μmole ml^{-1})	% cuticle degraded	Amino sugars (μmole ml^{-1})	% cuticle degraded
Protease	4.72 ± 0.56[a]	9.44	0.12 ± 0.034	0.53
Protease + chitinase	4.96 ± 0.63	9.92	0.62 ± 0.053	2.75
Chitinase	0.02 ± 0.004	0.04	0.42 ± 0.076	1.86
Chitinase + NAG'ase	0.02 ± 0.005	0.04	0.43 ± 0.082	1.90

[a]Mean values ± SE, N = 5. The results are representative of three similar experiments.

Hydrolytic enzymes have been detected in spores of fungal plant pathogens (e.g. Eberhart, 1961), and Soderhall *et al.* (1978) found protease in ungerminated and germinated spores of *Aphanomyces astaci*. However, the techniques used by Soderhall and his co-workers throws some doubts on the validity of their results (Charnley, 1984).

A. Release of enzymes from ungerminated conidia of *M. anisopliae* (ME1)

Conidia of *M. anisopliae* (ME1) collected from infected *Manduca sexta* larvae or from SDA plates were examined for enzymatic activity.

Several methods were tried to obtain dry spores which were free of mycelial contamination. The simplest and most successful involved applying an air stream on to heavily sporulating plates (14 d after inoculation) inverted over a filter paper in a buchner funnel connected to a vacuum pump. About 20 plates provided 1 g of spores by this means. Attempts to remove the numerous remaining spores from plates by suction or camel hair brushes resulted in significant mycelial contamination. Several strains of *M. anisopliae* (particularly RS324) produced spores which were too sticky to obtain completely free of mycelium without prior suspension and separation in aqueous media. This procedure may have leached enzymes from conidia or alternatively extracted enzymes present on mycelium or in the growth media. It is considered preferable to use dry spores particularly when enzymes might be located external to the cell membrane (Eberhart, 1961).

Conidia were harvested from *Manduca* larvae within 2 d of their appearance on the cuticle surface. Conidia had a low level of bacterial (*c.* 1-2 bacteria/spore) and mycelial contamination but the collective

volumes of contaminants was negligible in comparison with that of spores.

Conidia were examined for four enzyme activities: protease, carboxypeptidase, chitinase and NAG'ase (Table 32). NAG'ase was present in intact spores harvested from infected *Manduca* (Tables 32 and 33) and a large proportion of the activity was liberated on contact with water (Table 32). Far less NAG'ase was extracted from conidia from plates, which may reflect their (on average) older state (> 10 d) or be due to the different growth conditions experienced by the mycelium. After exhaustive washing of conidia from either source in water they still contained considerable NAG'ase activity which could be detected by suspending spores directly in the substrate, 0.1% *p*NP-β-N-acetylglucosaminide in Cit-PO_4 buffer (1 mM, pH 5.0) (Table 33). This activity was presumably either released by the weak buffer solutions or was bound to the cell surface by stronger bonds.

To determine the nature of the binding various chemical treatments were used to extract NAG'ase from spores derived from plates (Table 34). Substantially increased levels of NAG'ase over controls (washed in water) were obtainable by washing spores in 0.2 M K phosphate buffer which suggested some enzyme might be linked by electrostatic bonds. Tween 80 (1%) also released NAG'ase indicating that further enzyme was attached either on to membranes directly or internal to them (i.e. endocellular activity).

In contrast to NAG'ase, protease activity was similar irrespective of the source of conidia. Protease was extracted from plate spores by

water though this left considerable quantities of cell bound activity (Tables 32, 33 and 34). Liberation of enzyme was slightly increased (*c.* 130%) by washing in dilute (0.02 M) or concentrated (0.2 M) buffer indicating some weak ionic binding. Tween 80 released more protease than did the buffer suggesting the involvement of membranes in the binding or containment of some of the enzyme.

Mercaptoethanol (in 0.02 M buffer) did not release substantially more NAG'ase or protease than unamended buffer solutions (Table 34) indicating that neither enzyme was bound by disulphide bonds.

The assays for chitinase and carboxypeptidase were not sensitive enough to accurately determine the small amounts of enzyme involved. Incubation periods had to be extended over several hours to demonstrate that trace activities were present (Table 32); the slight degradation of colloidal chitin may have been caused by NAG'ase rather than chitinase activity. The low levels or absence of chitinase in spores may reflect its inducible character. Unfortunately sporulation on chitin agar medium was too poor for comparative experiments with spores from SDA.

Presumably conidial walls like those from hyphae contain both chitin and protein, therefore the cell-bound enzymes identified in this study may have been left over from the process of sporulation. The association of wall lytic enzymes and sporulation has been well documented in fungi (Bergkvist, 1963a,b; Chen *et al.*, 1968; Hagihara, 1960). In addition several groups of workers have suggested that lytic enzymes, including proteases, may be important in germination of bacterial and fungal spores (e.g. Sierra, 1967; Leighton and

Stock, 1970). However the incorporation of PMSF (1×10^{-4} M), an effective inhibitor of the extracellular alkaline protease (see Table 20), failed to inhibit either germination or growth in a medium containing 1% NAG/basal salts. If this selective protease inhibitor had prevented germination it could have indicated the importance of proteolysis.

Control experiments were conducted in which spores were suspended in casein solutions and enzymolysis compared with and without added PMSF. 71% inhibition of proteolysis occurred compared with 84% inhibition of cell-free enzyme (culture filtrate) indicating that the protease may have received some slight protection within cell walls. This residue of proteolytic activity retained by spores may have been sufficient for its role, if any, in germination. In addition, under appropriate conditions *M. anisopliae* (ME1) produces at least one other endo-protease (pH opt. 5.5) and two exo-peptidases which could potentially participate in germination.

B. Distribution of enzymes in preparations of disrupted conidia

After disruption of conidia by carborundum and sonication (Materials and Methods) enzymes were found in both the soluble fraction and attached to cell walls (Table 35). In contrast with growing hyphae (Sect. II, 7B) there was little difference in the relative distribution of protease and NAG'ase - about 40% of both activities were wall-bound.

8. Location of cuticle-degrading enzymes on hyphae

Ultrastructural evidence suggests that enzymic degradation of

Table 32. Release of enzymes from ungerminated spores of *M. anisopliae* (ME1) collected from infected *Manduca* (A) or SDA plates (B).

		Protease (µg trypsin equivalents ml^{-1})	Carboxypeptidase (n moles PA $ml^{-1}h^{-1}$)	Chitinase (nmoles NAG $ml^{-1}h^{-1}$)	NAG'ase (n moles PNP $ml^{-1}h^{-1}$
A	Water washed	0.04	Tr	2	48.51
	Buffer washed	0.06	Tr	2	42.76
B	Water washed	0.02	0	0	0.36
	Buffer washed	0.03	Tr	Tr	1.62

Conidia (200 mg) were washed by successive centrifugation in distilled water (15 mls) and K phosphate buffer (15 mls, 0.2 M, pH 7.0). The supernatants were concentrated (x8) in collodian tubes embedded in polyethylene glycol (M.Wt. 20,000) and used as the sources of enzyme activity. Tr = trace.

Table 33. Direct measurement of spore bound enzyme

Source of conidia:	*Manduca* larvae	SDA plates
Enzyme		
Protease[a)]	39	21
NAG'ase[b)]	82	8

a) Water-washed spores (20 mg) were incubated in 3 ml 1% buffered casein (1 mM, K phosphate, pH 7.0). After 1 h incubation TCA soluble products were determined with ninhydrin (see Materials and Methods). Results are expressed in nmoles $ml^{-1}h^{-1}$.

b) Water-washed spores (20 mg) were suspended in 3 ml of Cit-PO_4 buffer (1 mM, pH 5.0) containing PNP-β-N-acetylglucosamine (1 mg/ml). Colour development was as described in Materials and Methods.

Results are expressed in nmoles $ml^{-1}h^{-1}$.

Table 34. The effect of various extraction media on release of bound enzymes from ungerminated spores.

Extraction media	Protease (%)		NAG'ase (%)	
1) H_2O	100		100	
2) K phosphate buffer (0.02 M, pH 7.0)	129		220	
3) K phosphate buffer (0.2 M, pH 7.0)	133		409	
4) Buffer (0.02 M, pH 7.0) plus Tween 80 (1%)	171	0.51 µg[a] trypsin equivalents	506	4.09nmoles[a]
5) Buffer (0.02 M, pH 7.0) plus 2-mercaptoethanol (0.02M)	130		265	

a) Maximum activities expressed as enzyme units per 200 mg spores.

Table 35. Distribution of enzymes in preparations of disrupted conidia.

Source of conidia	Manduca larvae		SDA plates	
Enzyme:	Protease (µg trypsin equivalents $ml^{-1}h^{-1}$)	NAG'ase (nmoles PNP $ml^{-1}h^{-1}$)	Protease	NAG'ase
Soluble	0.12 (57.14%)	98.64 (60.75%)	0.10 (58.82%)	3.32 (54.84%)
wall-bound	0.09 (42.86%)	63.72 (39.25%)	0.07 (41.18%)	2.73 (45.16%)

Conidia (200 mg) were suspended with 20 mg Carborundum (600 mesh) in 20 ml distilled water and sonicated at 8 ± 3°C for 45 min. The pellet was washed by successive centrifugations in distilled water (5 x 15 mls) and incubated for 1 h in 15 ml K phosphate buffer (0.2 M, pH 7.0) to extract wall-bound enzymes. Supernatants were concentrated (x5) in collodian tubes embedded in polyethylene glycol (MWT 20,000) and used as sources of enzyme activities.

Tables 32 to 35. The results are the means of at least 3 replicates. Each experiment was repeated three times using spores from fresh plates.

insect cuticle by invading fungi is rather localised (Zacharuk, 1970c). That this apparent immobilisation of pathogens extracellular enzymes might be accounted for by binding of enzymes to the fungal cell-wall has not been previously considered. However, binding of enzymes to hyphae is a well documented phenomenon amongst saprophytic and plant pathogenic fungi (Lisker *et al.*, 1975; Eberhart *et al.*, 1970; Polacheck and Rosenberger, 1978; Barash and Klein, 1969). Location of activity and the nature of binding can be established by assay of culture fluids and by various chemical treatments to extract enzymes from hyphae. The techniques involved include incorporation of high levels of salts to desorb ionically-bound protein, mercaptoethanol to release enzymes covalently linked by disulphide bonds and solubilisation with detergents (Barash *et al.*, 1969; Polacheck *et al.*, 1978; Cooper *et al.*, 1981; Lisker *et al.*, 1975).

A. Release of cell-bound protease and NAG'ase from mycelium of *M. anisopliae* (ME1)

Release of cell-bound enzymes by various chemical treatments is summarised in Table 36. Attachment of both enzymes to pure cell walls (prepared as described in Materials and Methods) appeared to be by ionic bonds; these were relatively weak as low ionic concentrations (0.02 M) were almost as effective as high levels (0.2 M) in releasing enzyme.

With intact mycelium detergents caused a relatively small release of protease, though the anionic detergent sodium dodecyl sulphate (SDS) and the cationic detergent Cetyltrimethylammonium bromide (CTAB) released more than the neutral detergent Tween 80 (T80). However, detergents, particularly SDS and CTAB had a very pronounced

effect on NAG'ase releasesuggesting that a large part of the total cell bound enzyme was attached to membranes (probably by non-covalent linkages to charged lipids) or was contained within membrane structures. NAG'ase was also released by the sulphydryl reagent mercapto-ethanol, therefore some of the enzyme may also be bound to cells by S-S bonds (however see Sect. III, discussion).

The effect of washing whole mycelia with increasing buffer concentrations is shown in Fig. 52. Release of protease increased with increasing buffer concentration up to 0.2 M. Higher concentrations were required to release an equivalent proportion of NAG'ase which suggests it is the more tightly bound of the two enzymes. The liberation of enzymes did not apparently require *de novo* protein synthesis or active transport across cell membranes as neither cyclohexamide (30 µg/ml) nor the metabolic inhibitor sodium azide (10^{-3}M) altered the release. Some liberation may have been attributable to osmotic effects, as glucose (0.1 M) resulted in more protease (122%) and NAG'ase (152%) than water controls, though this was less than the activities released by 0.05 M KCl (Table 36).

B. Distribution of enzymes in disrupted hyphae of *M. anisopliae* (MEl)

After disruption of growing hyphae (from 3 d 1% NAG/basal salts cultures) enzymes were found in both the soluble fraction and attache d to cell walls (Table 37). However there was a substantial difference in distribution of the two enzymes; NAG'ase showed a higher percentage (13.21%) of bound to total enzyme than protease (2.86%). Enzymes released from cell walls (the wall bound fraction) with K phosphate buffer (0.2 M, pH 7.0) had a markedly higher specific activity than enzymes in the soluble fraction presumably because

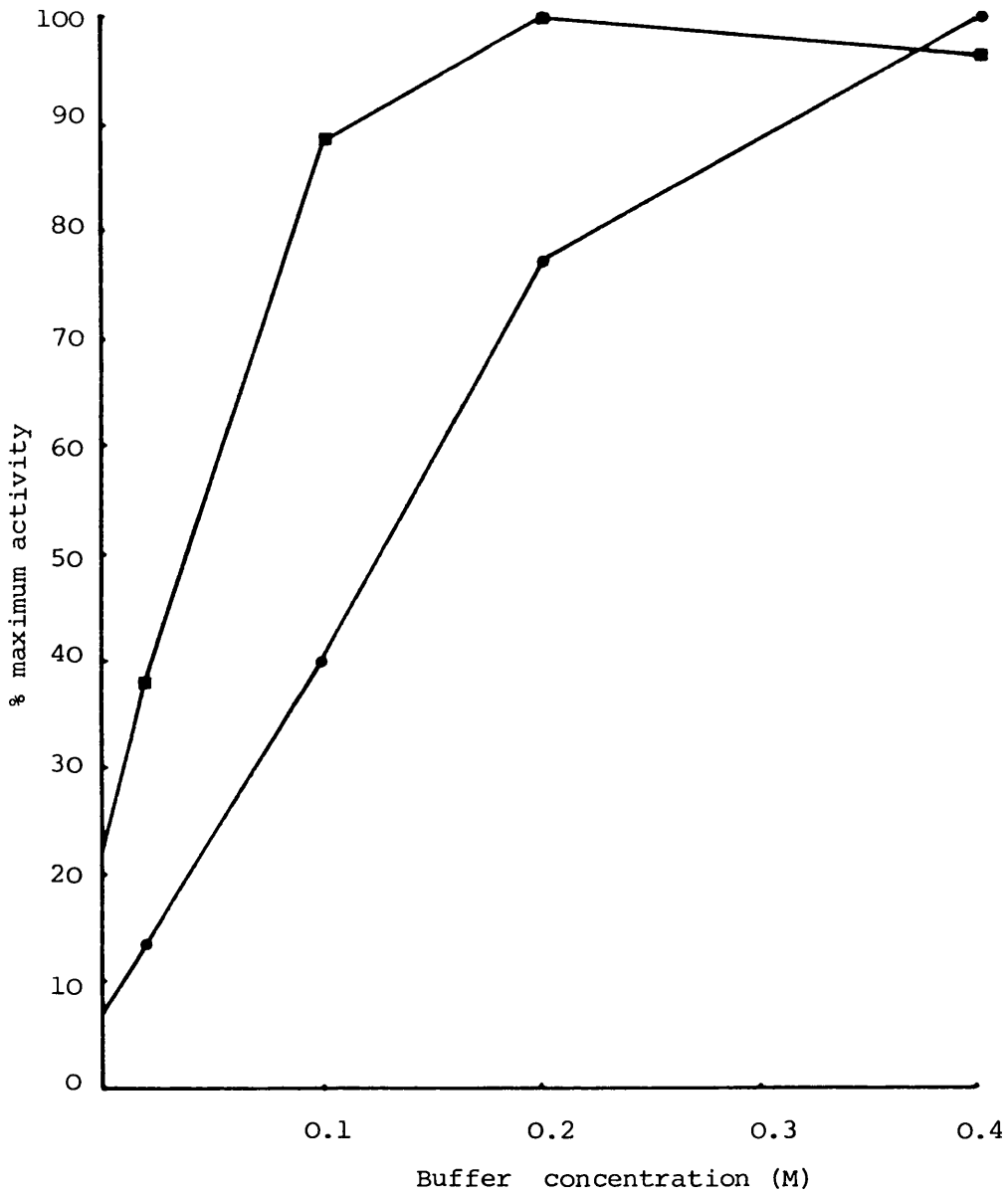


Fig. 52. Effect of K phosphate buffer concentration (pH 7.0) on release of enzymes from mycelium of *M. anisopliae* (MEl). (■—■) protease (opt. pH 8.2), 13.12 OD_{595} h/lOO mg d.w. (●—●) NAG'ase, 117.4 nmoles PNP released $ml^{-1}h^{-1}$ lOO mg. d.w.

Each point is the mean of 3 replicates. The results are representative of three experiments.

Table 36. The effect of chemical agents on release of enzymes from mycelium and pure cell walls of *M. anisopliae* (ME1)

A) Intact mycelium

Extraction medium	Protease		NAG'ase	
	released enzyme[a]	effect on soluble enzyme activity[b]	released enzyme[a]	Effect on soluble enzyme activity[b]
H_2O	100	100	100	100
T_{80} 1%	119	93	391	102
SDS 0.2%	143	97	2,190	93
CTAB 0.2%	164	89	4,014	86
M 0.02 M	116	93	2,570	103
Glucose 0.1 M	122		152	
KCl 0.05 M	154		216	
B (0.02M, pH 7.0)	165		208	
B " " + T_{80} 1%	167		466	
B " " + SDS 0.2%	178		2,342	
" " " +CTAB 0.2%	200		4,324 (386.57 nmoles PNP)	
" " " +M 0.02M	155		2,624	
" (0.2M, pH 7.0)	465 (0.32 µg trypsin equivalents)		1,085	

/contd...

Table 36 continued

B) Pure cell walls

Extraction medium	Protease released	NAG'ase released
H_2O	100	100
B (0.02 M, pH 7.0)	169	705
B (0.02 M, pH 7.0) + CTAB 0.2%	163	698
B (0.02 M, pH 7.0) + Me0.02 M [d]	161 (1.09 µg trypsin[c] equivalents)	739
B (0.2 M, pH 7.0)	189	972 (1731.8 nmoles[c] PNP)

B = K phosphate buffer, T_{80} = Tween 80, SDS = sodium dodecyl sulphate, Me= 2-mercaptoethanol, CTAB = cetytrimethylammonium bromide.

a) Percentage enzymatic activity as compared with that in mycelium shaken with distilled water (100%) (Each result is the mean of at least 5 replicates).

b) The effect on enzyme activities and assay sensitivity of the chemical agents was measured by comparing the activity of enzyme solutions after incubation with or without the agents for 30 min.

c) Maximal activities expressed as enzyme units per 100 mg dry wt. fungal matter.

d) 2-Mercaptoethanol was tested only once against pure cell walls, all other treatments were repeated at least three times.

Mycelium (1.4 g) from 3 d 1% NAG cultures was suspended in distilled water and sonicated. The pellet was washed by 2 successive centrifugations in distilled water and the combined supernatants used for an estimate of soluble enzyme. The pellet was washed finally with K phosphate buffer (0.2 M, pH 7.0) for 5 h to give an estimate of cell-bound enzyme. Supernatants were concentrated (*c.* x5) in collodian tubes embedded in polyethylene glycol (MWT 20,000) and used as sources of enzyme activity. Enzyme units are expressed as total enzyme present in supernatants or culture fluid. The results are representative of 3 similar experiments.

Table 37. Distribution of bound and extracellular protease and NAG'ase activities in disrupted mycelium

Fraction	Total enzyme				Specific activity	
	NAG'ase (µmoles PNP)	% Total	Protease (µg trypsin equivalents)	% Total	NAG'ase	Protease
Wall	256.0	13.2	9.3	2.9	189.7	6.8
Soluble	721.3	37.2	92.3	28.8	20.2	2.6
Extracellular (culture medium)	961.0	49.6	218.4	68.3	102.8	23.4

the latter contained high levels of endocellular proteins.

C. Origin of wall-bound enzyme

It is possible that at least part of the enzyme in the wall fractions is an artifact of hyphal breakage and subsequent binding. To test whether soluble enzymes will bind to hyphae, a lyophilised finely ground preparation of walls was exhaustively treated with K phosphate buffer (0.2 M, pH 7.0) and compared with untreated walls for ability to bind extracellular enzymes from a chitin culture. As shown in Fig. 53, the presence of cell walls reduced soluble enzyme activities (Column B) particularly when the walls had been treated to remove ionically bound proteins. Furthermore the activity could be recovered by washing with buffer (Column C) indicating that activities were ionically bound to the cell wall fragments. The fact that isolated cell walls had a greater affinity for soluble protease than NAG'ase, yet a higher proportion of the total NAG'ase was cell-bound (Table 37) suggests that the distribution of enzymes shown by Table 37 is due to an initial unequal distribution of enzymes in cells rather than being merely an artifact of hyphal breakage.

The affinity of cell walls for enzymes raises the possibility that *in vivo* ionically bound enzymes may result from binding of extracellular enzymes to wall components, assuming that sites are available on wall surfaces. However it was demonstrated in a series of experiments that extracellular enzymes are not a prerequisite for cell bound activity.

It had been found in experiments on the regulation of protease synthesis that it was possible to suppress extracellular production

completely by growing mycelium in a simple medium containing a carbon source such as glucose and high levels of NH_4Cl. To determine whether bound enzymes were also repressed, mycelia grown for 3 d in a medium containing glucose (1.5%) and NH_4Cl (0.3%) were transferred either to identical (complete) media or incomplete media lacking a nitrogen source (i.e. de-repressing). After 8 h in the incomplete medium the pathogen produced both cell-free and bound protease (0.32 OD_{595} $h^{-1}mg^{-1}$). In the complete medium, although no extracellular protease was produced, low levels of ionically bound enzyme were present (0.12 OD_{595} h^{-1}mg dry $wt.^{-1}$). This demonstrates that cell bound activity does not exclusively derive from extracellular protease. Extracellular and cell bound NAG'ase activities were found in both culture conditions.

One possible explanation for this result is that production of the extracellular and bound protease fractions are under different regulation. If this were the case levels of bound enzyme could conceivably be correlated with some other criterion such as mycelial dry weight. This was tested using growth media containing various levels of alanine which partially repressed the production of extracellular protease, while simultaneously enhancing the growth rate. Activities of cell bound fractions of both enzymes were correlated with extracellular enzyme levels rather than with amounts of mycelium (Fig. 54).

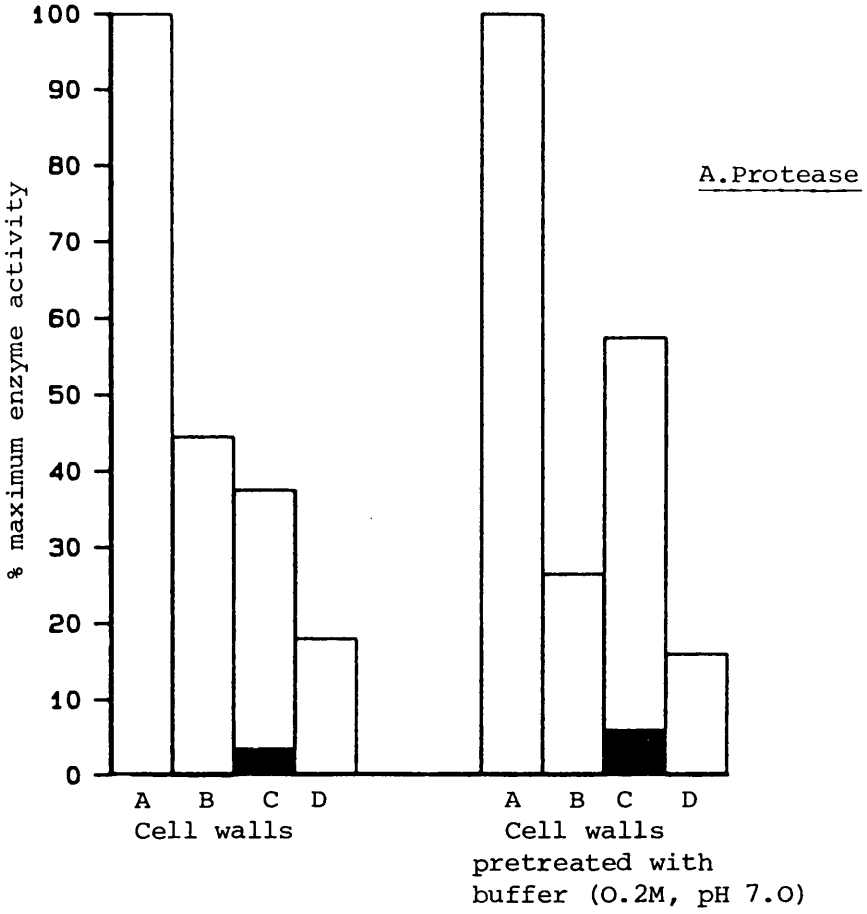
In the third experiment the effect of repeated washings of mycelia and cell walls was tested (Fig. 55). Washing cell walls with K phosphate buffer (0.2 M, pH 7.0) at $4^{o}C$ for 2 h extracted all

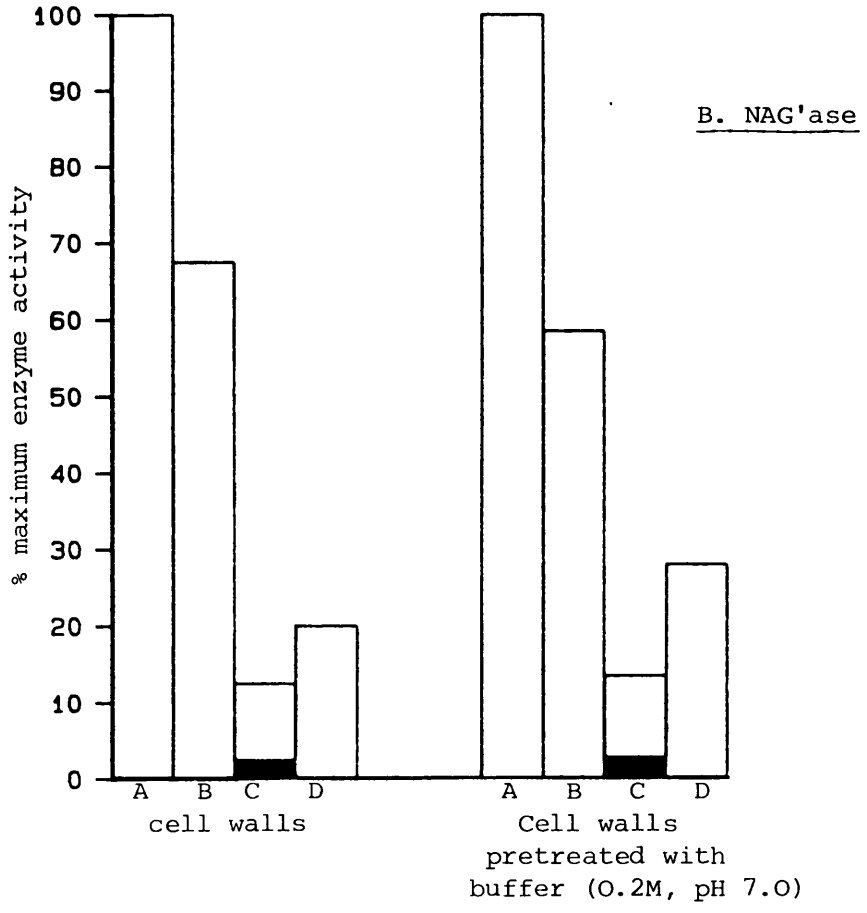
Fig. 53. Binding of protease and NAG'ase by cell walls isolated from *M. anisopliae* (MEl).

Ordinate: Column (A): activity of control enzyme solution (100%). Column (B); percentage soluble enzymatic activity remaining after 10 min incubation of control enzyme (2 ml) with 50 mg freeze-dried ground cell walls. The lower shaded section of Column (C) shows the activity of enzyme released from cell walls by washing in water, the upper section of the column represents activity of ionically bound enzyme desorbed from washed walls by incubation for 15 min with K phosphate buffer (0.2 M, pH 7.0). Column (D): column A minus combined activities of (B) and (C) represents inactivation or non-ionic binding. Enzymes obtained from *M. anisopliae* (MEl) grown on chitin-salts medium were dialysed and diluted so that activities used were (A) protease (2.8 µg trypsin units) (B) NAG'ase (0.54 units). Units described in Materials and Methods.

The experiment was repeated three times with similar results. Fresh enzyme and wall preparations were used on each occasion.

Fig. 53.


100
90
80
70
60
50
40
30
20
10
0
% maximum enzyme activity
A.Protease
A B C D
Cell walls
A B C D
Cell walls pretreated with buffer (0.2M, pH 7.0)


100
90
80
70
60
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40
30
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0
% maximum enzyme activity
B. NAG'ase
A B C D
cell walls
A B C D
Cell walls pretreated with buffer (0.2M, pH 7.0)

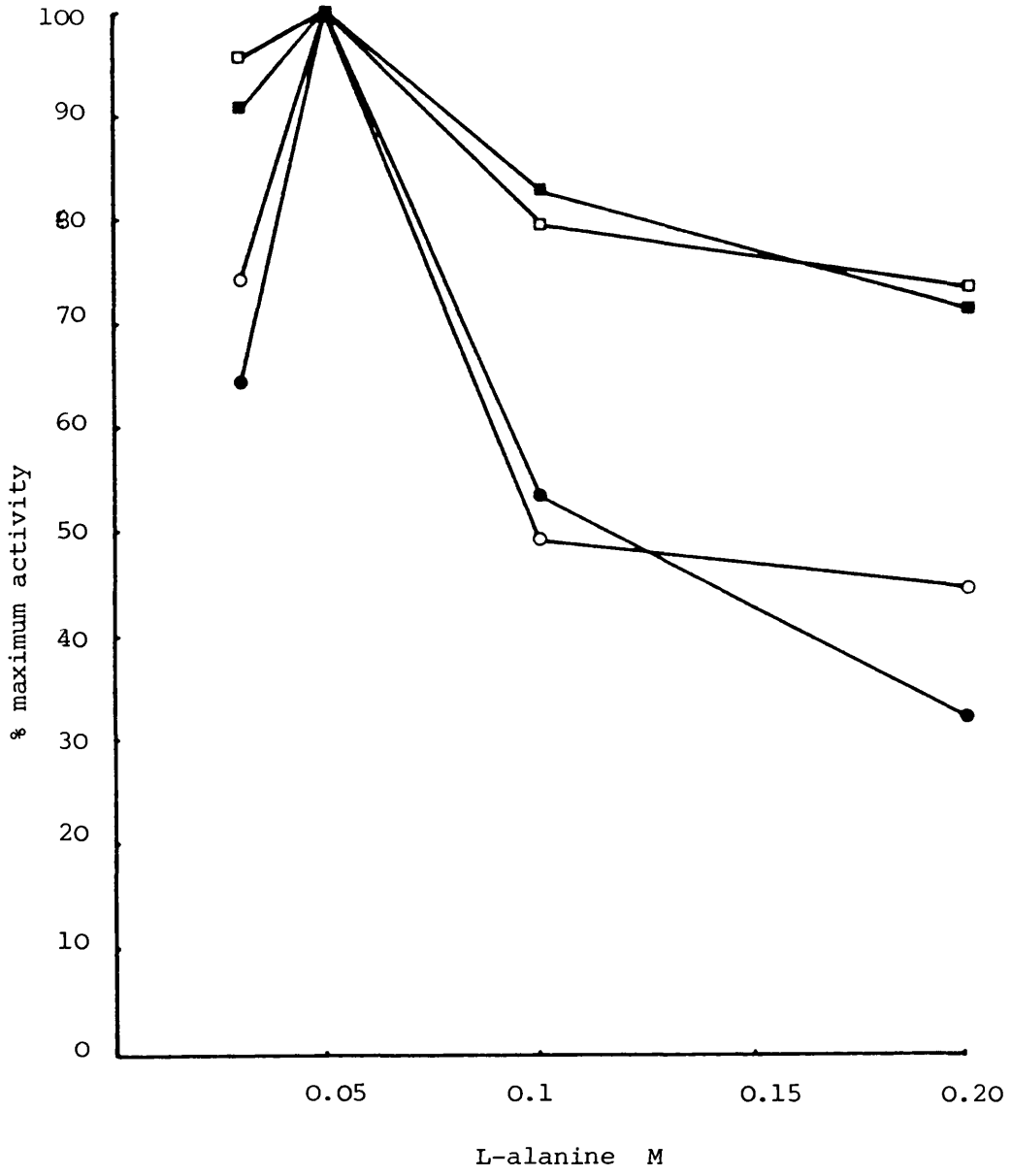


Fig. 54. Production of extracellular and bound protease (pH 8.2) and NAG'ase by *M. anisopliae* (ME1)grown in basal salts medium containing different concentrations of L-alanine. Maximum enzyme levels expressed as activity per mg d.w. of fungi are (■—■) extracellular NAG'ase (18 nmoles $ml^{-1}h^{-1}$), (□—□) bound NAG'ase (15.7 nmoles $ml^{-1}h^{-1}$), (●—●) extracellular protease (1.1 OD_{595} $ml^{-1}h^{-1}$) (○ ○) bound protease, 0.29 OD_{595} ml h^{-1}). Each point is the mean of 3 replicates. The results are representative of 3 similar experiments.

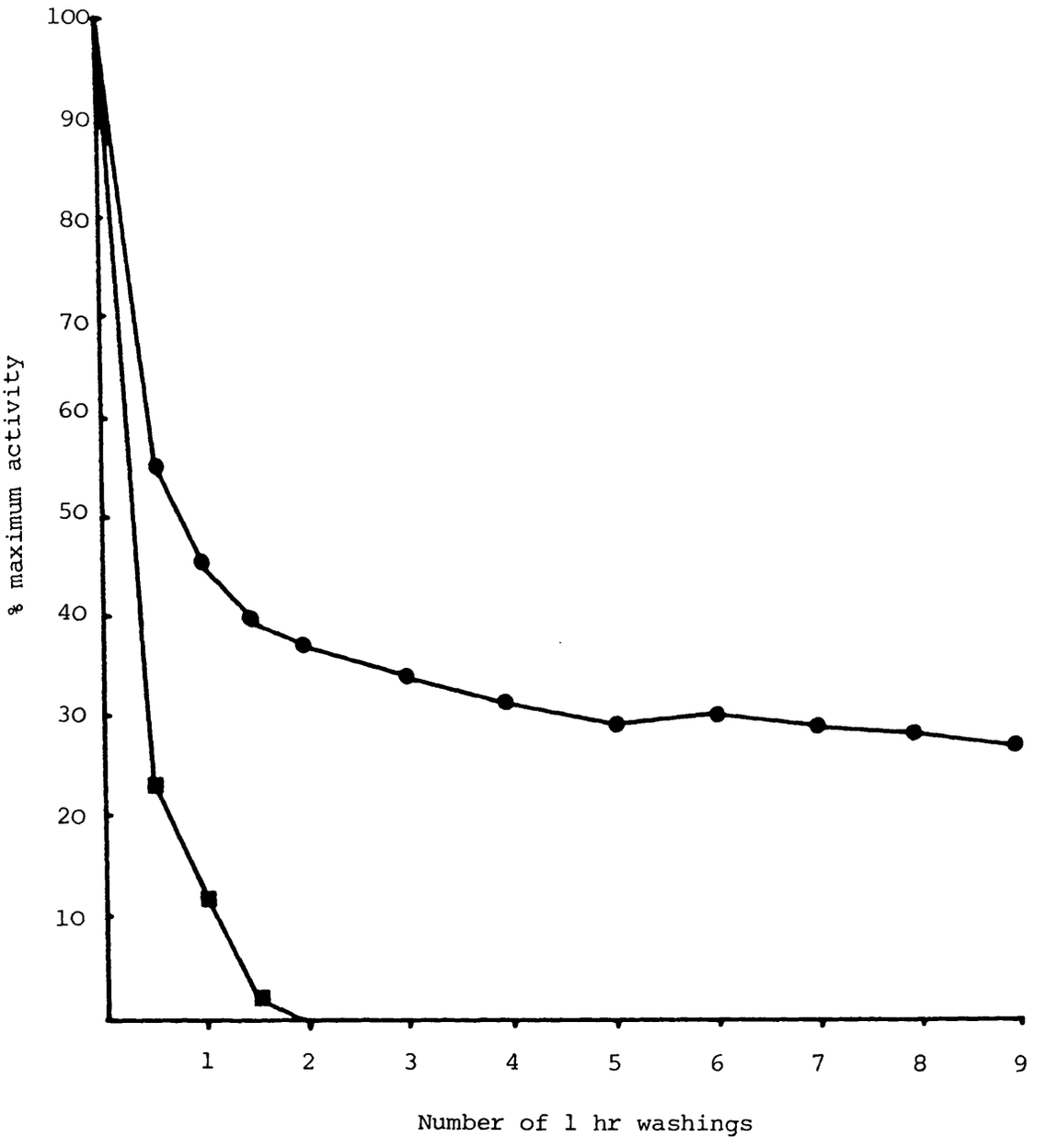


Fig. 55. Effect of number of washings by K phosphate buffer (0.2 M, pH 7.0) on protease released from mycelia (●—●) and isolated cell walls (■—■).

Each point is the mean of three replicates. The results are representative of 2 similar experiments.

available protease. However protease continued to be released at a slow rate from mycelium even after washing for 24 h. This suggests that enzyme released from the wall was slowly being replaced by pre-formed protease inside the cell. The process appeared to be passive and did not require *de novo* protein synthesis, as neither Na azide nor cyclohexamide affected the pattern of release. A similar pattern of continuous release by mycelia has been observed with bound cellulase in *Rhizoctonia solani* (Lisker *et al.*, 1975).

In conclusion it appears that even under repressing conditions, basal levels of protease are produced which is not released but attaches ionically to sites in the cell wall. When conditions allow, synthesis of large quantities of protease eventually saturates these sites and allows the release of enzyme to an extracellular location.

D. Autolysis of purified cell-walls

It was found previously (Sect. I.6A) that crude chitinase hydrolysed chitin in disrupted mycelia. An experiment was conducted therefore to see whether in common with some other microorganisms (e.g. Polacheck and Rosenberger, 1974) wall-bound enzymes could cause autolysis of isolated walls. It was found that purified walls released amino-acids, reducing sugars, and amino sugars when incubated at 30°C in 1 mM K phosphate buffer (pH 6.5) (Fig. 56). However the amounts liberated were very small. It was calculated from the levels of reducing sugars and amino acids released that after 9 h only 0.35% (dry/wt) of the cell wall had been solubilised. No soluble compounds were released from walls heated at 80°C (10 min).

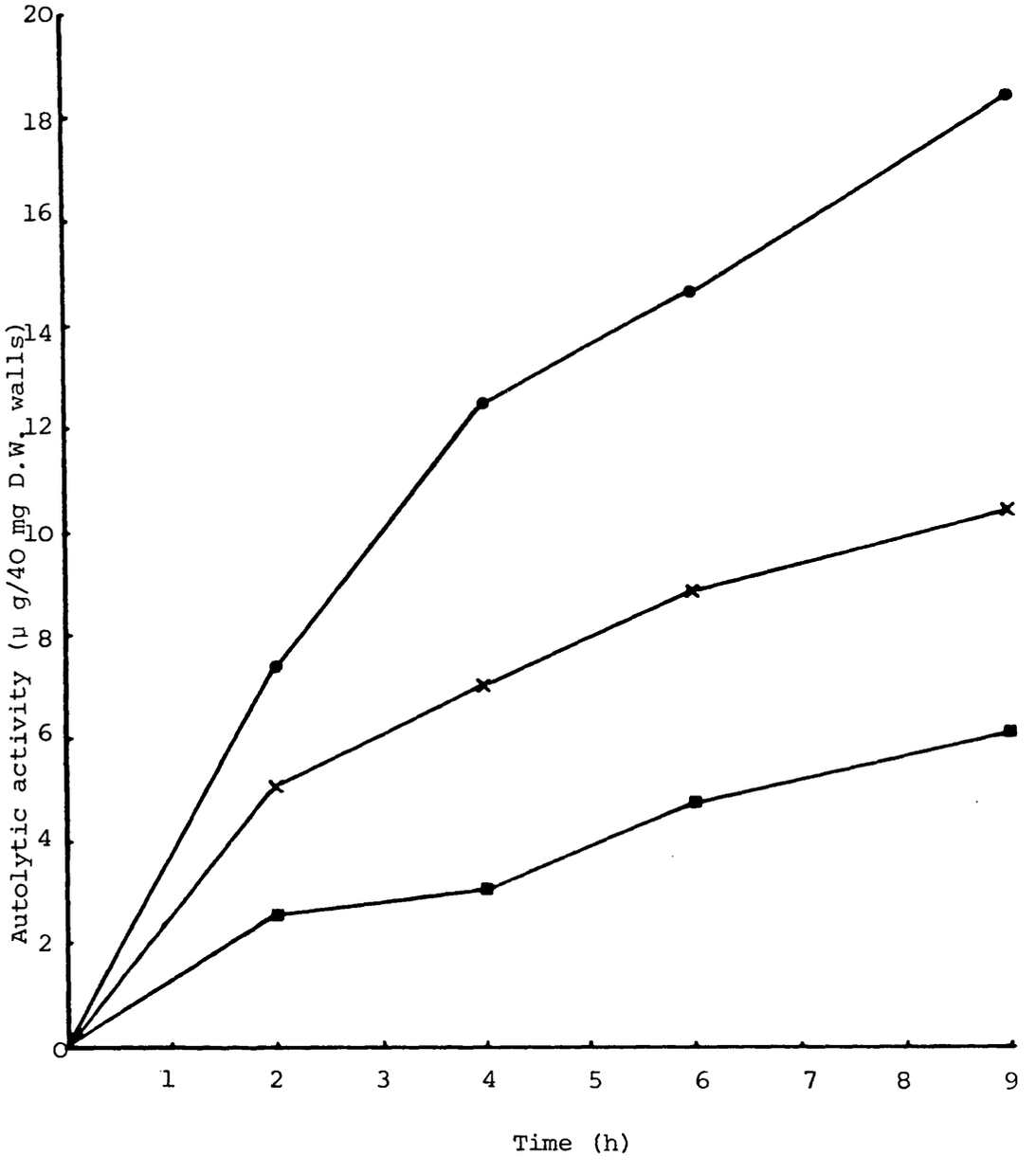


Fig. 56. Autolytic activity associated with cell walls (40 mg) suspended in 4 ml K phosphate buffer (0.01 M, pH 7.0). (×) amino sugars (Smith *et al.*, 1979); (●) reducing sugars (Nelson, 1944); (■) amino acids (ninhydrin). Each result is the mean of three replicates. The experiment was repeated 3 times (using different wall preparations) with similar results.

The reaction products of autolysis demonstrate that wall-bound enzymes can digest both protein and carbohydrate wall components; presumably therefore they may have a function in modifying wall structures during growth as suggested for *Neurospora crassa* (Mehta and Mahadevan, 1975).

9. Catalase and Peroxide formation

In Sect I. it was reported that all the pathogens produced an enzyme with peroxidative activity against pyrogallol but not with guaiacol. From this it was concluded that the enzyme was a catalase. Catalase is generally involved in the protective destruction of H_2O_2 generated during cellular metabolism, ionising radiation and by the dismutation of superoxide radicals (Chance and Oshino, 1971; Masters and Holmes, 1977). Although there have been several previous attempts to detect H_2O_2 formation by fungi (Lyr, 1956; Lyr, 1958; Lyr and Ziegler, 1959) only one has been successful (Koenigs, 1974). He found that H_2O_2 production by wood-rotting fungi may be involved in degradation of polysaccharides in secondary walls. As H_2O_2 is a vigorous reagent and potentially reactive against numerous host components including cuticle polymers, an attempt was made to determine whether free extracellular peroxide is produced by entomopathogens.

Extracellular production of H_2O_2 was detected by growing several strains on a heated haemoglobin medium (HbA) (Materials and Methods). Variations of this media have been used successfully in several studies on bacteria (e.g. Whittenbury, 1964) and in Koenig's (1974) study of wood rotting fungi. Peroxide production was inferred when a green discolouration developed beyond or under colonies growing on

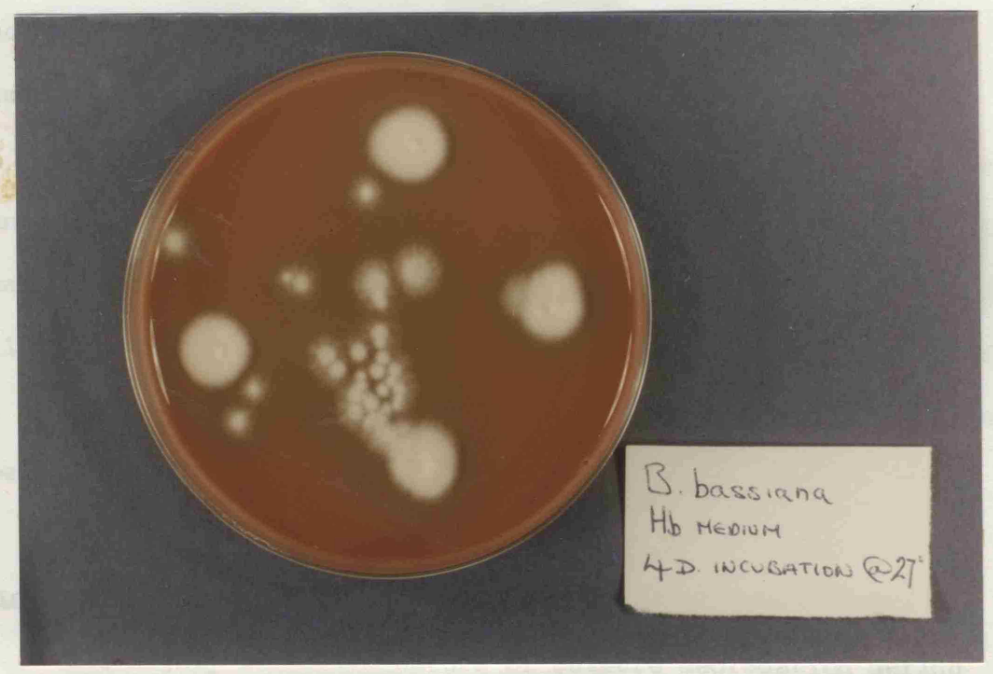
the HbA (Gordon *et al.*, 1953; Koenigs, 1974). On this basis *B.bassiana* and *V. lecanii* produced H_2O_2 within 2 d of inoculation. Of the isolates of *M. anisopliae* tested MEl, RS549, RS23 and RS298 produced H_2O_2 by 3 d while RS324 showed no reaction until 4 d. Except for RS324 and RS549, all isolates produced reaction zones outside colony margins (Table 38). *V. lecanii* and *B. bassiana* were particularly high producers of H_2O_2 (Plate 54).

To investigate the validity of assuming the green discolouration was due to H_2O_2 production the effect of peroxide on Hb spectrum (360 nm to 660 nm) was compared with the spectrum for Hb utilised in growth media for MEl, *B. bassiana* and *V. lecanii* (see Materials and Methods). Both H_2O_2 and the pathogens lowered the soret (405 nm) and methemoglobin (630 nm) peaks while no new peaks were found at 618 nm (sulfhaemoglobin) or 610 nm (alkaline haematin). These results confirm the effects of H_2O_2 on Hb described by Koenigs (1974) and confirm that the oxidant is produced by the pathogens.

A. Production of Catalase

The ability of the pathogens to produce catalase on Hb was recognised visually by effervescence on the addition of H_2O_2 (10 vol) to mycelia. No effervescence occurred on adding H_2O_2 to Hb media which rapidly turned green. All the pathogens possessed H_2O_2 splitting activity, which probably serves to protect the fungi from the toxic effects of peroxide. The catalase presumably remains in the vicinity of the mycelium by being at least partially endocellular or wall-bound. Further protection could be derived from having the enzymes responsible for H_2O_2 production (mostly oxidases) external to the

Plate 54. H_2O_2 production by *B. bassiana* grown for 4 d at 27°C on Hb media. Green reaction zones indicate peroxide formation.



plasma membrane. There are reports in some fungi of extracellular glucose oxidases (Gancedo *et al.*, 1967) which could account for the extracellular location of H_2O_2.

B. Degradative action of H_2O_2

As H_2O_2 can degrade several polymers including cellulose (Halliwell, 1965) and pectin (Whistler, 1959), a preliminary study was conducted on its effects on cuticle and cuticle components. Reaction mixtures comprised 4 ml H_2O_2 (0.05%), 4 ml 0.2N sodium acetate - 0.2 N acetic acid buffer (pH 4.2) (Halliwell, 1965) containing 5 mg substrate and 0.2 ml water or 10 mM $FeSO_4$. Over a 24 h period (30°C) the turbidity of suspensions of colloidal chitin and cuticle remained constant which suggested no significant solubilisation had taken place. However with iron as a catalyst, melanin (either alone or complexed with chitin) was dissolved and bleached within 1 h. In the absence of iron the reaction was much slower with melanin being dissolved but not bleached after 12 h.

10. Interactions between host cuticle and pathogen enzymes

The highly localised nature of cuticle degradation during penetration (Zacharuk, 1970c) may be related not only to the biochemistry of the pathogen's enzymes and cell wall, but also to the ability of the insect cuticle to inhibit enzyme activities or to limit their diffusion through the cuticle.

The similarities between fungal cell walls and cuticle (both are composite structures containing protein and chitin) and the affinity of the former for pathogen enzymes suggested a study to

Table 38. Production of H_2O_2 by entomopathogens on bovine haemoglobin agar (HA). 6 d after inoculation. 3 replicates/ test.

Reaction ratio: $\frac{\text{diam. of colony + green halo}}{\text{diam. of colony}}$

Species	Growth (mm)	H_2O_2 production (reaction ratio)
B. bassiana	16.7	2.56
V. lecanii	7.2	3.24
M. anisopliae ME1	12.9	1.48
RS324	11.0	1
RS549	24.8	1
RS298	15.0	1.87
RS23	22.6	1.19

determine whether restriction of enzyme activity could result from immobilisation by components of insect cuticle. In addition to this possibility some components of insect cuticle such as quinones can inhibit many enzymes (Hoffman-Ostenhof, 1963), and even the polymerised form, melanin, may inhibit microbial proteases and chitinase (Kuo and Alexander, 1967; Bull, 1970). As melanin is produced in response to wounding or at infection sites by both *Schistocerca* and *Manduca* its effect on enzymes was determined.

A. Binding or inactivation of fungal enzymes by cuticle components

Protease, carboxypeptidase, chitinase and NAG'ase activities were compared before and after incubation with dry or hydrated locust cuticle (presoaked in water for 4 h) to determine any loss of activity. The results with cuticle in either condition were essentially similar (Fig. 57).

The presence of cuticle invariably reduced (> 50%) both soluble enzyme activities and protein levels (determined by change in OD at 280 nm) indicating adsorption onto the cuticle. The time for adsorption with culture filtrates, purified protease or a semi-purified preparation of chitinase was very rapid, being complete within the shortest time of measurement (< 3 min). Adsorbed enzymes were firmly bound as very little (< 5%) could be eluted by repeated water washings.

However the proteolytic enzymes were almost completely desorbed by washing in K phosphate buffer (0.2 M, pH 7.0) which indicates attachment by non-specific ionic bonds. In contrast, chitinolytic activity was not recoverable which suggests either inactivation by inhibitors or more specific binding to the cuticle.

The extent of binding of enzymes to locust chitin was studied as a model for cuticular chitin unmasked by fungal proteases (Fig. 58). Chitinase (semi-purified using column chromatography) had a very high affinity for the chitin. Thus 5 mg of chitin adsorbed more than 97% of the enzyme from 3 ml of solution (containing 8.4 units) and adsorption was completed (as judged by change in OD at 280 nm)

within 3 min. The chitinase was not recovered by washing in 0.2 M buffer indicating that binding is not a simple charge effect. It was found however that activity returned to a dialysed supernatant after the chitin had been hydrolysed by the enzyme. Furthermore, heat denaturation of the chitinase (60 min, 100°C) decreased the amount adsorbed from 97% to 43% and 2/3 of this could be desorbed with buffer. These results suggest that a specific enzyme substrate bond is formed which is dependent on the structure and activity of the chitinase. This affinity for chitin was utilised as a means of purification (Materials and Methods).

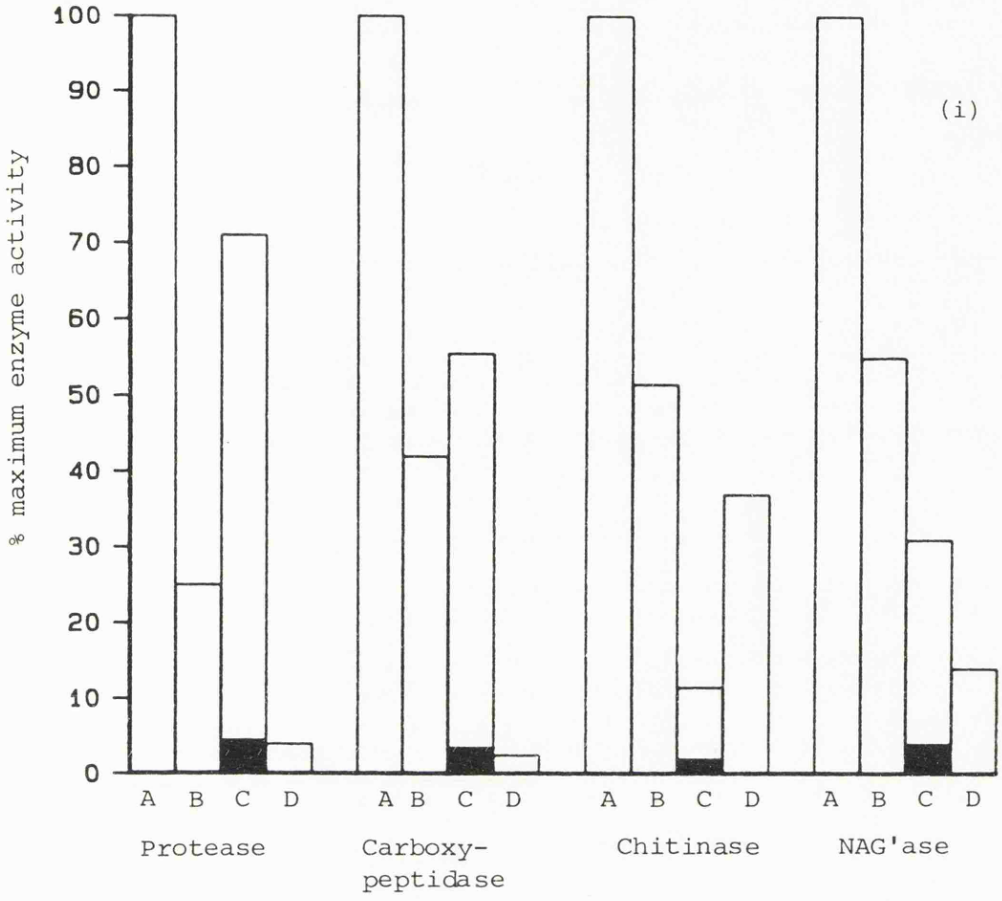
The NAG'ase showed far less affinity than chitinase for the chitin (Fig. 58). Using 100 mg of chitin and 3 ml of partially purified NAG'ase *c.* 85% of soluble enzyme bound and about half of this was recovered by washing in buffer. That a substantial part of the activity was not recoverable indicates some degree of enzyme substrate binding even though activity against locust chitin is extremely low (Table 20). The loss of activity is unlikely therefore to be due to reaction product inhibition; this is supported by the lack of increased activity following dialysis.

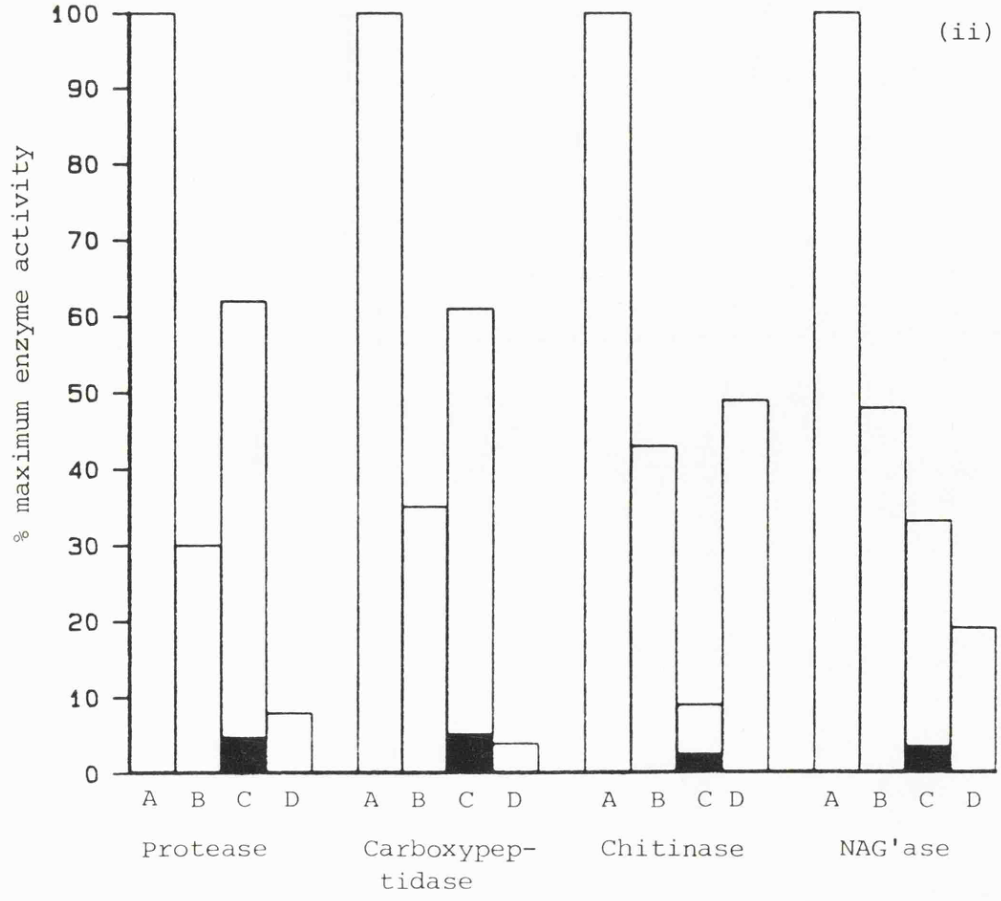
The alkaline protease whether purified (Fig. 58) or in culture filtrate showed some affinity for chitin. As with other substrate-enzyme combinations adsorption took place within 3 min. Almost complete recovery of enzyme was achieved with 0.2 M buffer indicating that binding was by ionic bonds.

Fig. 57. Binding of 4 fungal enzymes by freeze dried (i) and hydrated (ii) locust cuticle.
Ordinate: Column (A): activity of control enzyme solutions (100%). Column (B);percentage soluble enzymatic activity remaining after 10 min incubation with cuticle. The lower shaded section of Column (C) shows the activity of enzyme released from cuticle by washing in water, the upper section of the column represents activity of ionically bound enzyme released from cuticle by incubation for 15 min with K phosphate buffer (0.2 M, pH 7.0). Column (D); Column A minus combined activities of (B) and (C) represents inactivation or non-ionic binding.

Each result is the mean of at least 3 replicates. The experiment was repeated three times with similar results. Fresh enzyme and cuticle preparations were used on each occasion.

Fig. 57.


(i)
% maximum enzyme activity
100
90
80
70
60
50
40
30
20
10
0
A B C D
Protease
Carboxy-peptidase
Chitinase
NAG'ase


(ii)
% maximum enzyme activity
100
90
80
70
60
50
40
30
20
10
0
A B C D
Protease
Carboxypep-tidase
Chitinase
NAG'ase

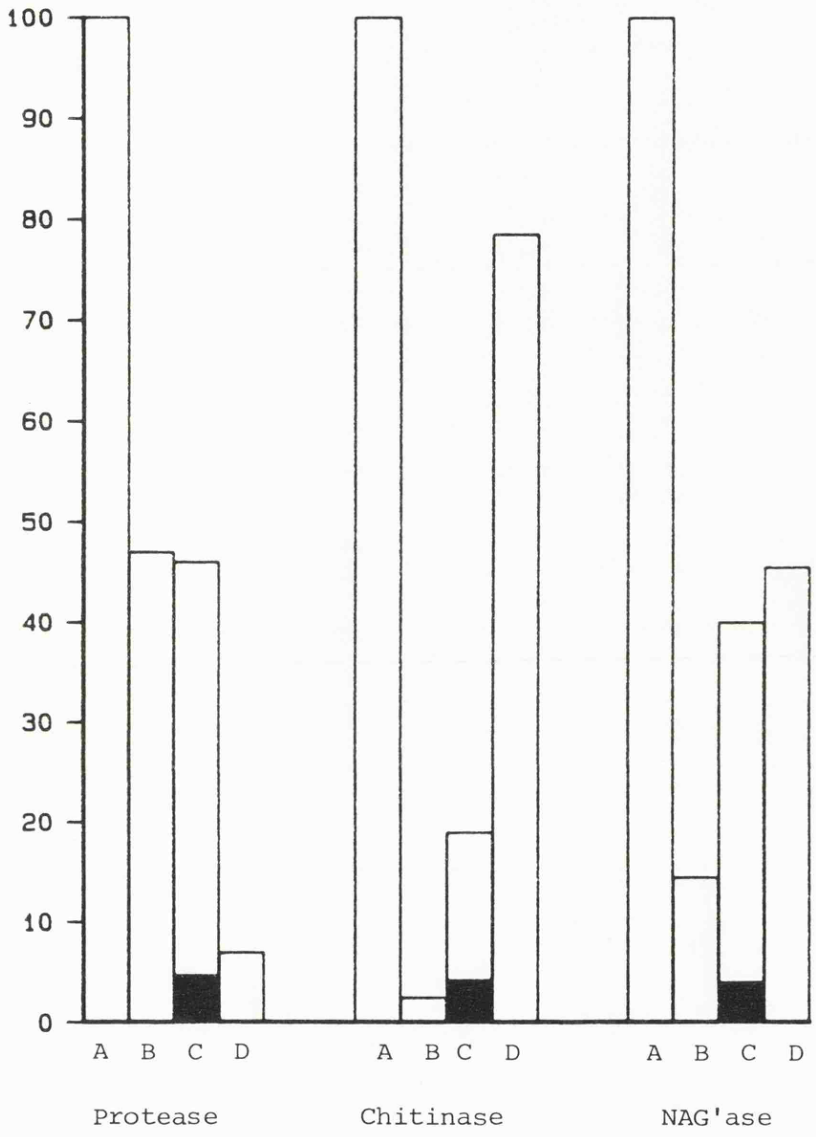


Fig. 58. Binding of three fungal enzymes by ground locust chitin. See Fig. 57 for column designations. Enzyme activities and amount of chitin present are: protease 24.6 µg trypsin units; 100 mg chitin; Chitinase 8.4 units, 5 mg chitin NAG'ase 4.2 µmole PNP $ml^{-1}h^{-1}$, 100 mg chitin. Each result is the mean of at least 3 replicates. The experiment was repeated twice with similar results. Fresh enzyme and cuticle preparations were used on each occasion.

B. The effect of melanin on enzyme activity

According to Andersen (1980) the method of cuticle preparation used throughout this study does not extract the phenolic compounds and their derivatives, which can be inhibitory to some enzymes (Hoffman-Ostenhof, 1963). As there was no evidence of inhibition of soluble activities or enzymes bound to cuticle it seems unlikely that phenolics cause any significant inhibition of activity in cuticle. However, localised production of inhibitory quinones around infective structures may occur as evidenced by the deposition of melanin (Gotz and Vey, 1974).

Preliminary experiments demonstrated that soluble melanin (0.25 mg/ml) (Materials and Methods) had no effect on either protease or chitinase activity. Experiments were performed therefore to determine whether pre-incubating casein or chitin with melanin to form complexes (Materials and Methods) affected their susceptibility to enzymolysis. However, even with the high melanin to substrate ratio of 1:10 there was only a marginal decline in protease ($\bar{x}$ decline 3.7%) and chitinase ($\bar{x}$ decline 6.2%) activities. Degradation of both casein and chitin-melanin substrates released solubilised melanin into the assay media but this did not interfere with the rate of reaction.

DISCUSSION II

The presence of at least two and probably three extracellular endoproteases in *M. anisopliae* MEl cuticle culture filtrates (Section I) was confirmed by iso-electric focussing. The alkaline protease could be resolved into two components (pI 4.5 and pI *c.* 9.5) which eluted together as a single peak (M.Wt. 25 kilo daltons) on gel chromatography. In several of its properties the major activity resembles other alkaline proteases such as protease I of *Aspergillus oryzae* (Bergkvist, 1963 a,b) a 24 kilodalton protease of *Scopulariopsis breviscaulis* (Singh and Vezina, 1971) and a 22 kilodalton protease (CA-7) from *Aspergillus oryzae* (B-1273)) (Ives and Tosoni, 1967). Thus it exhibits different pH optima for digestion of various proteins; casein degradation by MEl protease was maximal at 9.0 (for protease I, 8.0 - 8.5 (Bergkvist, 1963b); *S. brevicaulis* (10.5 - 11.0 (Singh and Vezina, 1981)), but for haemoglobin it is 8.2 (for protease I 7.6 and *S. brevicaulis* 7.0 - 9.5). Like *S. brevicaulis* protease (Singh and Vezina, 1971) the MEl enzyme exhibited esterase activity and was strongly inhibited by sulfonyl fluoride but not soybean trypsin inhibitor.

The MEl protease was relatively stable over a wide range of pH values on the acidic side of the iso-electric point, but was more readily inactivated at pH values above pI 9.5. The converse occurs with a *Penicillium* protease (pI 4.5) which was most stable at pH values above the isoelectric point (Singh and Martin, 1960). The protease was stable at 30°C to 40°C and is likely therefore to remain active under most conditions which would also allow fungal growth.

Kucera (1980) recently isolated two proteinases, PI and PII, from bran cultures of a different strain of *M. anisopliae* (strain 42). PII showed activity *c.* pH 9.0 (vs. haemoglobin) had a molecular weight of 71,000 and was inhibited by sulfhydryl reagents, but not by PMSF Evidently therefore this enzyme is markedly different from those produced by the isolate used here. PI was inhibited by PMSF but for a serine enzyme had the unusually low pH optimum of 6.5. Its molecular weight (35,000) was also considerably higher than that of the MEl serine protease. Subsequently, Kucera (1981) reported that when St 42 was grown on a variety of N sources only wheat bran allowed production of a third protease (pH optimum, *c.* 7.0). Its narrow requirements for production suggest similarities to the minor protease (pH 5.5) of MEl.

It would appear to be not unusual for different strains of a single species to possess markedly different proteases: well characterised examples are *S. brevicaulis* (Miyake *et al.*, 1958; 1960; Singh and Vezina, 1971) and the bacterial pathogen of fish *Aeromonas salmonicida* (Shieh and Maclean, 1975; Mellergaard, 1983). In the case of *M. anisopliae* too little is known of the role of extracellular processes in pathogenesis to assess whether strain differences in properties of proteases have adaptive significance or relate to pathological manifestations. However *prima facie* the comparatively low molecular weight of the MEl alkaline protease would seem favourable for diffusion through the cuticular matrix.

The MEl alkaline protease had activity against all the proteins examined including keratin and collagen which are generally resistant

to proteolysis due to their configurations and cross-linking (Pearse, 1972). It is possible however that collagenase activity could have been merely on the ends of the molecules where the amino acid sequence is different from the main helical portion and more vulnerable to enzyme attack (Pearse, 1972). The ability of the protease to degrade elastin is particularly interesting as this protein contains large amounts of alanine and glycine (Partridge, 1962) which also predominate in locust cuticle. The protease did not however degrade peptides of alanine or glycine, and it released peptides from locust cuticle which were not subsequently degraded to amino acids (over a 24 h period). The author is not aware of any information as to the efficacy of uptake of peptides into cells of imperfect fungi, however it seems likely that exo-peptide hydrolases such as the amino peptidase and carboxypeptidase (both of which degrade the trimer G-G-P will be necessary for efficient amino acid utilisation.

Like other serine hydrolases, MEI alkaline protease possessed esterase activity. Both crude filtrates and purified protease of *M. anisopliae* (MEl) preferentially hydrolysed the ester TAME which is specific for trypsin-like enzymes. However there was also a weaker activity against BTEE, a specific synthetic substrate for chymotrypsin. Crude culture filtrates from other strains of *M. anisopliae* showed rather similar specificities indicating that this may be a species characteristic. In contrast, filtrates of *B. bassiana* and *V. lecanii* preferentially hydrolysed BTEE indicating a preference for aromatic amino acid residues. Serine proteinases possessing chymotrypsin and trypsin-like specificities are widespread among micro-organisms with the former apparently predominating (Morihara

and Tsuzuki, 1969). The distinction is not absolute as some of the filtrates possessed significant activity against both aliphatic (i.e. trypsin-like) and aromatic (i.e. chymotrypsin-like) residues. With crude filtrates the minor activities could have derived from separate enzymes, but use of the pure *M. anisopliae* (MEl) alkaline protease (pI 9.5) confirmed that single fungal proteases can have broad specificities. Relatively non-specific proteases may be common among microbes as 5 chymotrypsin-like proteases from unrelated species of bacteria and fungi all showed activity against aliphatic amino acid residues (Morihara *et al.*, 1969). Both trypsin and chymotrypsin are capable of degrading a wide variety of protein types, and differences in specificity of the pathogen's enzymes did not apparently affect the ability of crude filtrates from *M. anisopliae, B. bassiana* and *V. lecanii* to hydrolyse locust cuticle (Table 28). Furthermore Smith *et al.* (1981) found that a variety of commercial preparations including trypsin or chymotrypsin were equally effective in the degradation of *Heliothis zea* cuticle. In contrast Yamazaki (1972) found trypsin cf chymotrypsin far more effective at hydrolysing non sclerotized pupae of *Bombyx mori*, so the possibility cannot be ruled out that the specificity of a pathogen's proteolytic enzymes may be a factor limiting its host range.

As well as endoproteases some fungi produce peptide exo-hydrolases (e.g. Plumbley and Pitt, 1979). Although aminopeptidase activity has been identified in *Verticillium cinnabarium, Candida albicans* and *Fusarium oxysporum* (Male, 1971) peptide exo-hydrolases have not apparently been isolated and characterised in imperfect fungi. Column chromatography and flat bed iso-electric focussing of *M.*

anisopliae (MEl) culture fluids revealed a single enzyme with activity against both leucine and alanine naphthylamide. Semi-purified enzyme eluted from a Sephadex G-100 column had no activity against casein or hide protein azure and did not possess carboxy-peptidase activity against NCBZ-glycyl-phenylalanine or hippuryl-L-arginine. It did however hydrolyse the peptide glycylglycyl phenylalanine which establishes the enzyme as a true aminopeptidase and not an arylamidase (Prescott and Wilkes, 1966). On the information now available the enzyme is most properly categorised as an amino-peptidase (aminopolypeptidase) (EC 3.4.11.2) rather than a leucine aminopeptidase (EC 3.4.11.1), as hydrolysis was greater when the residue was L-alanine. This preference for L-alanine was not exclusive to MEl as culture filtrates from 5 other strains of *M. anisopliae* hydrolysed alanine residues between x3.9 (RS 298) and x9.5 (RS23) times faster than leucine. It is noteworthy that alanine is a major component of insect cuticle, and for MEl at least (Sec. 1) supports better growth than leucine.

The requirement for heavy metal groups for maximal amino-peptidase activity was demonstrated by dialysis against EDTA. In addition the inhibition of hydrolysis of L-alanine naphthylamide by L-alanine indicates an additional form of control for this enzyme besides CR. Amino acids have also been reported to inhibit amino-peptidases from *Phoma medicaginis* (Plumbley and Pitt, 1973). The neutral pH optimum is similar to the *P. medicaginis* aminopeptidases (Plumbley and Pitt, 1973) but is lower than that from *A. oryzae* (Nakadai *et al.*, 1973). The molecular weight of the MEl aminopepti-dase was estimated as *c.* 35,000. Molecular weights for aminopeptidases have been calculated as low as 26,500 from *A. oryzae* (Nakadai

et al., 1973) or as high as 400,000 in *Bacillus stearothermophilus* (Roncari and Zuber, 1969). The relatively low molecular weight of the ME1 enzyme reflects its extracellular location. The only aminopeptidases known to the author with similarly low molecular weights (*Aeromonas proteolytica*, MWT 29,500, Prescott and Wilkes (1966); *A. oryzae*, MWT 26,500, Nakadai *et al.* (1973); *A. parasitica*, MWT 32,000; Lehmann and Uhlig (1969)) were all found in culture filtrates.

Most interest in microbial peptide exo-hydrolases has been directed towards aminopeptidases, and except for yeasts, relatively few studies have been done on enzymes which hydrolyse peptides with free COOH groups. The spectroscopic assay used in this study with CBZ-glycyl-phenylalanine as substrate and Co^{2+} as activator is claimed to be specific for carboxypeptidase A activity (Appel, 1974). Fractionation of *M. anisopliae* (ME1) cuticle culture medium by column chromatography produced a single carboxypeptidase A peak which had no activity against the carboxypeptidase B substrate hippuryl-L-arginine or amino acid naphthylamides. It did however, hydrolyse the peptide glycylglycylphenylalanine. Several reports suggest that strong chelating agents inhibit carboxypeptidase A activity (Folk *et al.*, 1960; Wintersberger *et al.*, 1962), while not affecting catheptic carboxypeptidases which are inhibited by sulfhydryl reagents (Fruton *et al.*, 1941; Greenbaum and Sherman, 1962). Inhibition by both agents as found with the ME1 enzyme is infrequent, although it has also been reported for an enzyme from brewers' yeast (Felix *et al.*, 1966) and a metal sulfhydryl complex has been described in bovine pancreatic carboxypeptidase A (Vallee, Coombs and Hock, 1960). Carboxypeptidases isolated from plant

tissues and yeasts are frequently described as serine hydrolases (reviewed in Ryan, 1973) but PMSF had little effect on the fungal enzyme at the concentrations used. The inhibition of the fungal carboxypeptidase by EDTA as well as the marked effect of Co^{2+} and ionic strength all confirm a similarity with carboxypeptidase A (EC 3.4.12.2) (Lumry *et al.*, 1951; Folk and Gladner, 1960; Vallee *et al.*, 1960), rather than with the catheptic carboxypeptidases (EC 3.4.12.12) which generally occur in plants, other fungi and bacteria (Appel, 1974; Ryan, 1973).

The molecular weight of the enzyme was calculated *c.* 26,500 which is smaller than carboxypeptidases from other sources such as bovine carboxypeptidases A, 34,600 (Bargetti, 1963) and the fungal catheptic carboxypeptidase of *Phymatotrichum omnivorum,* 31,400 (Prescott and Boston, 1967). Some large molecular weight carboxy-peptidases (> 80,000) are composed of subunits (Ryan, 1973). It is possible therefore that it represents a monomer of a larger enzyme but this is unlikely as column chromatography produced only one activity peak.

The esterase activities of *M. anisopliae, B. bassiana* and *V. lecanii* are represented by numerous iso-enzymes. In *M. anisopliae* all bands were inhibited by PMSF indicating that they are β-esterases - the most prominent group of "non-specific" esterases (Heymann, 1980). However on the basis of their reactions with naphthyl esters the isoenzymes appeared to have different substrate specificities. A similar phenomenon has also been described in rat and pig liver β-esterase isoenzymes (Arndt *et al.*, 1978; Junge *et al.*, 1979). Among the major groups classified as β-esterases are serine proteases

and many lipases (Heymann, 1980; Aldridge and Reiner, 1972). The alkaline serine protease produced by *M. anisopliae* (ME1) had considerable esterase activity against PNP propionate or PNP caprylate and like other β-esterases (Benoehr *et al.*, 1966) hydrolysed tributyrin. Protease may therefore be a significant contributor to total esterase activity - at least against simple synthetic esters. However, as column iso-electric focussing produced only three protease peaks the multitude of bands shown on iso-electrofocussing plates cannot be ascribed to isoenzymes of endo-proteases. Some carboxypeptidases (Snoke *et al.*, 1949) aminopeptidases and lipases (Heymann, 1980) also hydrolyse esters. However inhibition of all bands by PMSF and the lack of any activity against long chain naphthyl esters indicate that neither peptidases nor lipase activity were interfering with the determination of fungal esterases. Although the possibility of other hydrolytic enzymes being involved cannot be excluded, most of the iso-enzymes were probably β-type carboxyesterases (EC 3.1.1.1). It is noteworthy that the majority of bands fell in the pI range 4.5 to 6.5 generally associated with this class of enzyme (Heymann, 1980).

Esterases catalyse many enzymatic reactions though preferentially hydrolysing aliphatic or aromatic esters and amides (Shnitka, 1974; Heymann, 1980). The considerable heterogeneity of esterases could account for their collective lack of specificity. Multiple enzyme strategies are believed to play an important role in the ability of an organism to adapt to different environments (Moon, 1975; Somero, 1975), presumably including that provided by an insect host. It is however extremely difficult to assign a role in pathogenicity to a

diverse and heterogeneous group such as carboxyesterases solely by their action on simple synthetic substrates. The activity against short and intermediate length aliphatic esters does not itself prove any role in nutrition or penetration especially in view of the apparent absence of such esters from insect cuticle. However it is possible that as with low molecular weight fatty acids (Blomquist, 1984) these compounds could be lost in the usual extraction and characterisation techniques. If esterase activity has any role in cuticle penetration it may involve breaking the ester bonds possibly present in cuticulin (Wigglesworth, 1970). As the model for cuticulin proposed by Wigglesworth (1970) resembles plant cutin (see literature review) this could represent a useful model substrate.

Column chromatography of *M. anisopliae* culture media showed that two distinct activities were responsible for catalysing the hydrolysis of chitin and chitobiose. The chitinase had many similarities with those found in other microbes. These properties include an optimum pH of 5.0 - 5.5 (Jeauniaux, 1963; Unestam, 1968) and the lack of any requirement for co-factors (Stirling, *et al.*, 1971) also the molecular weight of the chitinase (33,000) is similar to enzymes from *Streptomyces* (30,000; Jeauniaux, 1959b), *Serratia marcescens* (36,000; Lysenko, 1976) and *Phycomyces blakesleeanius* (30,000; Cohen, 1974). The enhancing effect of acetate in buffers has also been noted with chitinases from *P. blakesleeanius* (Cohen, 1974) and from a termite (Waterhouse, *et al.*, 1961) but *Lycoperdum* chitinase was apparently unaffected (Hackman and Goldberg, 1964). As with chitinase from *Aphanomyces* (Unestam, 1968) the enzyme was stabilised against extremes of pH and temperature by the presence of its substrate.

Chitinase fractions eluted from a Sephadex G-100 column had activity against chitosan as well as crystalline and colloidal chitin. Since the chitinase gave a relatively broad peak with column chromatography the possibility that it existed in multiple forms with different specificities cannot be excluded. Relatively few studies have been conducted on the products of chitin hydrolysis and the mode of chitinase action is currently in dispute. Stirling *et al.* (1979) has suggested that the end product of chitinase attack is chitobiose and the appearance of mono or trisaccharides is due to contaminating chitobiase activity. However pure preparations of chitinase from *V. albo-atrum* or tomato plants (Pegg and Young, 1982) and a spider (Mommsen, 1980) have shown that NAG can be either the sole reaction product or a major end product along with di and trisaccharides. It seems most likely that both the nature of the chitin substrate and the bond preferences of individual chitinases determine how much NAG is produced (Jeuniaux, 1963; Powning and Irzykiewcz, 1965). The chitinase from *M. anisopliae* (MEl) had no activity against chitobiose only trace activity against chitotriose but preferentially cleaved chitotetraose in the middle bond. In spite of this it had only a limited ability to produce oligosaccharides from chitin and NAG was the major (*vs.* colloidal chitin) or only product (*vs.* crystalline chitin) detected following chitin hydrolysis. These results suggest that a classical multichain attack involving single random breaks is unlikely to be involved, especially as the tight binding of chitinase and insoluble substrate (Fig. 58) will prevent the necessary diffusion of enzyme from molecule to molecule. Similar results concerning reaction products were found by Berger and Reynolds (1958) and Pegg *et al.* (1982) who suggested that the

inaccessibility of internal glucosidic bonds means the enzyme acts as an exo-chitinase on insoluble chitin. An alternative possibility is that the reaction proceeds largely *via* a single chain processive mechanism similar to that described for several other polysaccharides (Cooper *et al.*, 1978). This involves random (i.e. endo-acting) hydrolysis of a bond and successive hydrolytic cuts of adjacent susceptible bonds so that a single macromolecule is completely degraded before a new one is attacked. Such a mechanism, especially if it involved simultaneous digestion of several parallel chains, could result in the rapid degradation of chitin fibrils and in addition produce monomers for nutrition and induction of further enzyme synthesis.

The NAG'ase fraction isolated by column chromatography possessed simple glycosidase activity against PNP acetylglucosaminide and hydrolysed di, tri and tetrasaccharides as well as possessing possible trace activity against colloidal chitin. In this respect the enzyme differs from the chitobiase of *S. griseus* which could not split the tetramer or chitodextrins (Berger and Reynolds, 1958) and resembles more the enzyme from *Candida albicans* (Barrett Bee *et al.*, 1982). The NAG'ase also possessed activity against *P*NP-β-N-acetylgalactosaminide. NAG'ase from a number of unrelated species possess both activities (Woollen *et al.*, 1961). Mixed aminopolysaccharides containing NAG and N-acetylgalactosamine have a widespread distribution in lower plants and throughout the animal kingdom where they are found in structural materials, intercellular cements and biological lubricants (Powning and Irzykiewicz, 1964).

The NAG'ase was calculated to be between 110-120,000 daltons. Although NAG'ase's seem rather variable in their substrate specificities, enzymes from other sources all have similar molecular weights, ranging between 110,000 and 140,000 (Spindler, 1976; Mommsen 1978; Kimura, 1977; Brun *et al.*, 1976; Reyes and Byrde, 1973; Mega *et al.*, 1970). The optimum pH (*c.* 5.0) for NAG'ase activity resembled that of the chitinase. This also compared with most other NAG'ases which show maximal activities in the range 4.3 to 6.3 (Powning and Irzykiewicz, 1964; Pugh *et al.*, 1957; Spindler, 1976; Berger *et al.*, 1958).

NAG'ase was significantly inhibited by its reaction product NAG and possibly also by the corresponding di and trisaccharides. However NAG neither inhibited chitinase activity nor reversed enzyme absorption onto chitin even though the monomer inhibits a chitinase from *Lycoperdon* (Tracey, 1955). Chitobiose and chitotriose had a pronounced inhibitory effect on chitinase confirming a report by Molano *et al.* (1977) for inhibition of *Streptomyces* chitinase by the dimer. These observations merit further work on the effects of inhibitors to determine whether inhibition is competitive, non-competitive or mixed. The results from this would confirm whether chitobiose is acting as a reaction product (non-competitive) or substrate (competitive) inhibitor of chitinase. It is noteworthy that cellobiose inhibits most cellulases. Consequently a suggested role of cellobiases is to remove potential cellulase inhibitors (Reese, 1977). It would seem that MEl NAG'ase is well adapted to function in this role for chitinase.

Although *in vitro* production of extracellular enzymes by

entomopathogens has been the subject of several studies, to date only impure commercial enzymes (Samsinakova *et al.*, 1971; Smith *et al.*, 1981) or a culture filtrate from *B. bassiana* (Samsinakova *et al.*, 1981) have been used on cuticle to assess the relevance of enzymes to pathogenicity. In the current study the potential of fungal enzymes to degrade locust cuticle was suggested by the release of degradation products during hydrolysis by culture filtrates from *M. anisopliae*, *B. bassiana* and *V. lecanii*, and confirmed by the action of purified enzymes of *M. anisopliae* (ME1) on cuticle.

ME1 protease released peptides containing all of the amino acids found in intact cuticle, which included several that support good growth of *M. anisopliae* (Sect. I). Protease also released small quantities of amino sugars (Table 31), which were presumably set free after solubilisation of surrounding protein. This observation suggests that some chitin fibres may be short and unconnected in the protein matrix. Chitinase also released some NAG from cuticle (equivalent to 3-4% of total cuticle chitin) indicating that at least part of the chitin in the preparation was accessible. Staining of cuticle fragments by wheat germ agglutinin showed that NAG polymers were exposed at the cut edges of the fragment possibly providing substrate for the chitinase to act on without the need for penetration of the cuticle *per se*. Evidently therefore isolated cuticle fragments should not be regarded as having chemical and physical characteristics identical with that of cuticle *in situ*. The activity of chitinase was however comparatively low unless the cuticle had been previously modified by treatment with protease. This suggests that most of the chitin in the cuticle was masked by protein, an interpretation consistent with the known structure of insect cuticle

(Neville, 1975; Jeuniaux, 1984) and in agreement with results o b-tained using commercial enzymes on unsclerotized cuticle (Samsinakova *et al.*, 1971; Smith and Grula, 1981). Fluorescence microscopy confirmed these results and demonstrated early degradation of protein in cuticle incorporated into cultures, which resulted in gradual exposure of chitin to enzymolysis. The lack of synergism between either protease or chitinase with NAG'ase does not rule out this possibility occurring *in vivo* where wall-bound enzyme on penetrating hyphae may be brought into direct contact with cuticle chitin.

Exuviae particles (sclerotized cuticle) were far more resistant to enzymic degradation than whole cuticle. However, exuviae supported good growth of *M. anisopliae* (MEl) (Sect. I), and enzymolysis was evidently sufficient to provide a source of C and N. Nevertheless, even after several days growth there was comparatively little overall disintegration of exuviae fragments as judged by fluorescence staining. Lipke and Geoghegen (197la) found a similar resistance to enzymolysis (commercial enzyme preparations) in sclerotised cuticle from *P. americana* and *Sarcophaga bullata* which they explained as a generalised protection by H bonds (see literature review). It is also interesting to note that moulting fluid which contains a 'trypsin-likep enzyme and chitinase (Bade and Stinson, 1978) only degrades unsclerotised cuticle and leaves the future exuviae intact.

As fungal enzymes cannot apparently alter the integrity of sclerotized proteins their role in penetration is likely to be greatest in soft cuticle. This is supported by ultrastructural evidence which suggests that growth through exocuticle generally

involves a stepwise progression of hyphae along lamellae until the endocuticle is reached, after which progression to the epidermis is more or less direct (McCauley *et al.*, 1968; Robinson, 1966; Zacharuk, 1970c). The "stepwise" mode of progression suggests that hyphae are mechanically cleaving their way along lines of least resistance in the cuticle rather than enzymatically degrading it. It cannot be ruled out however that sufficient digestion of protein might occur to loosen cuticular lamellae allowing an easier mechanical passage (Gabriel, 1968 a,b) also the extent of growth on exocuticle *in vitro* confirms the potential of enzymes for obtaining nutrients during penetration of sclerites.

Although the entomopathogens used in this study produced a variety of extracellular cuticle-degrading enzymes there is ultrastructural evidence that even in endocuticle the degradative effect of pathogen enzymes are limited to the vicinity of fungal structures (Zacharuk, 1970b,c). Presumably there are mechanisms which either result in inactivation or retention of enzymes to hyphae. Such retention could be of benefit to the pathogen as products of the enzyme action would be localised around hyphae for easy uptake. One hypothesis to account for the localised nature of degradation is that the cuticle acts as a molecular sieve, such that the intermolecular spaces may be too small to allow enzyme diffusion. It seems that although both chitinase and protease are small (Stokes radius, 24.5 Å and 20.9 Å respectively) they are in theory too large to penetrate unaltered cuticle layers or to enter the water filled canals (*c.* 6.5 Å diameter) that traverse some cuticle types (Phillips, 1964). The pores of the outer epicuticle (25 Å diameter, Locke, 1974)

may also restrict entry of enzymes. The enzymes therefore could only diffuse away from the hyphae *via* the epicuticular filaments, pore canals and dermal ducts. It is known that inorganic ions (Bruck and Komnick, 1971) and insecticides (Wigglesworth, 1942; Lewis, 1965) readily penetrate down these structures. Zacharuk (1970c) observed an accumulation of electron dense material in pore canals underneath penetration plates which could partly have derived from the fungi. There can be some 1,200,000 pore canals mm^2 of cuticle (David, 1967) which could provide effective means for the dispersal of fungal enzymes. The availability of these routes suggests that other mechanisms may exist to account for the limited mobility of pathogen enzymes. One possibility (discussed in Sect. III) is that enzymes may be localised and concentrated in the mucus that surrounds many fungal structures. Histochemical examination of some enzymes however (e.g. protease) suggested that they are bound directly to the cell wall. NAG'ase and to a lesser extent protease were released into water by conidia harvested from *Manduca* larvae. As spore walls contain both chitin and protein these enzymes may have been "remnants" from the process of sporulation. A similar explanation was proposed for the readily extractable diphosphopyridine nucleotidase on *Neurospora crassa* conidia (Zalokar and Cochrane, 1956). The ease with which some enzyme can be removed by washing in water or dilute buffer solutions and the degradation of enzyme substrates by non-germinated spores suggests that part of the enzyme activities are located at or near the surface of spores, and probably parallels the availability or release of enzyme under natural conditions. At least one third of the cell-bound protease or NAG'ase that could be released from conidia was located in purified cell walls(Table 35).Wall-bound protease provide spores

with the potential to obtain nutrients and effect preliminary modifications of the cuticle surface even before germination. This suggestion is supported by an observation that the epicuticle of *Pyrausta nubilalis* becomes granular and pale around non-germinated conidia of *M. anisopliae* (Wallengren and Johansson, 1929). Some apparent alteration in the appearance of locust cuticle around ME1 conidia may also be due to enzymolysis (Sect. III).

Some cell-bound enzyme was only released from intact spores after treatment of the cells with the surfactant Tween 80. This suggests a proportion of the enzyme is either lipid bound or endocellular, in the latter cases, this would imply inaccessibility to insoluble external substrates.

In mycelial preparations cell-bound enzymes were released in varying degrees by water, salt solutions, detergents and 2-mercaptoethanol. NAG'ase seemed more tightly bound to whole mycelia than protease since its release required either high concentrations of buffer ions or the disruption of membranes and disulfide bonds. These agents had far less effect on isolated cell walls so NAG'ase is probably mainly endo-cellular. The amount of ionically-bound fractions of both enzymes was correlated with the total amounts of each enzyme released into the medium rather than mycelial dry weight. Similarly, wall-bound cellulase has been correlated with extracellular levels of enzyme in *Rhizoctonia solani* (Lisker *et al.*, 1975). However, the ratio of cell-bound and extracellular fractions of enzymes, particularly protease, varied with the amount of carbon source used in the growth media; under certain conditions bound enzyme was present even when no extracellular enzyme

had been produced. Thus, as well as being important in interpreting pathogenesis, location of enzyme components is necessary for critical studies on regulation of enzyme synthesis and properties.

Fungal enzymes had a considerable affinity both for cell walls isolated from *M. anisopliae* and insect cuticle. This suggests that these enzymes are capable of a relatively non-specific binding to substrates. Binding of protease by cuticle resulted in immobilisation of over 70% of the enzyme activity, which *in vivo* could have a significant influence on the extent of cuticle degradation. Almost all the initial proteolytic activity was recoverable with 0.2 M buffer suggesting that the enzyme was ionically bound to cuticle rather than inactivated. However, buffer solutions failed to desorb chitinase from either cuticle or deproteinised chitin, suggesting enzyme inactivation or firmer binding. In view of the recovery of activity following solubilisation of chitin, and that denatured chitinase no longer showed irreversible binding to substrate it is apparent that specific enzyme substrate complexes were formed. Several chitinases bind irreversibly with their substrate (Bade and Stinson, 1981; Skujin *et al.*, 1973), however in contrast to the present work other enzymes; trypsin and chitobiase (Bade and Stinson, 1981) or ribonuclease (Skujin *et al.*, 1973) have been found to lack affinity for chitin. In this study both protease and NAG'ase ionically bound when relatively large quantities of chitin were used; although adsorption by NAG'ase was not completely reversible with salts. Similar results with binding of cuticular proteins to chitin were described by Hackman (1955). The conflict in results may be due to a failure by Bade and Stinson (1981) and Skujins *et al.* (1973) to take into account the larger quantity of

chitin required for maximal adsorption of enzyme proteins when ionic bonds only are involved.

The presence of phenolic compounds and cuticular oxidases in the insect integument (Anderson, 1980) frequently result in a localised melanization around penetrant hyphae (e.g. Gabriel, 1968a; Takahashi, 1958; Schabel, 1976a). Given the known susceptibility of micro-organisms and their enzymes to phenols it has been generally assumed that melanization reactions have antifungal effects (qv. Charnley, 1984), although in relatively few instances are these reactions enough to abort the infection process (e.g. Gotz and Vey, 1974; Brobyn and Wilding, 1977). A preliminary *in vitro* study with MEl protease and chitinase demonstrated no significant enzymic inhibition or shielding of substrate by melanin. The protease of *A. astaci* is likewise unaffected by melanin (Soderhall and Ajaxon, 1982). However Bull (1970) found protease and chitinase from a *Streptomyces* sp. were strongly inhibited whether the melanin was soluble or bound to substrate. Presumably enzymic resistance to melanin would have adaptive significance re pathogenesis. It should be emphasised that melanin-substrate complexes in insect cuticle may be more intimate than those manufactured in this study and consequently *in situ* melanin may offer greater physiochemical protection to cuticle components from enzymic degradation.

An analogous situation to melanization occurs in higher plants which produce lignin in response to wounding or infection (Ride, 1983). Lignified plant cell walls are virtually resistant to enzyme attack and fungal degradation *in vitro* (Ride, 1979, 1980). Also the low molecular weight phenolic precursors as quinones and free radicals available before their polymerization can inactivate cell

wall-degrading enzymes of a range of plant pathogens (reviewed by Friend, 1981 and Ride, 1983). However, the only study to date with an arthropod pathogen demonstrated that *A. astaci* protease was unaffected by melanin precursors (Soderhall and Ajaxon, 1982). Interestingly, it has been suggested that phenols esterified to carbohydrate in the plant cell wall may provide some protection from fungal enzymes (Friend, 1976). It should be worth investigating whether a similar situation exists with respect to cuticular chitin.

In all previous studies degradation of cuticle has been considered to result solely from enzyme degradation. However all strains of entomopathogens tested produced H_2O_2 which is a vigorous oxidising reagent with the potential to hydrolyse pectin (Whistler and Schweiger, 1959), cellulose (Halliwell, 1965) and polymers as complex as bacterial cell walls (Boevskaya, 1940; Cook and Quadling, 1962). Under the conditions tested H_2O_2 did not solubilise ground locust cuticle or chitin. However, this experiment should be regarded as preliminary and further work is warranted. Peroxide can oxidise phenols (Kar, 1937) and in this study H_2O_2 solubilised and bleached melanin. This action could potentially help in overcoming host defence mechanisms based on accumulation of phenolic products. Likewise the ability of H_2O_2 to oxidise small chain fatty acids (Kultyugin and Sokolova, 1936) may be significant considering their fungistatic action and superficial location on cuticles of some insect species (Smith and Grula, 1982; Kerwin, 1982). The small size of the H_2O_2 molecule should enable rapid diffusion through cuticle and potentially therefore could also act as a toxin before the fungus has completed penetration.

SECTION III

SECTION III

Production of Cuticle-degrading Enzymes during Penetration

In Sections I and II, it was shown that entomopathogenic fungi produce in culture enzymes capable of digesting the major components of unsclerotized insect cuticle. It is necessary, however, to establish that these enzymes are produced *in vivo* before their role (if any) in pathogenesis can be elucidated.

Two methods were used. In the first, samples of inoculated cuticle were examined by light microscopy and scanning electron microscopy. Extracellular and cell-bound enzymes produced during adhesion and germination of conidia were then localised histo-chemically. The second approach involved extracting from infected cuticle and assaying quantitatively for activity of cuticle-degrading enzymes. The availability of a large number of spectrophotometric (*cf.* histochemical) techniques allowed determination of enzyme types and properties, enabling comparison of production *in vivo* and *in vitro*.

1. Symptomology of blowflies (*Calliphora vomitaria*) infected by *M. anisopliae* (MEl).

Dillon (1984) and Hassan (1983) have already established the pathogenicity of *M. anisopliae* (MEl) for *S. gregaria* and *M.sexta*. However, for many of the histological experiments in this project blowflies (*Calliphora vomitaria*) were more amenable to study as their transparent wings allowed the use of conventional light microscopy. To establish the pathogenicity of *M. anisopliae* (MEl) *cf.* blowflies, flies were placed in 5 jars (10 per jar) containing conidia (Materials and Methods) and the progress of disease followed.

The first symptoms were evident about 2 to 3 d after inoculation and included lethargy and an apparent lack of co-ordination. In several respects symptoms resembled those induced by synthetic neurotoxic insecticides (Wilkinson, 1976). After 5 d the flies became increasingly comatose but dissection at this stage revealed that fungal growth was limited to the cuticle and epidermis. This suggests that the symptoms were the result of fungal toxins rather than a direct result of mycelial growth in the insect. All the flies were dead after 6 d. Just prior to death, dissection revealed that the haemocoel contained blastospores and hyphal bodies composed of 3 to 12 cells. Fungal growth following death was very rapid. Within 24 h a thick tangled mass of mycelium formed on the basal surface of the epidermis and hyphae started to penetrate many of the internal organs. After 2 d fungal hyphae invaded the flight muscles and the cadaver began to desiccate and partially mummify. By this time hyphae were emerging through the cuticle, initially from the intersegmental membranes, particularly on the ventral surfaces (Plates 12 and 13) and through the arthrodial membranes of the legs and proboscis. The hyphae rapidly formed a white layer over the fly and after 1 d in humid conditions (> 95% RH) characteristic green conidia were produced extensively.

2. Behaviour of *M. anisopliae* (ME1) conidia germinating on blowfly wings

The behaviour of germinating conidia was followed *in vitro* on cuticle obtained from *Calliphora*, *Schistocerca* and *Manduca*. (Fig. 59) shows the pattern of germination of conidia on fly wings. It is evident that germination was not synchronous. Spores which had failed to germinate by 30 h were generally found clumped together but close

proximity did not necessarily preclude high germination (Plate 24).

The conidia of MEl are cylindrical with rounded poles, *c.* 4 µm x 2.5 µm. Prior to germ tube formation the spores increased in size to 3.5 µm x 8.5 µm and often exhibited a decrease in refractive index. Usually each conidium produced a single germ tube, slightly lateral to one of the poles (Plate 25). However bipolar germination sometimes occurred (Plate 15), and often the tubes were bifurcate (Plate 14) so that single conidia could give rise to several widely separated appressoria. The extent of pre-penetration growth of germ tubes varied considerably, even on apparently uniform cuticle from the same species e.g. germ tubes produced on fly wings ranged from 2 µm up to 200 µm in length (Plates 47 and 14).

On *Manduca* it was evident that the nature of the cuticle influenced hyphal growth, as germ tubes were far longer on segmental cuticle than on hair sockets where appressoria were produced almost immediately after germination (Plate 36). Conidia germinating on hard (sclerotized) segmental locust cuticle usually gave rise to appressoria at the end of very short germ tubes (Plate 26), though post-penetration (> 36 h) mycelial growth on the cuticle surface was substantial. In the few instances where extensive prepenetration growth occurred germ tubes were generally orientated in approximately straight lines unless about to produce appressoria or deflected by setae or folds (Plate 16). Thus the route of growth may follow topographical features of the cuticular surface. Penetration of socket cuticle of *Manduca* and hard cuticle of *Schistocerca* and *Calliphora* always involved the production of appressoria; in contrast germ tubes often penetrated soft *Manduca* cuticle direct.

Appressoria developed as terminal swellings, up to 7 μm diam. on germ tubes. The appressorial cells showed no evidence of pigmentation but were delineated from germ tubes by septa (Plates 22 and 23). They therefore resemble the "hyaline" appressoria of many plant pathogens (Emmett and Parbery,1975). Frequently the original appressorial cell proliferated to form an infection cushion (Plate 19) which occasionally took the form of a plate of cells (Plate 20). Long germ tubes frequently encountered each other (Plate 21) which often resulted in an appressorial complex (Plate 29).

The main role of appressoria is to provide a stable platform for host penetration. To achieve this, appressoria must attach firmly to the host surface and produce infection pegs that pierce the cuticle (Emmett and Parbery, 1975). Penetration filaments of *Metarrhizium* arose from near the terminal edge of the appressorial cell. The filaments usually, but not invariably, succeeded in penetrating the cuticle (Plate 22).When they failed, the filaments continued to grow along the surface (Plate 49 *cf.* Plate 41).

The adherence of appressoria to cuticle surfaces was demonstrated by applying a jet of water from a pipette onto an inoculated surface. This procedure dislodged some conidia (although others held fast) and most germ tubes, but had no effect on appressoria. A similar adhesive ability of appressoria of plant pathogens has been attributed to the presence of a mucilagenous sheath (qv Emmett and Parbery, 1975). In the present work a light transmissable pale blue (under conventional light microscopy) halo surrounding appressorial cells was sometimes apparent which may represent mucilage. Additional evidence for the presence of mucilage comes from scanning electron

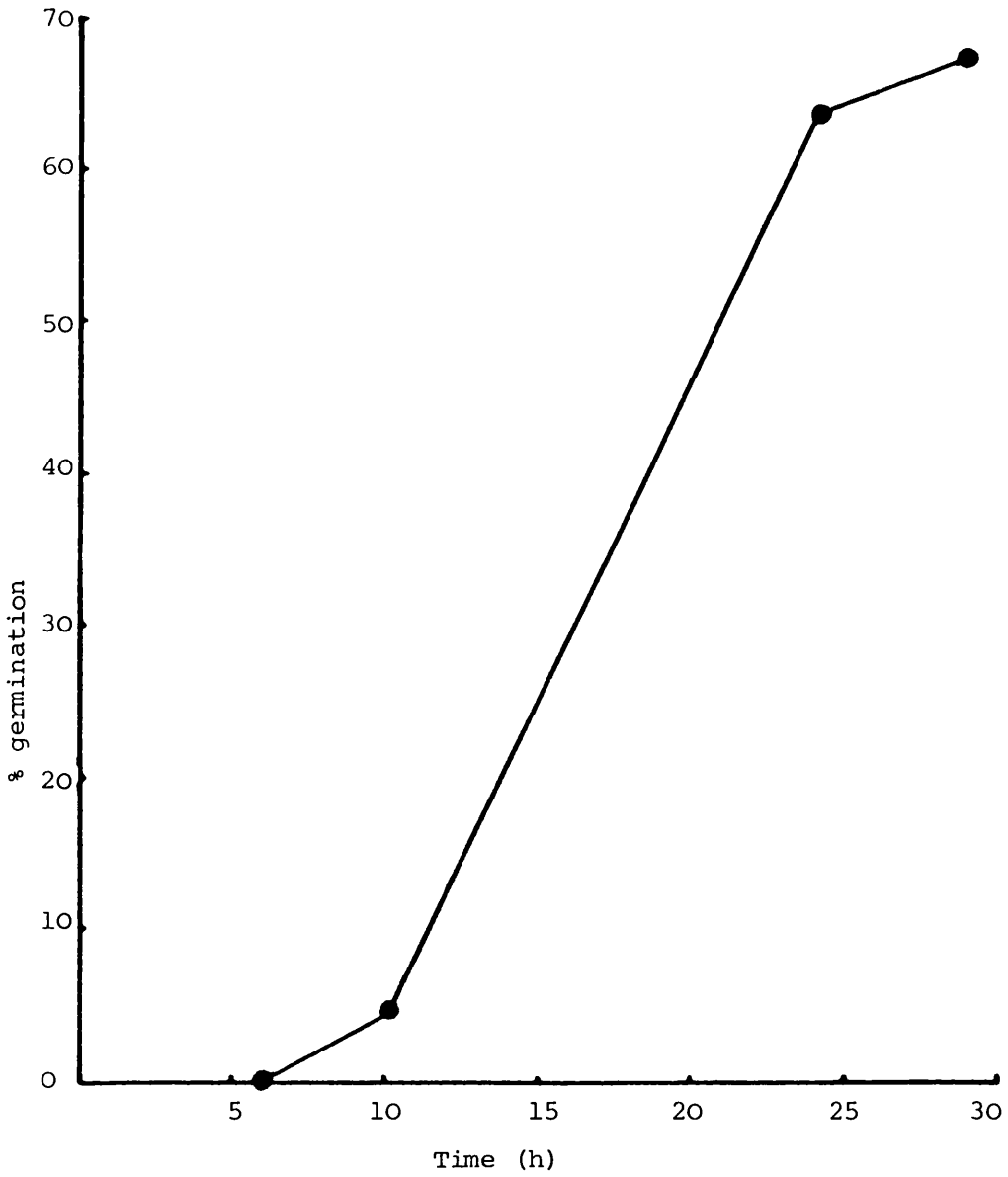


Fig. 59. Germination of *M. anisopliae* (MEl) conidia in blowfly wings.

Plates 14 to 23. Photomicrographs of *M. anisopliae* (MEl) incubated at 27°C for 24 h on excised wings of *C. vomitaria*.

Plate 14. Unipolar germination and bifurcate germ tube. Each germ tube has produced an appressorium.

Plate 15. Conidia and simple lobed appressoria. Note, bipolar germination of uppermost spore.

Plate 16. A compound appressoria formed by a bifurcated germ tube. The way germ tubes encircle setae suggests that their route of growth may follow topographical features.

Plate 17. Short bifurcated germ tube terminating in two appressoria. There is some indication of a mucoid sheath around the bilobed one. In the top left hand corner is an appressorial plate.

Plate 18. Compound appressoria formed of at least three cells. Note clump of ungerminated conidia in bottom right corner.

Plate 19. Compound appressoria with some indication of a mucoid sheath.

Plate 20. Appressorial plate apparently derived from a single conidia (top left of plate).

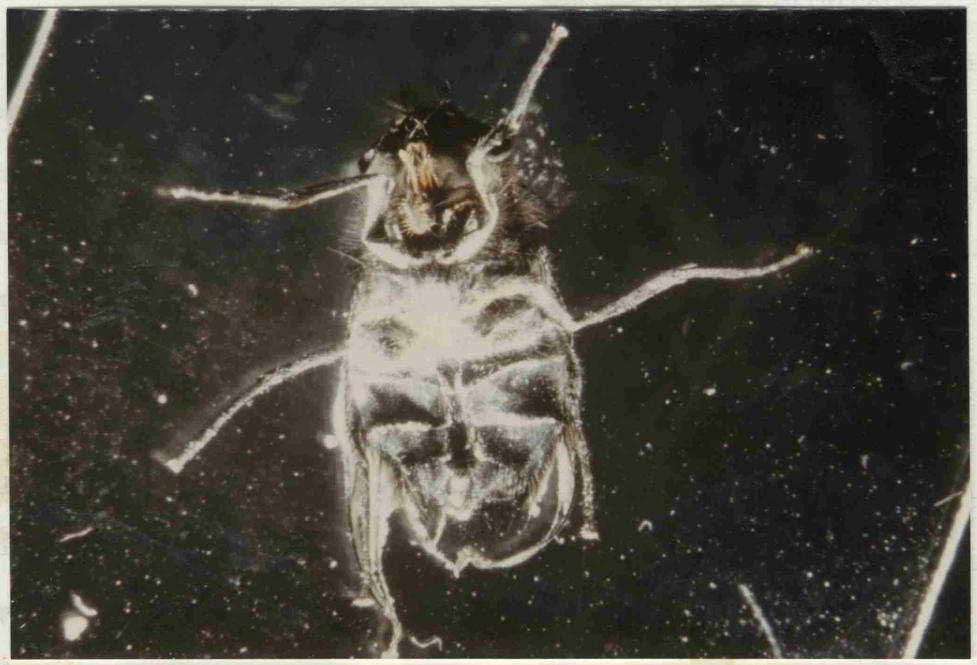
Plate 21. Compound appressoria formed from two hyphae.

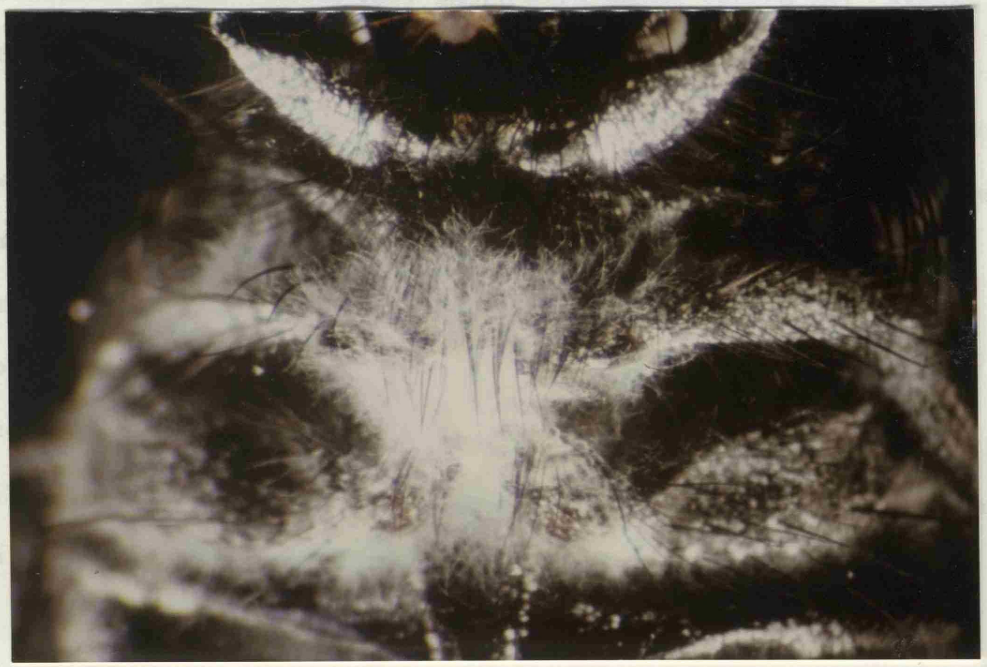
Plate 22. Conidium, germ tube and appressorium. Note penetration filament arising from near the terminal edge of the appressorial cell.

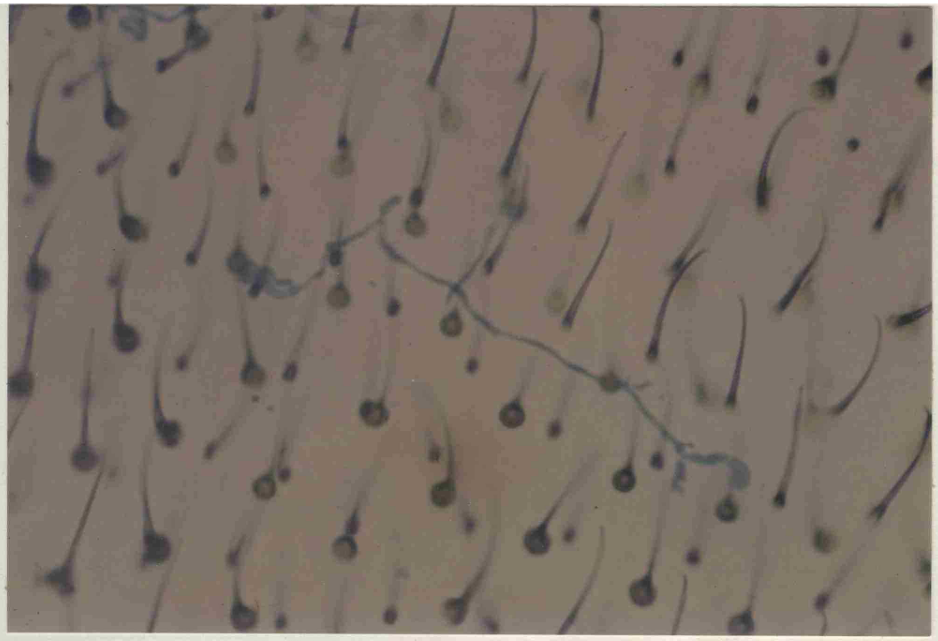
Plate 23. Conidium, germ tube appressorium and penetration filament Note the appressorium is delineated from the germ tube by a septum.

Plate 12. Ventral surface of *Calliphora vomitaria* 8 d after infection with ME1. White hyphae can be seen emerging, particularly through the segmental membranes (X6)

Plate 13. Close up of above specimen, showing thorax (X12).

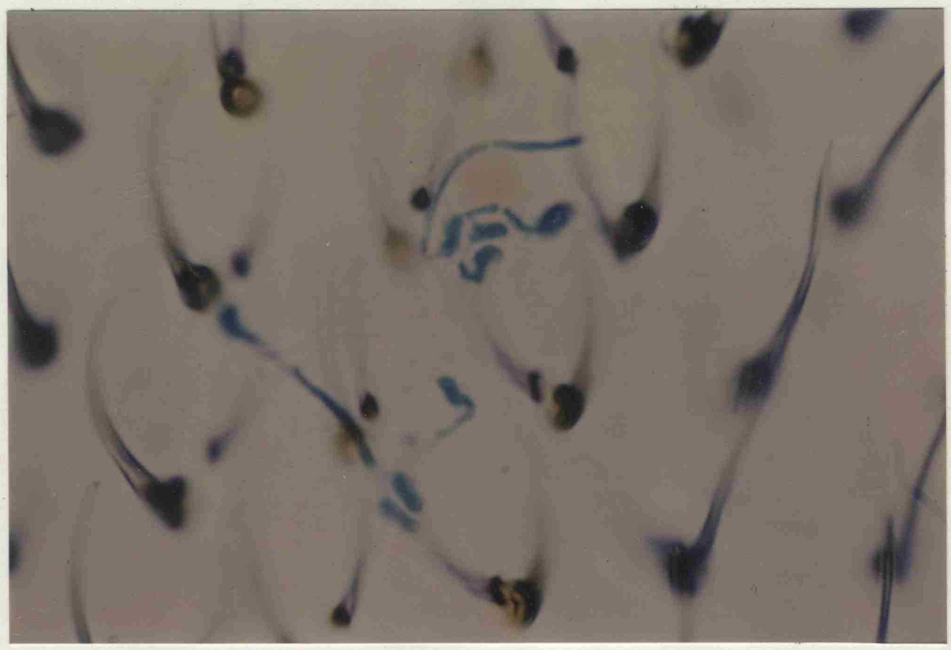






20μm

14



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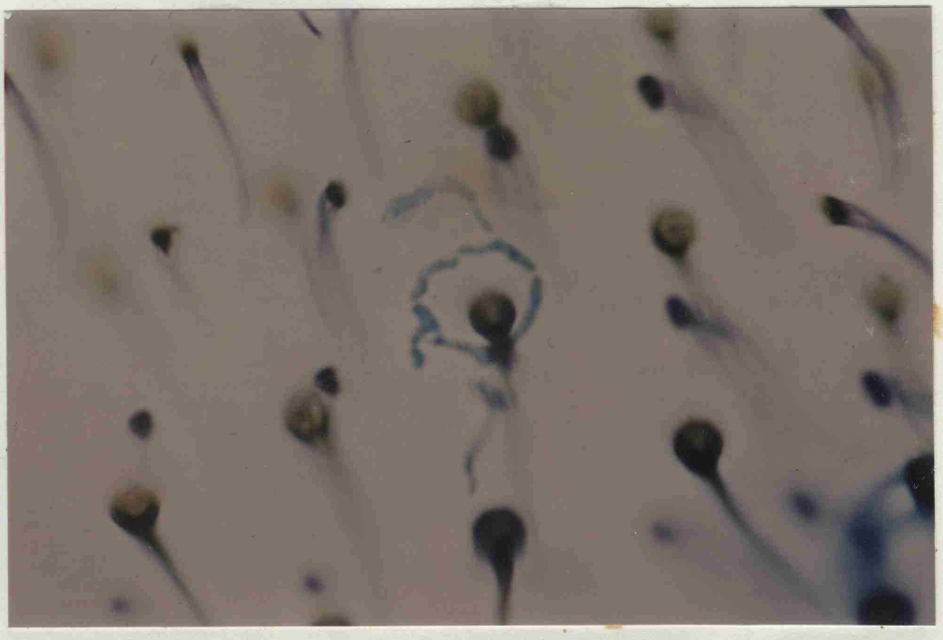

20μm

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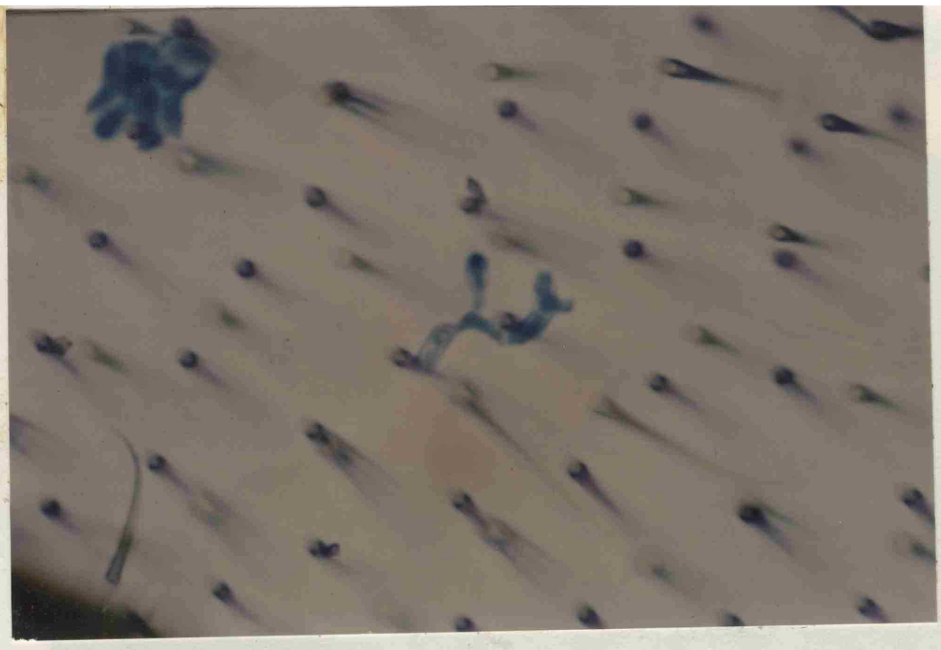

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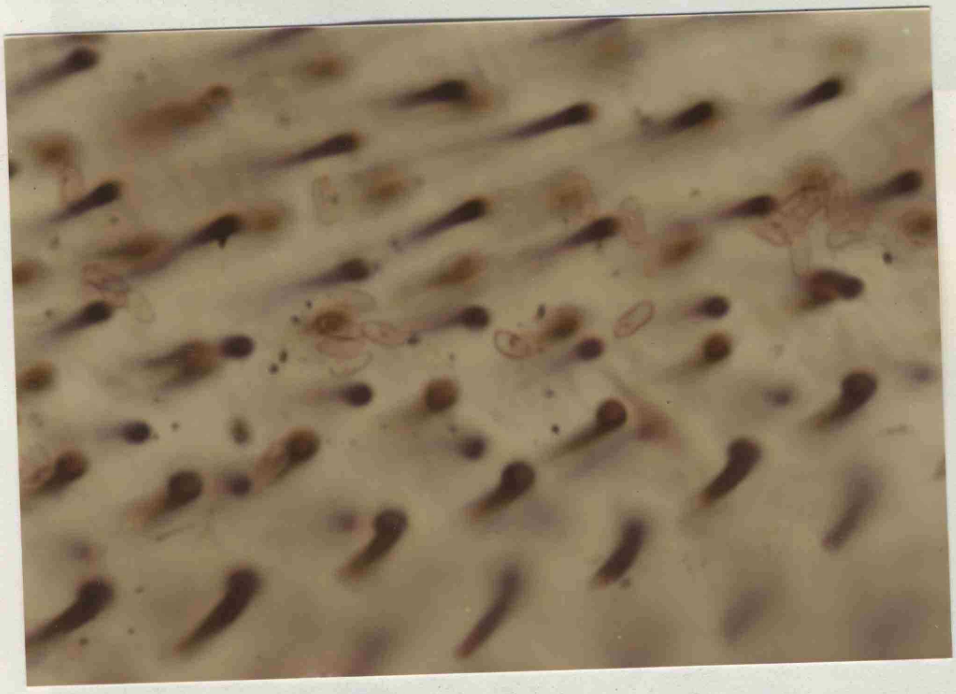

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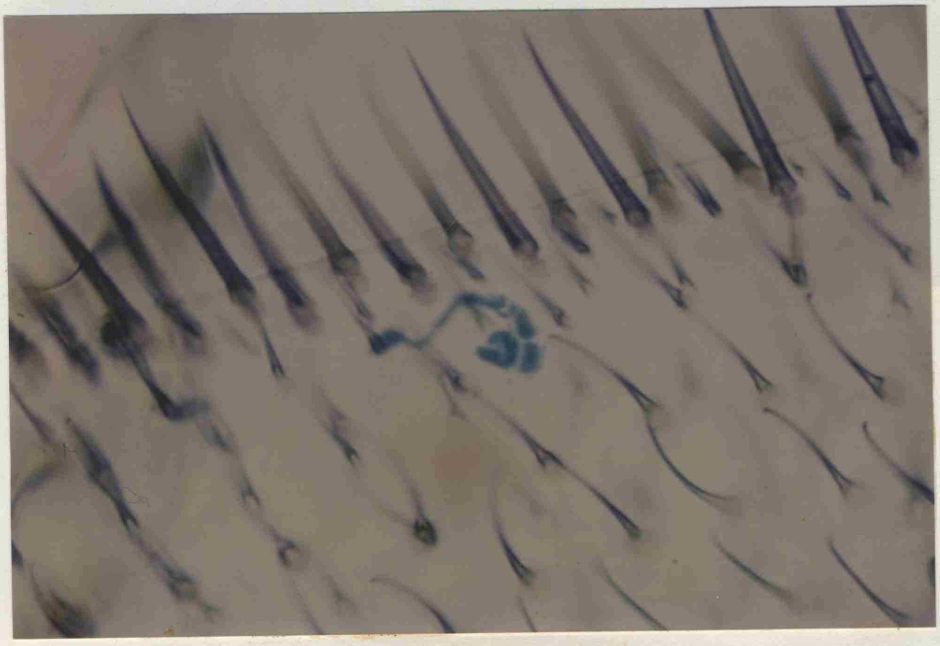

10μm

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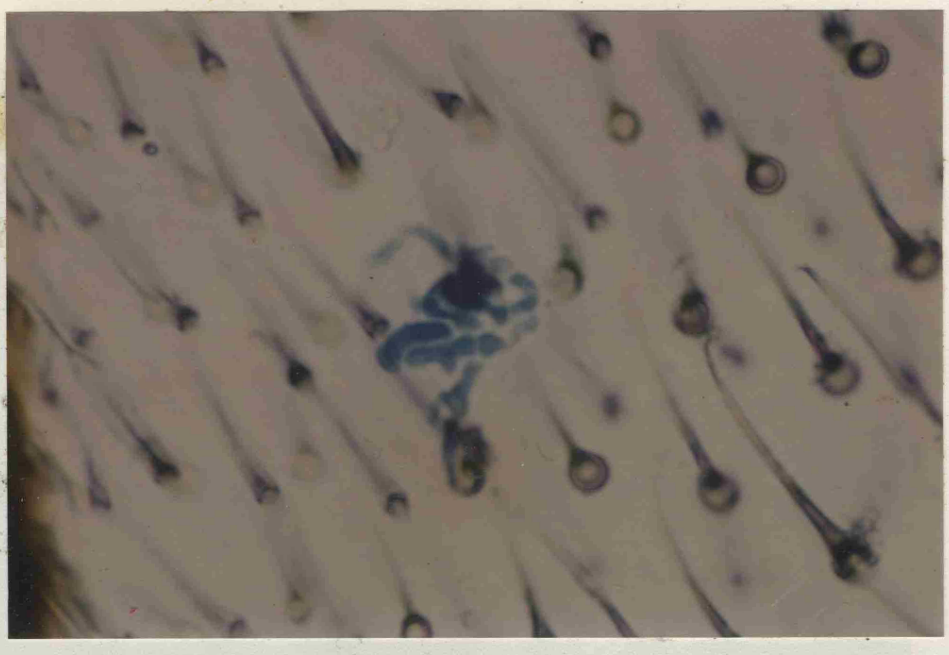

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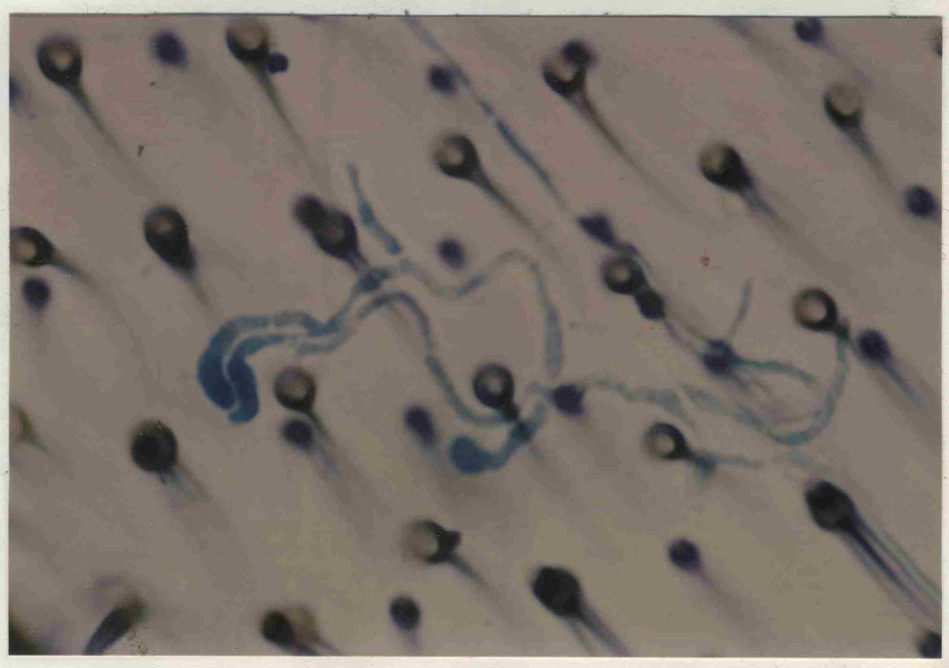

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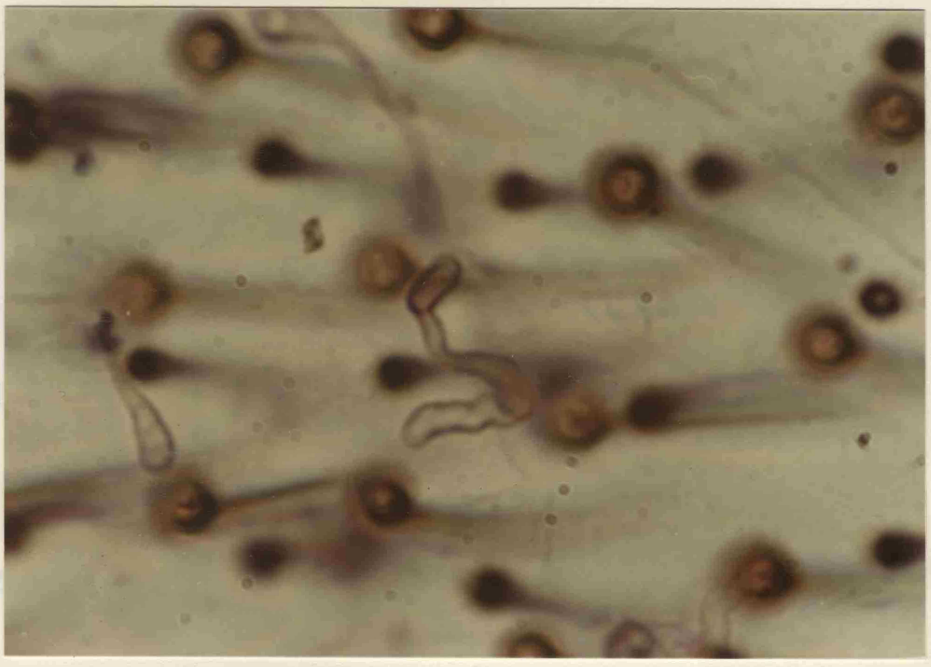

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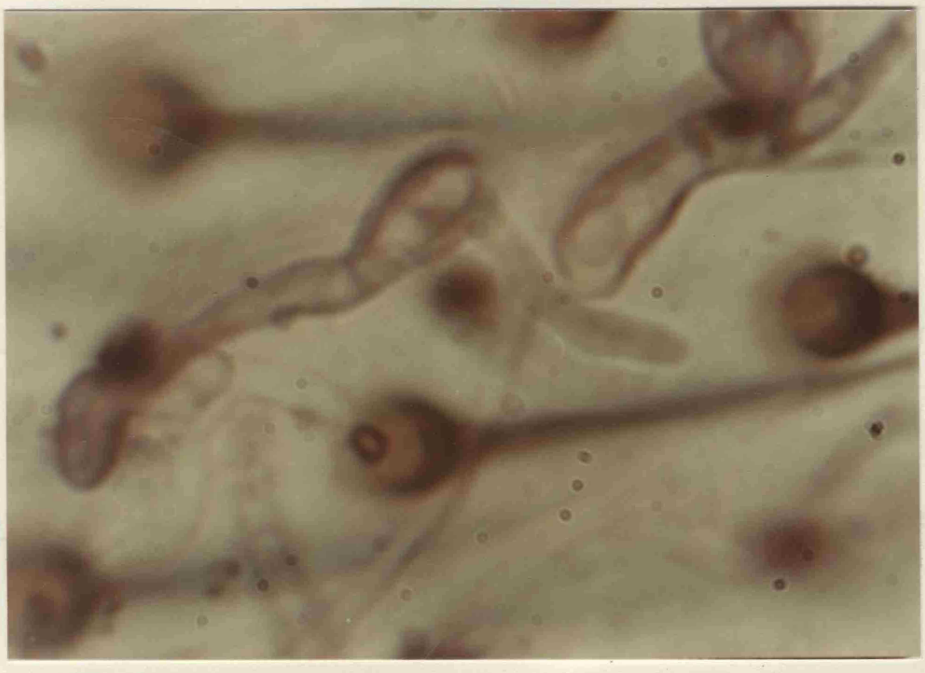

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Plates 24 to 33. Scanning electron micrographs of *M. anisopliae* (ME1) incubated (27°C) on excised integument of *S. gregaria*.

Plate 24. 16 h after inoculation. A cluster of conidia, adhering and germinating on sclerotized cuticle.

Plate 25. 10 h after inoculation. Germinating conidium. Note, germ tube produced lateral to pole.

Plates 26 and 27. 16 h after inoculation. Conidia, short germ tubes and appressoria. Note:

1. Appressoria covered in mucus,
2. Substantial clearing zones around conidia and appressoria.

Plate 28. 16 h after inoculation. Germinating conidia, showing germ tubes developing in close proximity.

Plate 29. 24 h after inoculation. Compound appressoria being formed by germ tubes from 3 conidia.

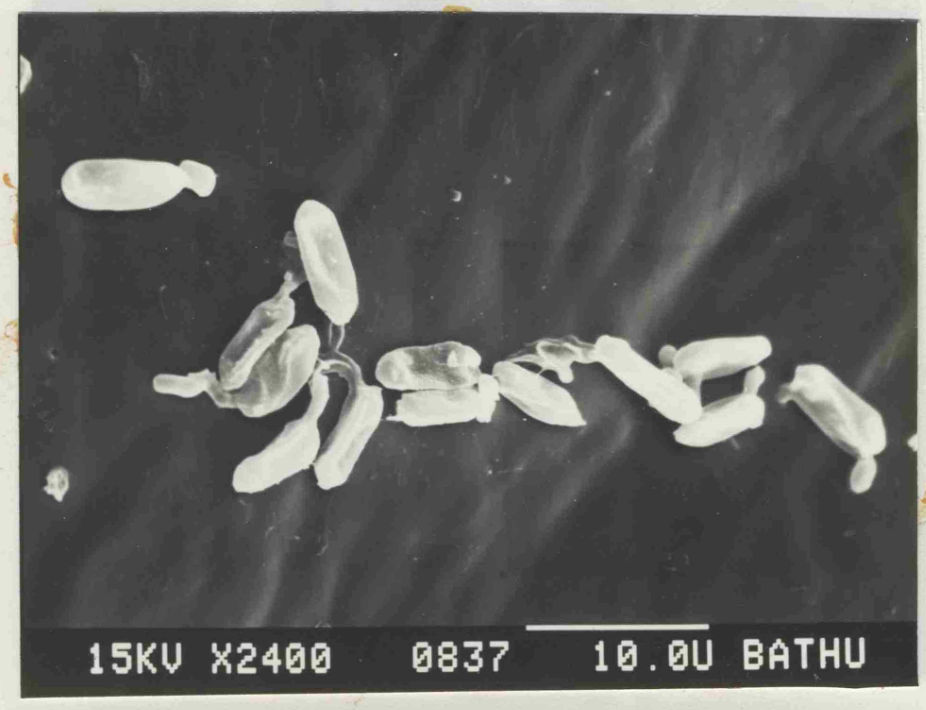
Plate 30. 16 h after inoculation. Hyphal tip, showing mucus production.

Plate 31. 24 h after inoculation. Hyphae connected to host cuticle by mucus.

Plate 32. 24 h after inoculation. Mucus synthesis by adjacent hyphae.

Plate 33. 40 h after inoculation. Undersurface of sclerotized cuticle, showing penetrating hyphae.

Plate 34. Scanning electron micrograph of *M. anisopliae* (RS324) incubated at 27°C for 24 h on sclerotized cuticle of *S. gregaria*. Note Bipolar germination with formation of 2 compound appressoria and mucus.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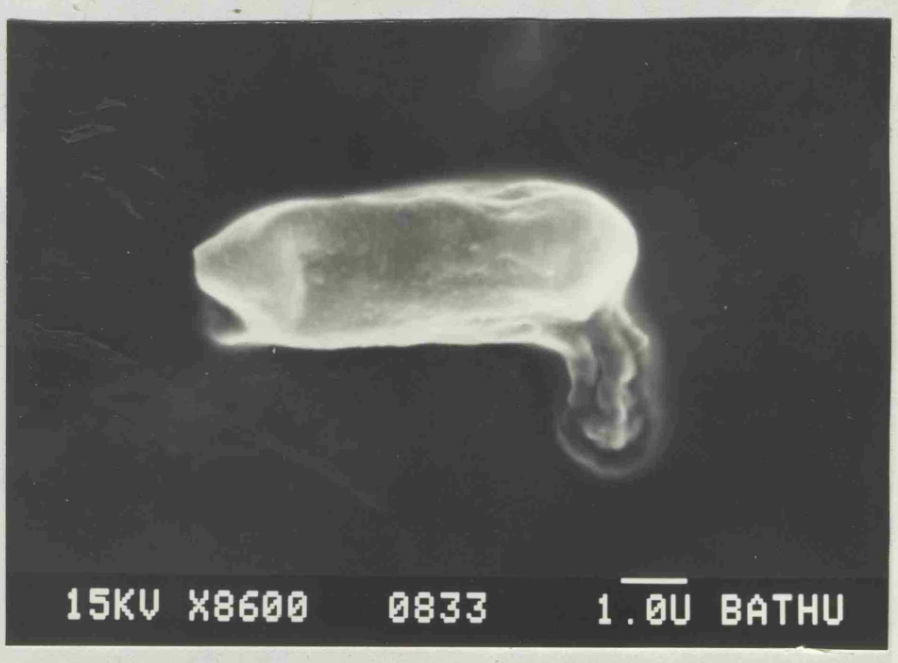
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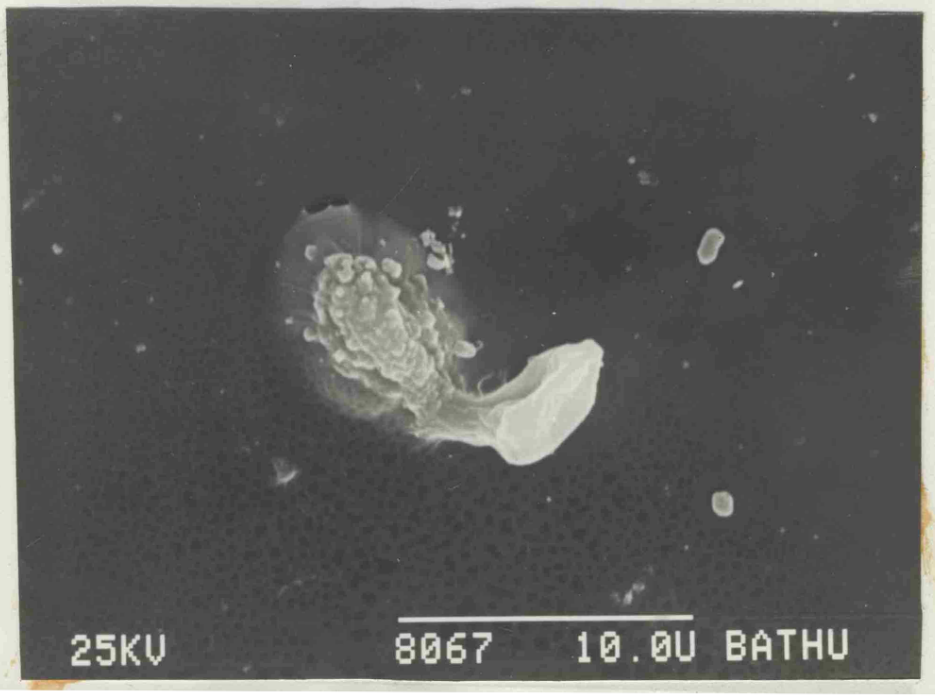
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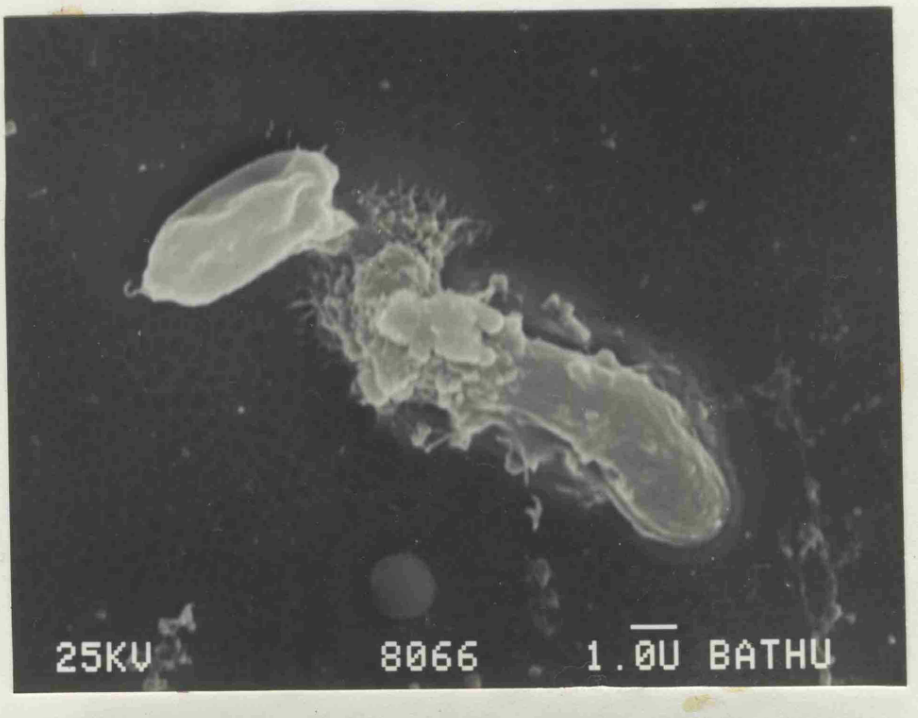
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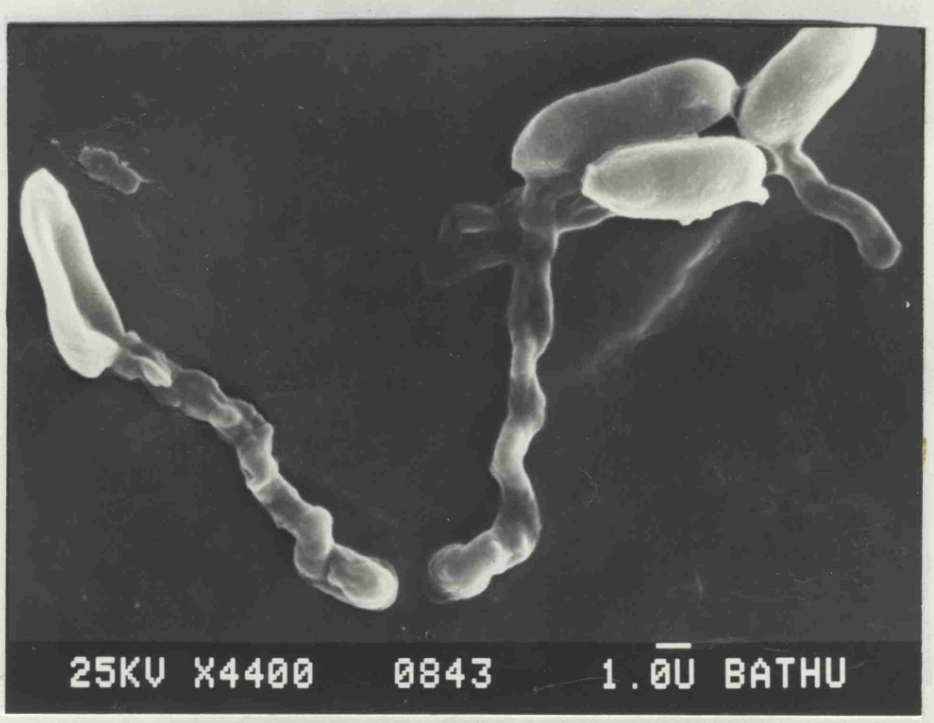
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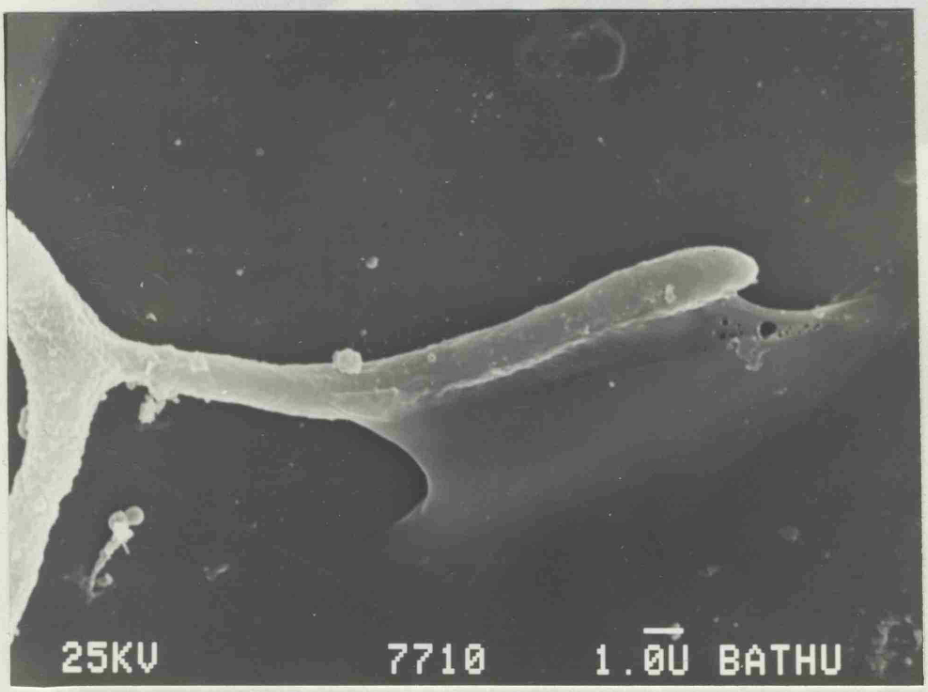
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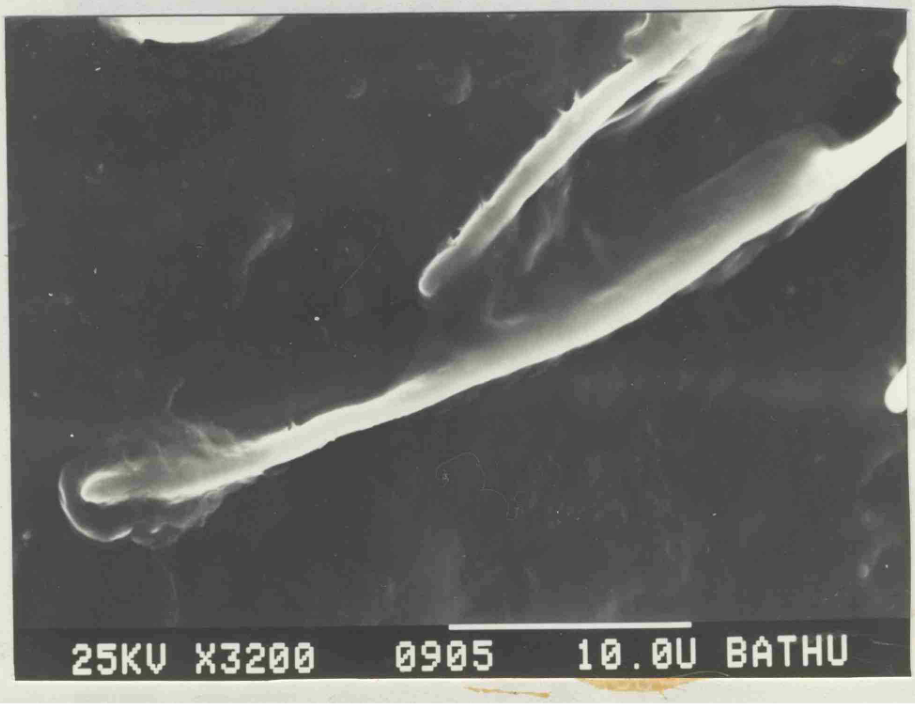
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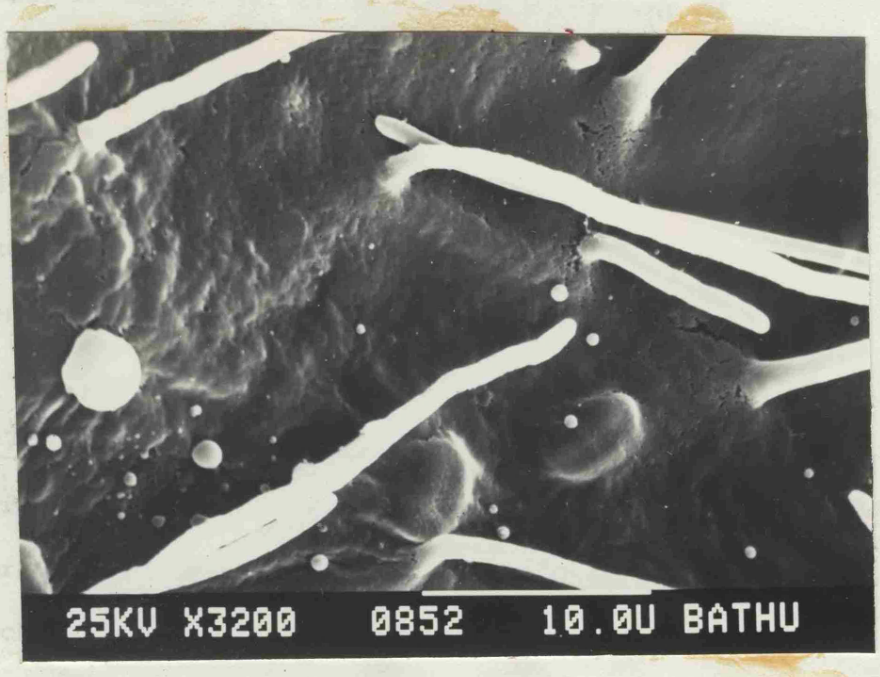

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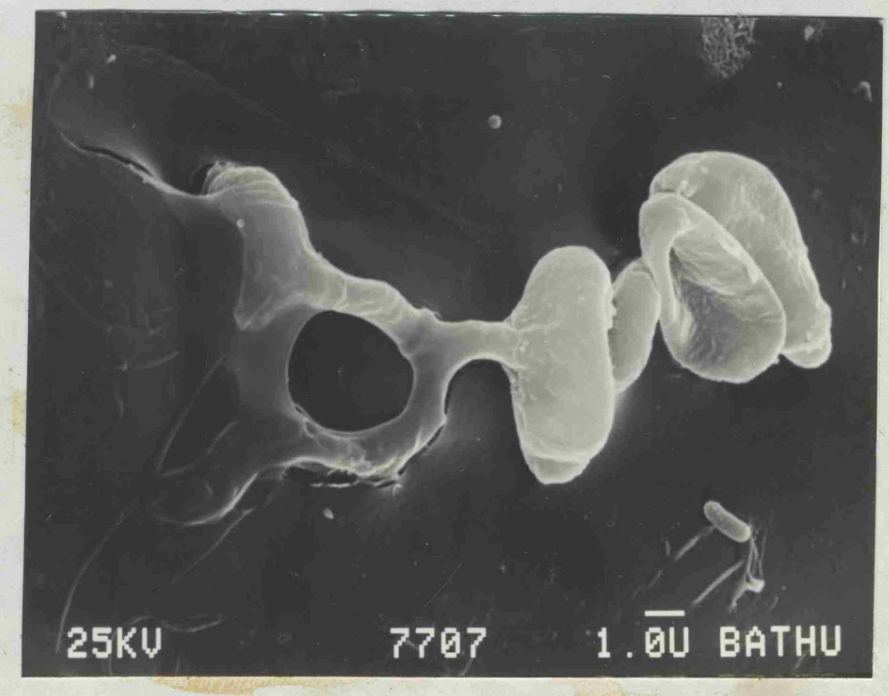

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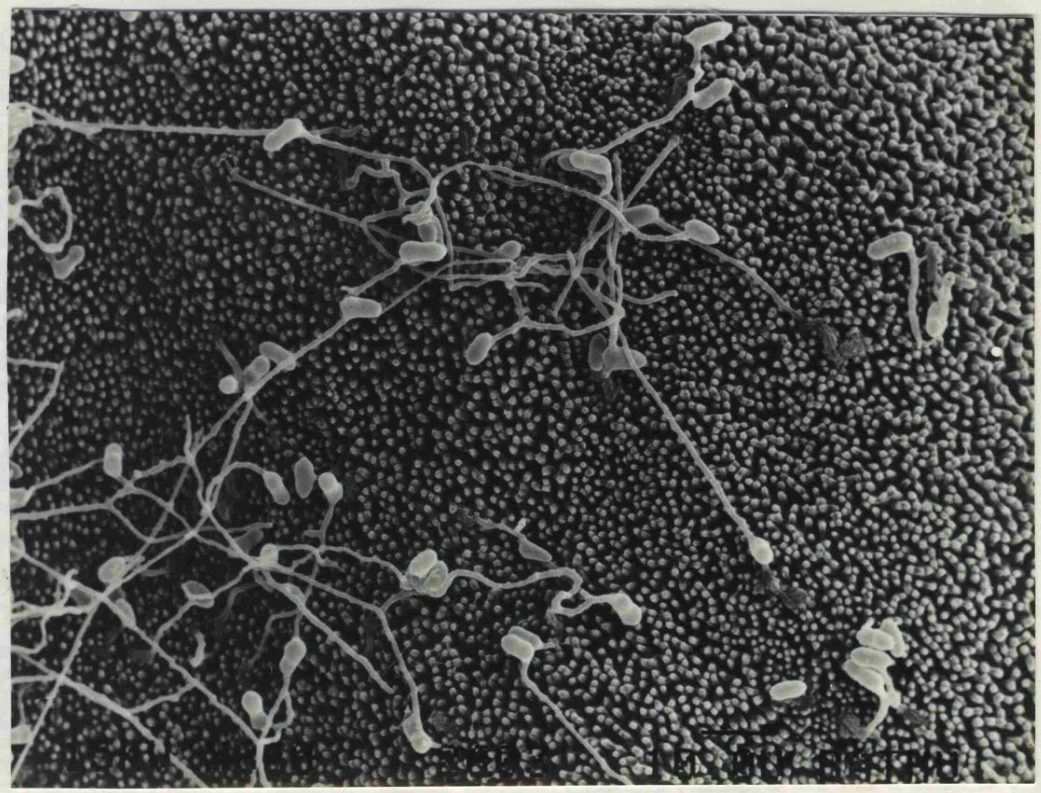
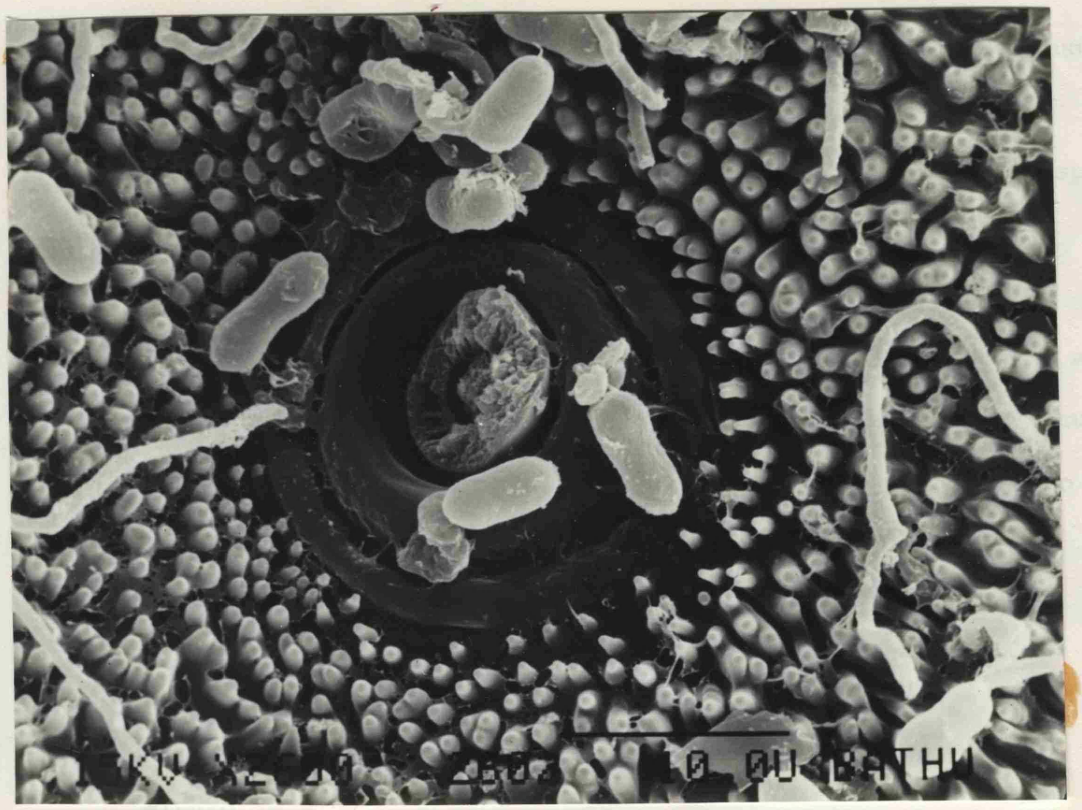
Plates 35 to 37. Scanning electron micrographs of *M. anisopliae* (ME1) incubated (27°C) on excised integument of *M. sexta*.

Plate 35. 24 h after inoculation. Note most germ tubes show extensive growth (up to 400 µm) before direct penetration or production of appressoria.

Plates 36 and 37. 24 h after inoculation. Hyphae growing errantly over the integument. Appressoria were more frequently produced by germ tubes in hair sockets *cf.* other regions of the cuticle.





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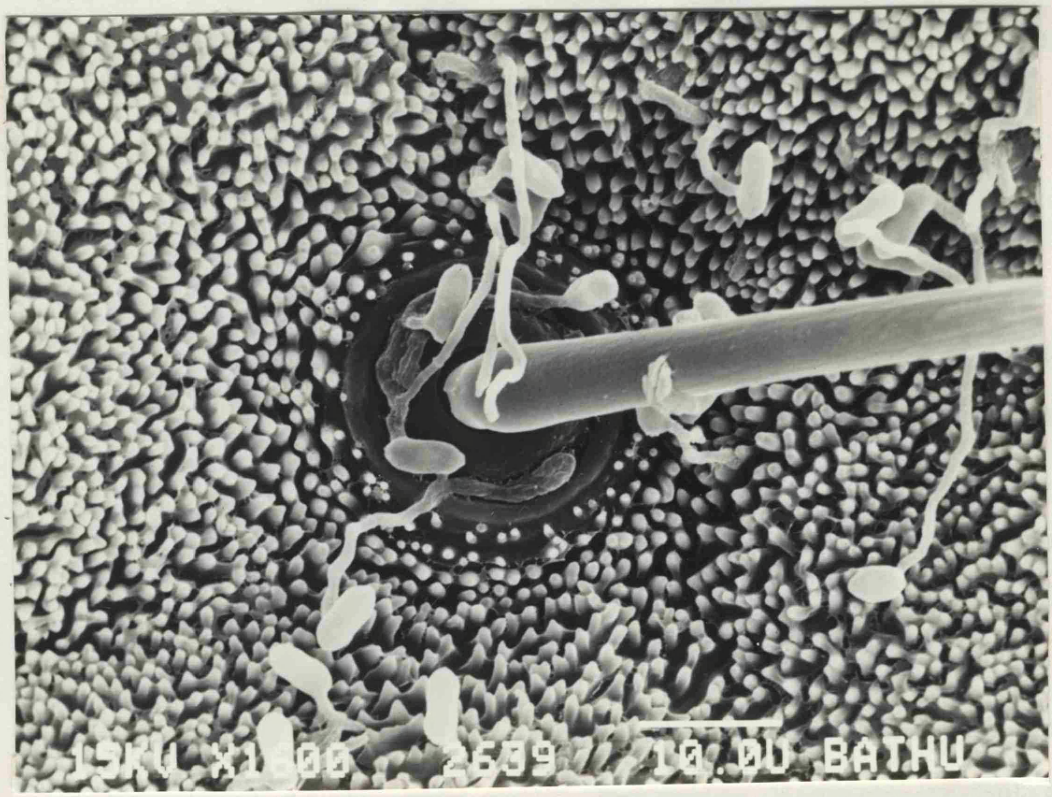


37

microscopy. Large amounts of a diffuse material were evident around hyphal tips and covering appressoria. A similar structure also connected developing hyphae with the host integument and filled spaces between adjacent fungal structures (Plates 26, 30, 31 and 32).

As well as acting as an adhesive, mucilage may also provide a suitable medium from which extracellular enzymes can act on the cuticle. Many of the SEM's of infected locust cuticle show what appears to be clearing zones in the epicuticle surrounding fungal cells, and secretions. These could result from enzymolysis or be merely an artifact of critical point drying (Plates 26 and 27).

Within 40 h of inoculation hyphae began to emerge directly from the apical surfaces of both locust segmental (Plate 33) and soft (non-sclerotised) arthrodial cuticle. This provides clear evidence that ME1 is capable of invasion *via* both sclerotised and unsclerotised cuticles.

3. Features effecting appressorium formation

A. Substrate hardness

Appressoria formed against glass surfaces (Plate 53) as well as on insect cuticle. They were never found on 1.5% agar media nor in shake cultures irrespective of whether growth occurred with a restricted or full supply of nutrients. These results suggest that thigmotropism may play a part in the induction of appressoria.

The effect of substrate hardness on the mode of hyphal growth was investigated with solid medium containing different concentrations

of agar. The results are illustrated in Plates 38 to 41 . Very few hyphae could be seen growing over the relatively soft surface of the 1.5% (w/v) agar. Most had successfully penetrated into the medium. The 3% and 5% (w/v) agar were evidently more difficult to penetrate as substantial surface growth occurred (Plate 39). In most cases penetration into the 5% (w/v) agar was *via* a multititude of side branches but these did not bear the swollen appearance of appressoria. Only a small proportion of the hyphae succeeded in penetrating 9% agar. Most grew on the surface and produced a large number of lobate appressoria. From each of these, filaments extended down into the media (Plate 40) or along the surface (Plate 41). The 9% agar medium was very hard (e.g. difficult to dent with a probe) and presumably correspondingly difficult to penetrate. However, as with fly wings (Plate 22) the penetrating filaments showed no signs of distortion or constriction as occurs during hyphal penetration of exocuticle (Robinson, 1966).

It is evident from these results that contact with a hard surface is an important inducement and perhaps a prerequisite for appressorial formation. This does not however rule out the possibility that other checks to germ tube elongation (i.e. the presence of growth inhibitors) may also be involved in appressorial formation *in vivo* (Emmett and Parbery, 1975; Charnley, 1984).

B. Light

Light directly influences appressorial formation in several plant pathogens (Emmet and Parbery, 1975). However, *M. anisopliae* (MEl) produced appressoria under constant light, constant dark or alternating dark (11 h) and light (13 h) periods.

The Effect of substrate hardness on appressorium formation

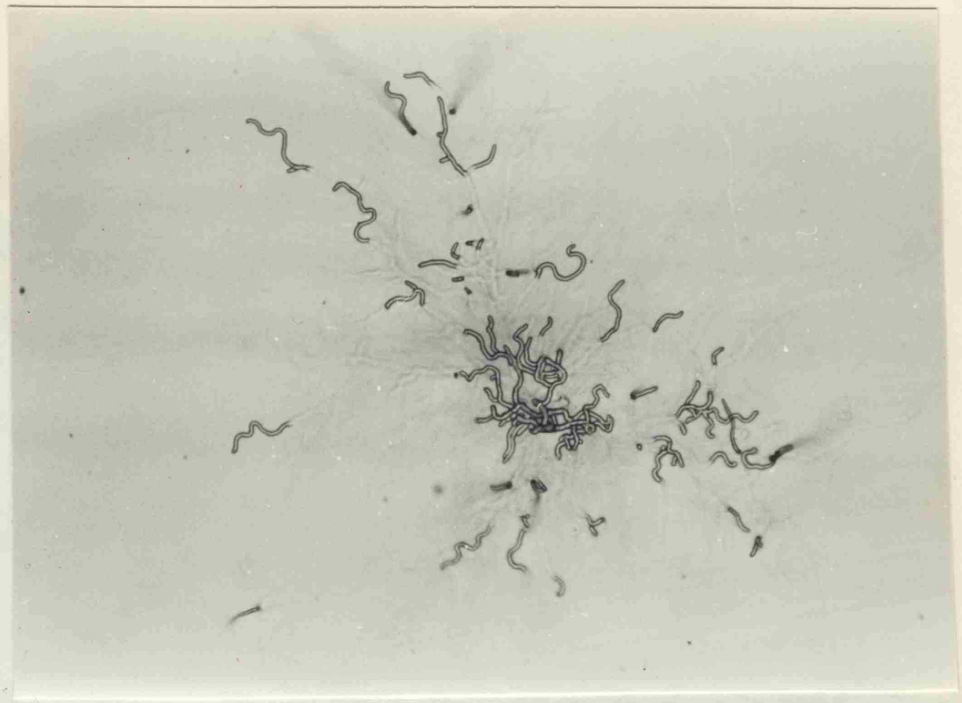
Plates 38 to 41. Growth of *M. anisopliae* (ME1) in solid basal salts medium containing 0.2% NAG and 1.5% - 9% agar.

Plate 38. Growth on media containing 1.5% agar. Note most growth submerged in agar.

Plate 39. Growth on 5% agar. Note hyphae extending down into the media from side branches.

Plate 40. Growth on 9% agar. Note appressoria like hyphae formed laterally against agar surface with hyphae extending down into the media.

Plate 41. Growth on 9% agar. Note a single mycelium showing a structure resembling an appressorial plate. From each appressoria penetration filaments extend down into the media or along its surface .



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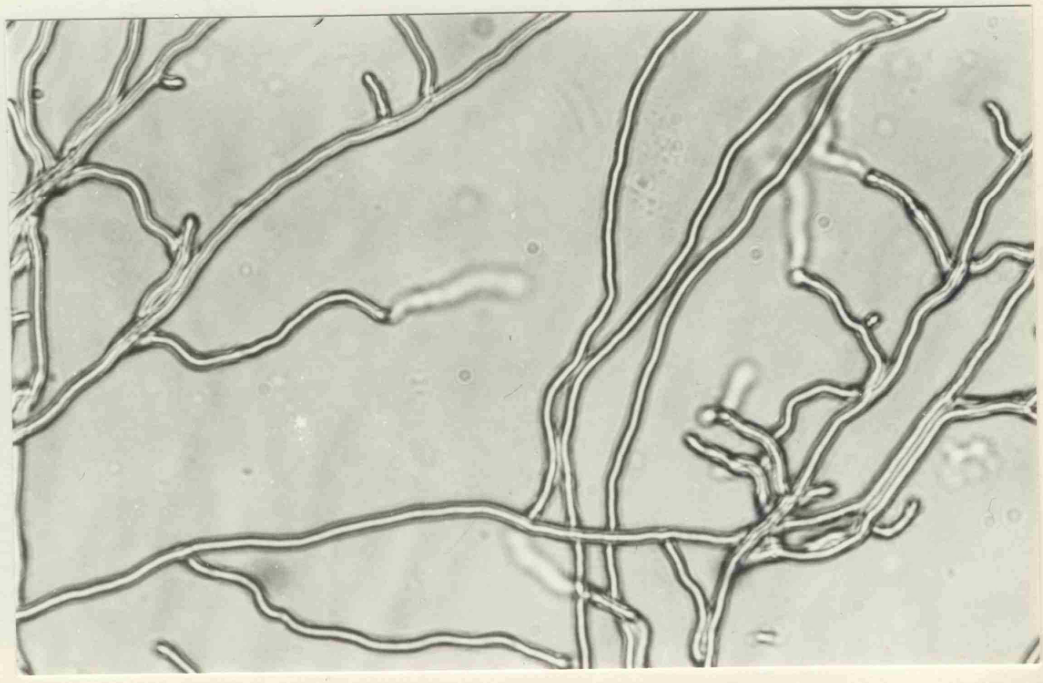

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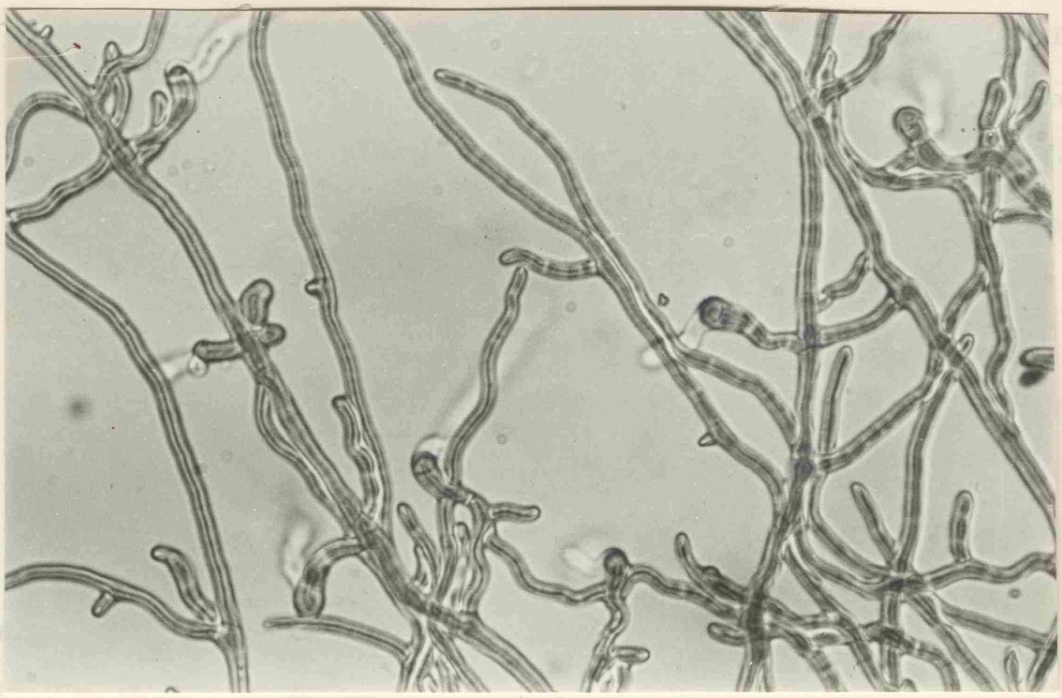

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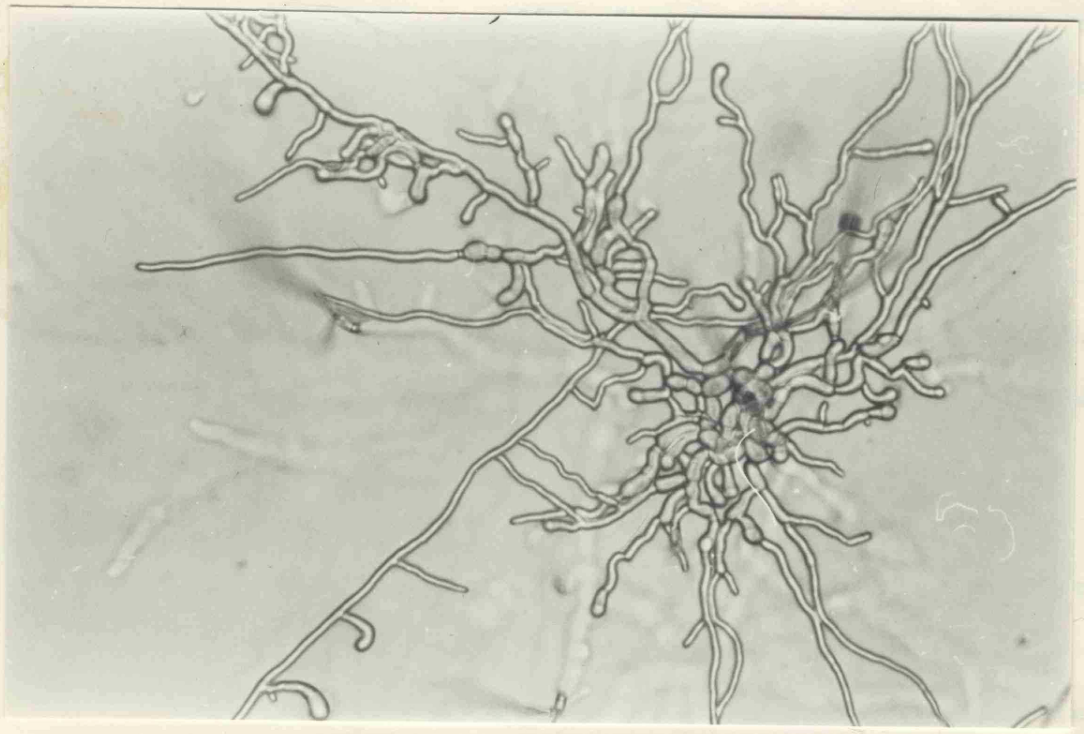

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4. Detection of enzymes produced on insect cuticle

Infected (*c.* 1,500 spores/wing) and control wings of *Calliphora vomitaria* were incubated for up to 40 h at 27°C and extracted by shaking in 0.2 M phosphate buffer, pH 7.0 (2 wings/ml) at 4°C for 1 h (Materials and Methods). The extracts were dialysed and concentrated before assay for enzyme activities.

No enzymes were detectable from uninfected wings during the course of the experiment or from infected wings within 8 h of inoculation (Fig. 60). The inability to detect bound enzymes from ungerminated conidia (*c.* 2.0% germination at 8h, see Fig. 59) was probably due to the miniscule weight of spores applied to cuticle surfaces. Among the first enzymes detected on infected cuticle were those of the proteolytic complex i.e. protease, aminopeptidase and carboxypeptidase); activities were evident *c.* 16 h after inoculation, coincident with the formation of large numbers of appressoria on the wing surface. The protease was identified as trypsin-like, based on PMSF (1×10^{-4} M) inhibition (residual activity, 18%), hydrolysis of the trypsin substrate TAME and an alkaline pH optimum (8.0 - 8.5). BTEE was not hydrolysed indicating that a chymotrypsin had not been produced. On the basis of these results the major protease activity is apparently comparable to the extracellular trypsin-like alkaline enzyme characterised from culture filtrates (Sect. II, 2H). Overnight dialysis against EDTA Na_2 (5mM) severely inhibited the aminopeptidase (> 80%) and to a lesser extent the carboxypeptidase (32%) thus distinguishing a major part of these activities from that of the protease which was unaffected. The relatively minor inhibition of carboxypeptidase suggests that the protease was responsible for a

proportion of the total (low) carboxypeptidase activity (see Sect. III, 2A).

NAG'ase and esterase activities were also obtained from wings 16 h after inoculation. Esterase production closely followed that of the protease and was also inhibited by PMSF (1×10^{-4} M). The NAG'ase however was produced at a slow rate until 24 h post inoculation reflecting the pattern of extracellular enzyme production in cultures grown on comminuted locust cuticle (Fig. 8).

Lipase activity (*vs* an olive oil emulsion) and chitinase were not detected during the 40 h of the experiment. The apparent absence of inducible (see Sect. I) chitinase may reflect the failure of the extraction medium to reach the chitinaceous (pro) cuticle. Also, *in vitro M. anisopliae* (MEl) chitinase binds to locust cuticle and chitin in a manner which cannot be reversed by high ion concentrations (Sect. II, 9A). In later experiments chitinase activity was assayed by measuring the release of chitin hydrolysis products from comminuted infected cuticle. Fly wings (32 h post-inoculation) were comminuted (Materials and Methods) and used simultaneously as enzyme source and substrate by suspending in buffer (2 comminuted wings/ml) at pH 5.0 (0.05 M Cit-PO_4 buffer) or pH 8.0 (0.05 M Tris buffer). The degradation products released from the wing cuticle by the extracted and bound fungal enzymes were determined at intervals up to 72 h. At the end of this period amino sugars (assayed by the methods of Reissig *et al.*, 1955 and Smith *et al.*, 1979) could not be detected, even when the incubation medium was concentrated x5-fold. This was not due to non-accessibility of chitin in comminuted fly wings, because dialysed

crude chitinase, released amino sugars (4 n moles $ml^{-1}h^{-1}$) from the fly wing-substrate. These results suggest that if chitinase is produced by penetration filaments it does not significantly degrade the fly cuticle. In accord with these results, extraction of comminuted cuticle with phosphate buffer (0.2 M, pH 7.0) failed to release chitinase. Lipase was also absent.

In contrast, amino acids were released (0.041 µmoles ml^{-1} and 0.012 µmoles ml^{-1} at pH 8.0 and pH 5.0 respectively) during 24 h incubation of comminuted infected fly wings. Extractions of the residual cuticle in K phosphate buffer (0.2 M, pH 7.0) released both exo- and endo-acting peptidases. Control wings, either uninfected or infected and heated (80°C, 10 min) contained no active enzyme and released only trace levels (< 0.002 µmoles ml^{-1}) of amino acids.

These results suggest that the proteolytic enzymes produced *in vivo* by *M. anisopliae* are capable of degrading the fly cuticle during penetration. As a proportion of the amino acids detected could possibly derive from fungal autolysis, the effect of a dialysed crude protease was tested against fly cuticle at pH 8.0. Significant degradation occurred with the release of 0.032 µmoles $ml^{-1}h^{-1}$ amino acids, indicating that wing-proteins were available for hydrolysis by fungal enzymes.

5. Histochemical localisation of cuticle-degrading enzymes

Attempts were made to demonstrate histochemically lipase/esterase, protease, aminopeptidase and NAG'ase during the pre-penetration growth of *M. anisopliae* (MEl) on blowfly wings. In the only comparable

studies known to the author Michel (1981, unpublished, reported in Fargues, 1984), demonstrated that conidia and/or appressoria of *B. bassiana* produced lipase/esterase and NAG'ase on the cuticle surface of *Galleria mellonella*. However, an attempt to demonstrate protease using N-benzoyl-DL-phenylalanine-β-naphthylamide was unsuccessful. Gabriel (1968) similarly obtained a positive histochemical result for "lipase" at the point of penetration by an *Entomorphthora* sp.

A. Protease

Evaluation of substrate

Of the four substrates tested only the two tripeptides, N-benzoyl-diglycyl-arginine-β-naphthylamide (BGGAN) and N-benzoyl-diglycyl-arginine-4-methoxy-β-naphthylamide (BGGAMN) were hydrolysed to yield coloured products when reacted with Fast blue BB or Garnet GBC (Materials and Methods). N-benzoyl-arginine-β-naphthylamide was not degraded even though, like the tripeptides, it is reported to be a substrate for trypsin (Nachlas *et al.*, 1964). Presumably this reflects a preference for relatively long peptides by the MEl protease.

There was likewise no hydrolysis of the chymotrypsin substrate N-benzoyl-phenylalanine-β-naphthylamide even with incubation periods $\leq$ 12 h. This is in agreement with results obtained testing enzymes produced *in vitro* against specific ester substrates for trypsin and chymotrypsin (Sect. II, 2H), but presumably the negative result could also have been influenced by chain length specificity.

No differences were detected between the sites (see below) of enzyme activity localised with BGGAN or BGGAMN when incubation periods

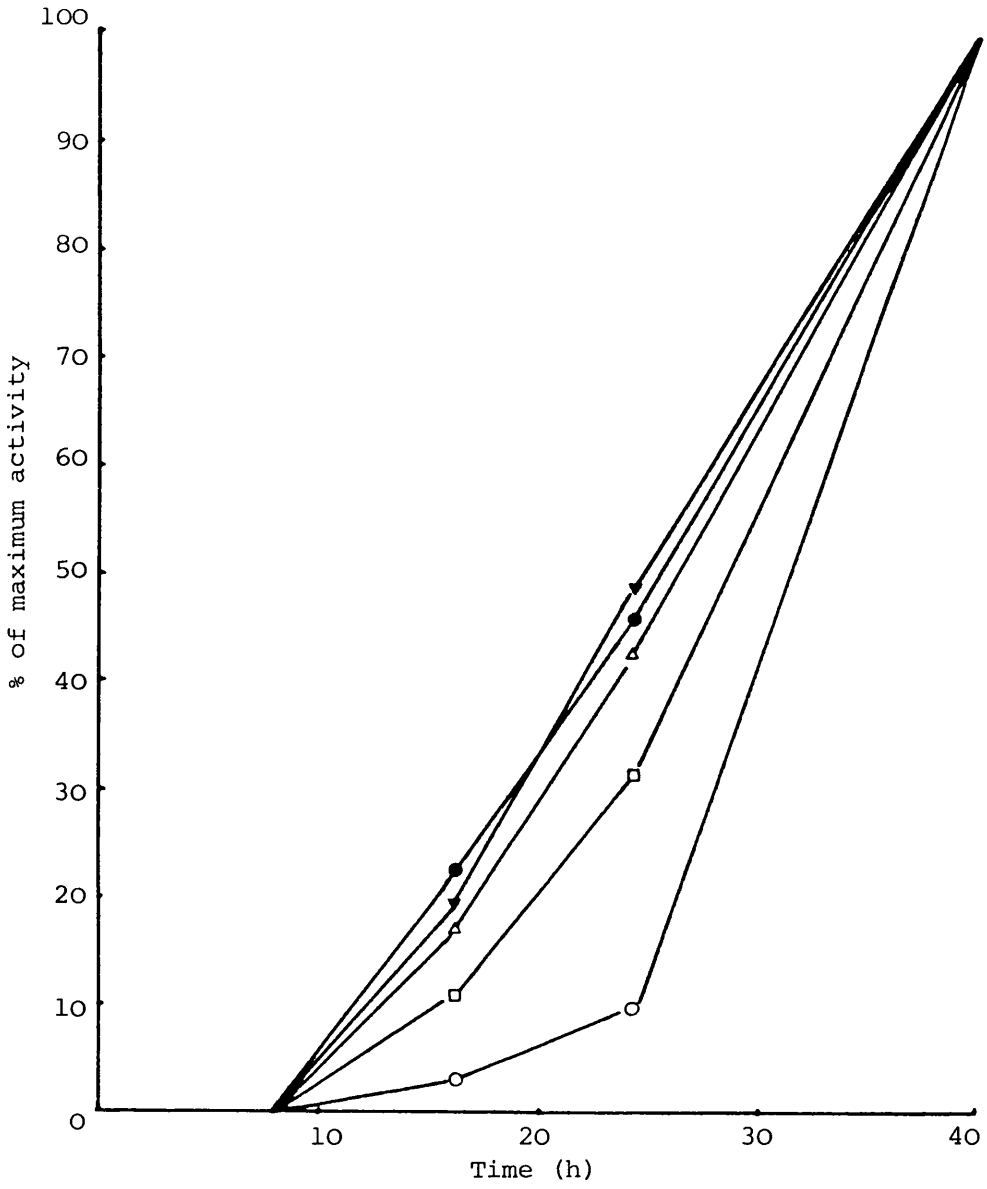

Fig. 60. Cuticle-degrading enzymes produced during penetration of blowfly wings. Enzymes were released by washing MEl-infected blowfly wings in 0.2 M K phosphate buffer, pH 7.0. Enzymes followed by maximum activity are: Protease (●—●) 1.2 µg trypsin equivalents $ml^{-1}h^{-1}$. Aminopeptidase (□—□) 0.11 µmoles $ml^{-1}h^{-1}$. Carboxypeptidase (△ △) 9.4 nmoles $ml^{-1}h^{-1}$. Esterase (▼—▼) 0.13 µmoles $ml^{-1}h^{-1}$. NAG'ase (○—○) 26.3 nmoles $ml^{-1}h^{-1}$. The experiment was repeated three times with similar results.

were less than 30 min (Table 39). After 60 min however the reaction product obtained with β-naphthylamide (BNA) showed additional staining of contiguous cuticular regions. It is known that BNA (breakdown product of BGGAN) couples less rapidly with diazonium salts than 4-methoxy BNA (breakdown product of BGGAMN) (Nachlas *et al.*, 1960) which suggests that the additional staining of the cuticle may be a diffusion artifact. Subsequent to this finding, BGGAMN was used exclusively. The diazonium salts fast blue BB and GBC both gave stable reaction products but a large degree of background staining occurred with the former in both treatments and controls when incubation periods exceeded 4 h. Copper chelation left the red reaction product of either diazonium salt almost unaltered in tone but its use in the assay technique was continued as chelation is reported to result in a more stable reaction product, insoluble in lipid (Nachlas *et al.*, 1960).

Detection *in vivo*

The activity of protease associated with the development of MEl on blowfly wings was followed from germination through the formation of appressorial plates to penetration (Table 39). Proteolytic activity against BGGAMN was detected on the surface of conidia and this increased in intensity during the swelling phase of germination. Additional activity was localised in discrete spherical deposits (0.3 μm to 3 μm in diameter) apparently contained within conidia and usually associated with emerging germ tubes. (Plate 42).In pre-appressorial germ tubes the enzyme activity remained particulate, apparently delimited by the diameter of the hyphae. However ,24 h after inoculation high levels of wall-bound activity were found

associated with all fungal structures (Plate 43a) but not in mucus. In older infections (> 40h) the enzyme was primarily localised on appressoria and appressorial plates suggesting that enzyme which earlier had been found bound to lateral walls may have been partially inactivated (Plate 43b). Often at this time extracellular enzyme appeared on the wing around centres of high protease activity, i.e. appressorial plates (Plate 44; 72 h post-inoculation protease activity had diffused into the agar surrounding the wing (Plate 45).

Pre-incubation for 30 min with PMSF (1×10^{-4} M) almost completely inhibited enzyme activity, a property which is shared with the alkaline serine protease from culture filtrates (Sect. II, 2A).

No enzyme activity was detected in infection structures on wings incubated in solutions devoid of either substrate or diazonium salts; or in heat-treated (80°C, 10 min) preparations incubated with substrate and chromagen.

B. Aminopeptidase

No differences were detected between the sites of enzyme activities localised with L-leucyl-4-methoxy-β-NA and L-alanyl-4-methoxy-β-NA. However staining with the L-alanyl derivative was in all instances far more intense, which is in agreement with results obtained using βNA substrates in the spectrophotometric assay of cuticle culture fluids (Sect. II, 3C).

Alanine aminopeptidase activity was apparent within 5 min incubation on some appressoria, all appressorial plates and in the

mucilage surrounding these structures (Plates 47 to 51 ; Table 39). Far weaker activities (only apparent after longer periods of incubation) were found in swollen conidia prior to germination, emerging germ tubes, small developing appressoria, appressoria which had not produced penetration filaments, and the lateral walls of unmodified hyphae. It may be particularly significant that filaments from appressoria which had apparently failed to penetrate the cuticle were surrounded by mucus containing enzyme activity (Plates 49 & 50). It seems likely therefore that successful penetration filaments also produce mucus and aminopeptidase.

Aminopeptidase production was also studied on surfaces such as glass. As on insect cuticle, high enzyme activity was associated with appressoria (Plate 53). This result confirms the constitutive nature of aminopeptidase (see Sect. I) and it follows that any influence the host surface has on enzyme production is likely to be indirect, i.e. through influencing appressorial formation.

In contrast to protease, preincubation for 30 min with PMSF (1×10^{-4} M) had no effect on aminopeptidase activity indicating that the histochemically-detected enzyme, like that found in culture fluids, was not a serine hydrolase. Controls containing heat-treated hyphae or devoid of the substrate or diazonium salt gave no colour even after 4 h incubation. Incubation periods were not extended beyond 4 h because of spontaneous degradation of the fast blue BB.

As *Manduca* cuticle could be more easily sectioned for microscopy than blowfly wings, it was used in experiments to locate aminopeptidase

on penetrating hyphae. These attempts were thwarted by the production of melanin which obscured the appearance of colour (Plate 52). Some preliminary experiments suggested that cardopin, an inhibitor of melanin synthesis (Reynolds, 1976) may be useful in future experiments.

C. Esterase

It is generally assumed that esters of short-chain fatty acids (C_2-C_4) will be preferentially hydrolysed by esterase, and long chain esters (C_8 and upwards) preferentially split by lipase (Shnitka, 1974).

Non-specific esterase activity was detected histochemically with α-naphthylacetate (C_2) and α-naphthylpropionate (C_3). No differences in enzyme localisation were detected with these substrates but staining with the propionate derivatives was in all instances far more intense. This is in agreement with results obtained using PNP esters in spectrophotometric assays of cuticle culture fluids (see Fig. 47).

Hydrolysis of α-naphthylpropionate was apparent after < 5 min incubation with pregerminating conidia, and appressoria. Staining of conidia was particularly intense (Plate 55) with activity distributed over the surface of the spore, although in some instances discrete spherical deposits of activity, apparently within conidia, were observed. It is possible that the high overall staining of the spore in most cases obscured the appearance of particulate activity.

In common with protease and aminopeptidase, germ-tubes showed only trace levels of esterase activity though some staining of septae

occurred. Appressorial cells showed a strong positive reaction apparently localised principally on cell walls (Plates 56 and 57). There was no evidence for the enzyme in appressorial mucilage.

Preincubation for 30 min with PMSF (1×10^{-4} M) almost completely inhibited esterase activity confirming it was caused by serine enzymes. No sites of enzyme activity were detected when preparations were incubated in solutions minus the substrate or diazonium salt, or when heat treated hyphae were incubated in complete media.

D. Lipase

Lipase activity was localised using α-naphthol AS nononoate (C_9) and Tweens.

Tween substrates

Tweens are soluble fatty acid esters of polyglycols and polysorbital. Fatty acids released by the action of lipase were precipitated as calcium salts; these were converted into lead salts and demonstrated as lead sulphide (Gomori, 1945). In preliminary experiments using 1% lead nitrate solution (Gomori, 1945), patchy non-specific lead depositions appeared on hyphae and cuticle. This problem was solved without apparently influencing enzyme localisation, by diluting the lead solution from 1% to 0.2%.

The extent of activity and the localisation of reaction products varied with the Tween substrate used. The precipitate obtained on hydrolysis of Tween 40 (saturated ester of palmitic acid) was patchily distributed in the vicinity of appressorial plates. Judging by levels

of precipitate, greater activity occurred against Tween 60 (saturated ester of stearic acid) and the precipitate was finer which probably gave better localisation than with Tween 40. Cytochemical localisation of enzyme was only obtained using Tween 80 (unsaturated ester of oleic acid). Crystals of lead sulphide, varying from <0.5 µm to 2.5 µm in diameter sometimes occurred in conidia and in almost all the appressoria that were observed (Plate 58). Usually, each appressorium contained a single crystal situated either centrally or near its extremity. Using Tween 80 no precipitate was ever found outside fungal structures. Presumably therefore either activity against Tween substrates 60 and 80 is due to differently located enzymes or there is some diffusion of the reaction products released from the former.

Preincubation for 30 min with PMSF (1×10^{-4} M) substantially reduced levels of precipitate indicating that activity against Tween 80 was due to serine hydrolase(s).

α-Naphthol-AS-Nonanoate substrate

Enzyme activity detected with α-naphthol-AS-nonanoate (C_9) and fast blue BB was limited almost entirely to conidia even when incubation periods were extended to 4 h. The enzyme(s) responsible therefore was almost certainly different from that which hydrolysed Tween 80.

It was suggested in Sect. II, 4C that enzymes capable of hydrolysing medium length (C_8-C_{10}) PNP esters *in vitro* were probably non-specific esterases as they showed only trace activity against a

C_{18} ester (Fig. 47). It seems probable therefore that activity against α-naphthol-AS-nonanoate detected *in vivo* was also produced by a non-specific esterase rather than a true lipase.

E. NAG'ase

Enzyme activity detected with α-naphthol-AS-βl-N-acetyl-β-D-glucosaminide and GBC was limited almost entirely to conidia (Plate 59), even when incubation periods were extended to 12 h.

Protease detection *in vivo*

Plates 42 to 46. Bright field light micrographs of *M. anisopliae* (ME1) infected blowfly wings, incubated in a medium containing β-GGAMN and GBC to demonstrate protease activity.

Plate 42. Germinating conidia, 16 h after inoculation. Sites of enzyme activity appear as discrete red deposits, from <0.3 μm to 3 μm in diameter. Incubation period, 45 min.

Plate 43a. 24 h after inoculation. Enzyme activity bound to cell walls. Note trace activity only of enzyme in appressorial mucus. Incubation period 10 min.

Plate 43b. 40 h after inoculation. Major enzyme activity localised in vicinity of appressoria and appressorial plates. Incubation period 10 min.

Plate 44. 72 h after inoculation. Enzyme activity localised in vicinity of appressoria and appressorial plates. Note appearance of apparent extracellular enzyme. Incubation period 10 min.

Plate 45. 72 h after inoculation. Extracellular enzyme activity localised in vicinity of wings on agar.

Plate 46. 32 h after inoculation. Infected wings heated (80°C, 10 min) before incubating (90 min) with substrate and chromogen.

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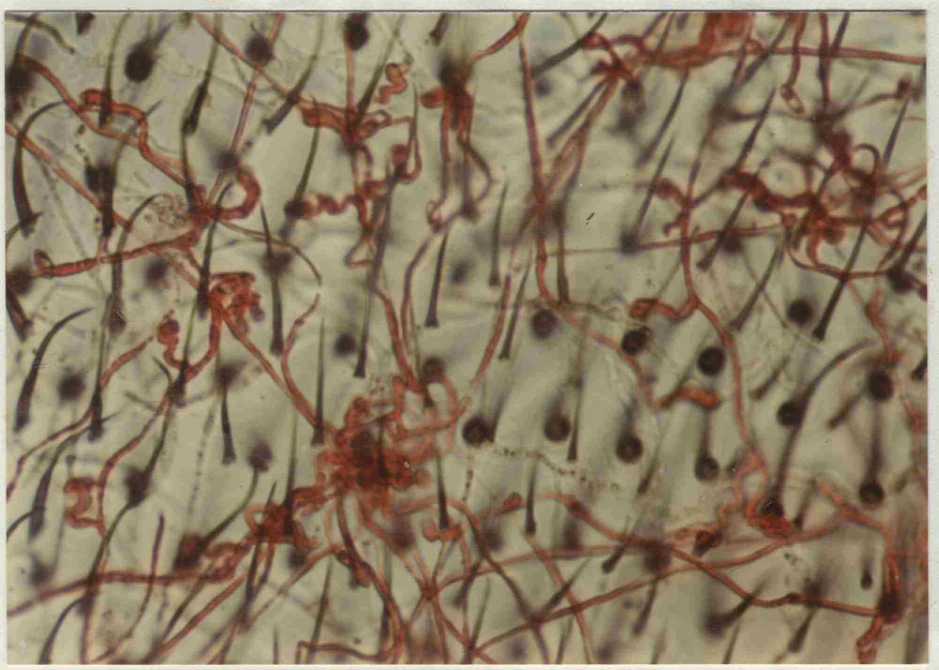

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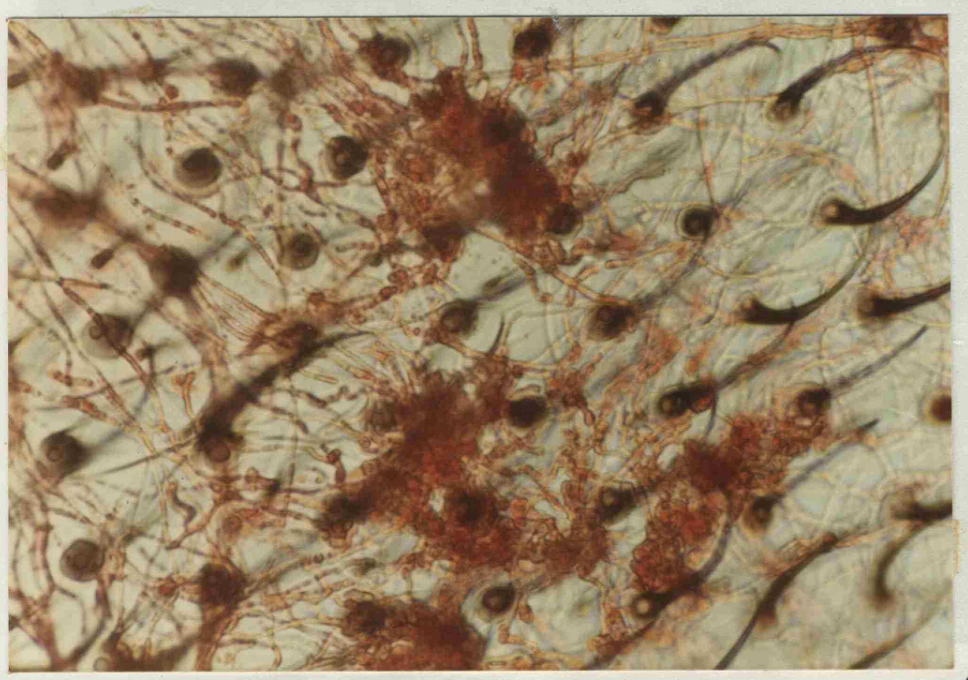

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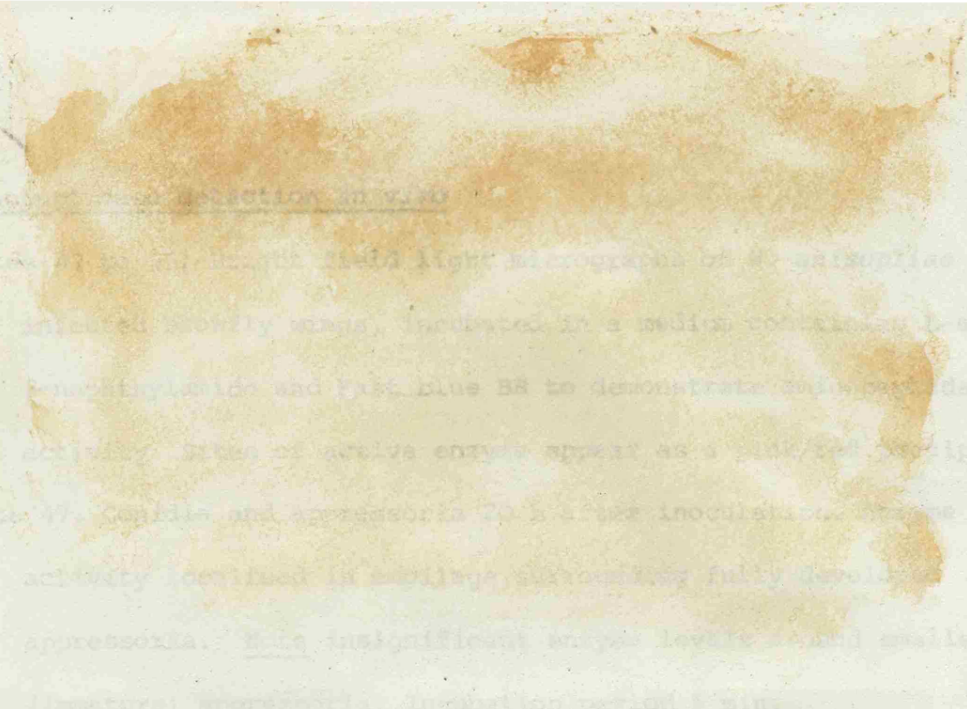

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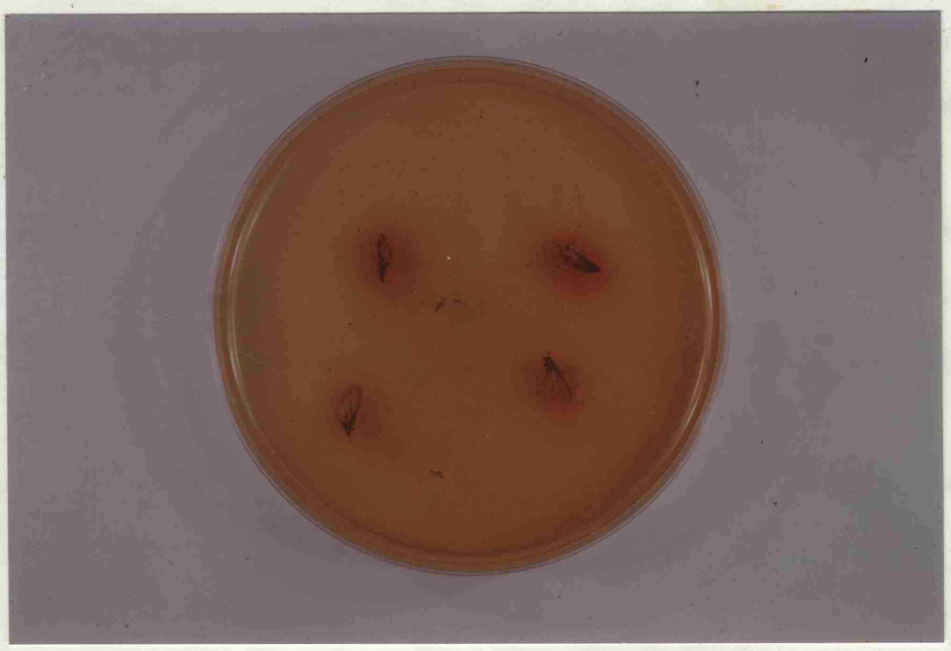

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Aminopeptidase detection *in vivo*

Plates 47 to 53. Bright field light micrographs of *M. anisopliae* (ME1) infected blowfly wings, incubated in a medium containing L-alanyl-β-naphthylamide and Fast blue BB to demonstrate aminopeptidase activity. Sites of active enzyme appear as a pink/red precipitate.

Plate 47. Conidia and appressoria 20 h after inoculation. Enzyme activity localised in mucilage surrounding fully developed appressoria. Note insignificant enzyme levels around smaller (immature) appressoria. Incubation period 5 min.

Plate 48. 20 h after inoculation. Enzyme activity localised in mucilage surrounding appressoria. Note little evidence of enzyme bound to cell walls. Incubation period 5 min.

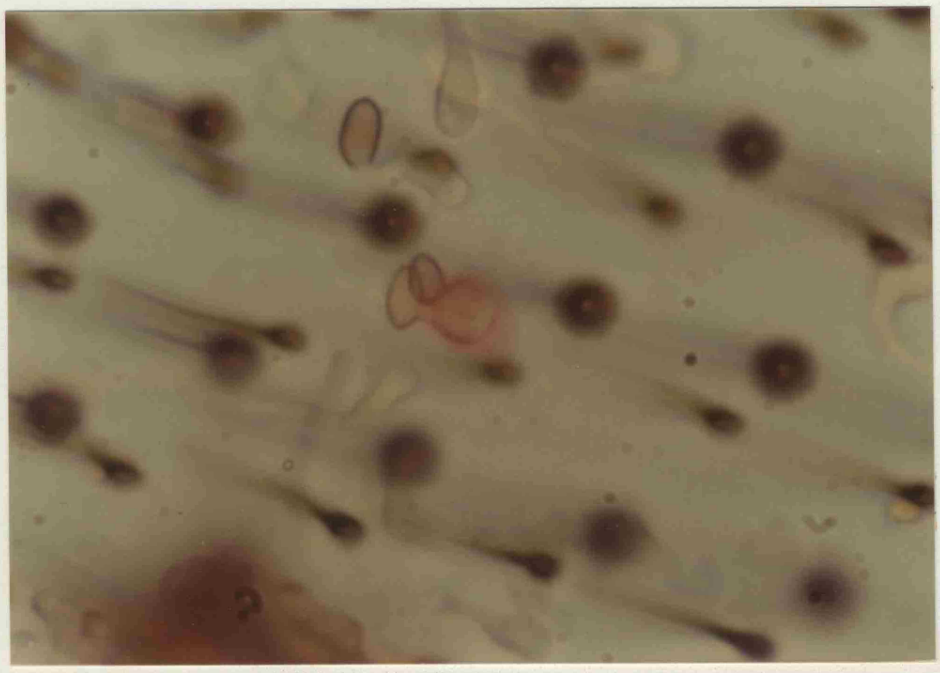
Plate 49. 32 h after inoculation. Note hyphae originating from appressoria which have apparently failed to penetrate the cuticle are surrounded by mucus containing considerable enzyme activity. Incubation period 5 min.

Plate 50. Hyphae originating from appressorium, 32 h after inoculation. Note enzyme is contained in mucus, with little evidence of wall-bound activity. Incubation period 5 min.

Plate 51 32 h after inoculation. High levels of enzyme activity localised on appressorial plates. Incubation period 5 min.

Plate 52. Transverse section (10 μm) of *Manduca* cuticle (cut using a cryostat) showing direct penetration by germ tube. Aminopeptidase activity is located in conidia. Note apparent cuticular "defence" reaction with melanin obscuring the penetrant hyphae. Incubation period 45 min.

Plate 53. Hyphal growth against glass surface, 72 h after inoculation. Note Enzyme activity localised on appressoria. Incubation period 5 min.



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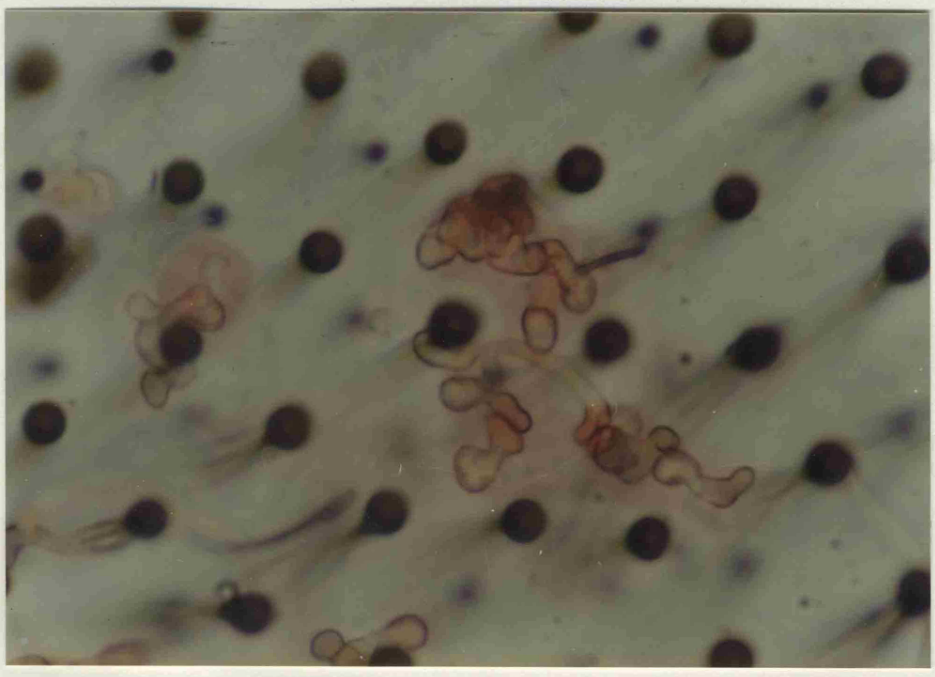

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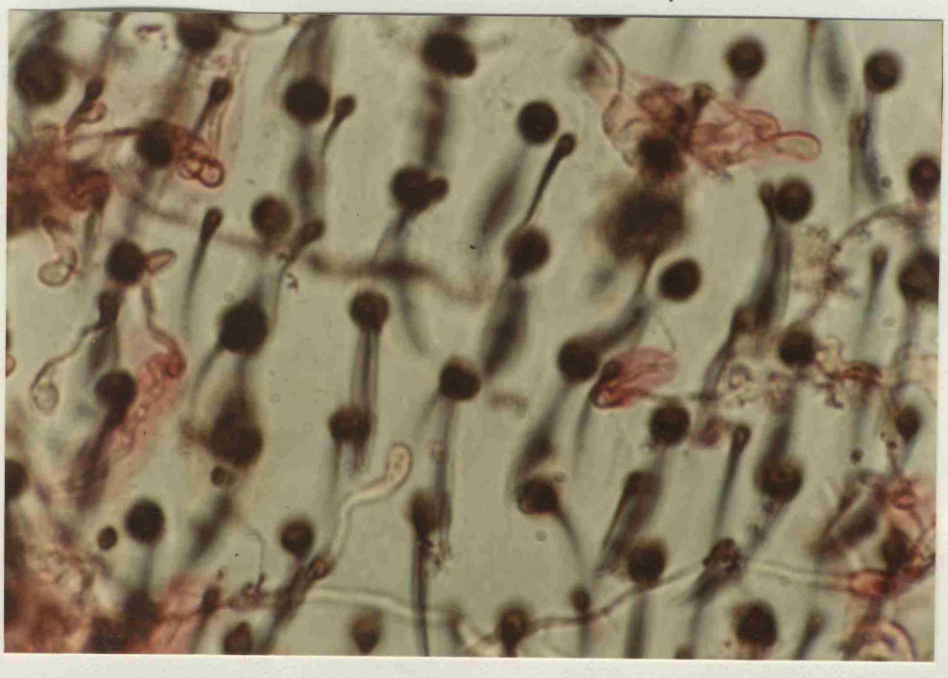

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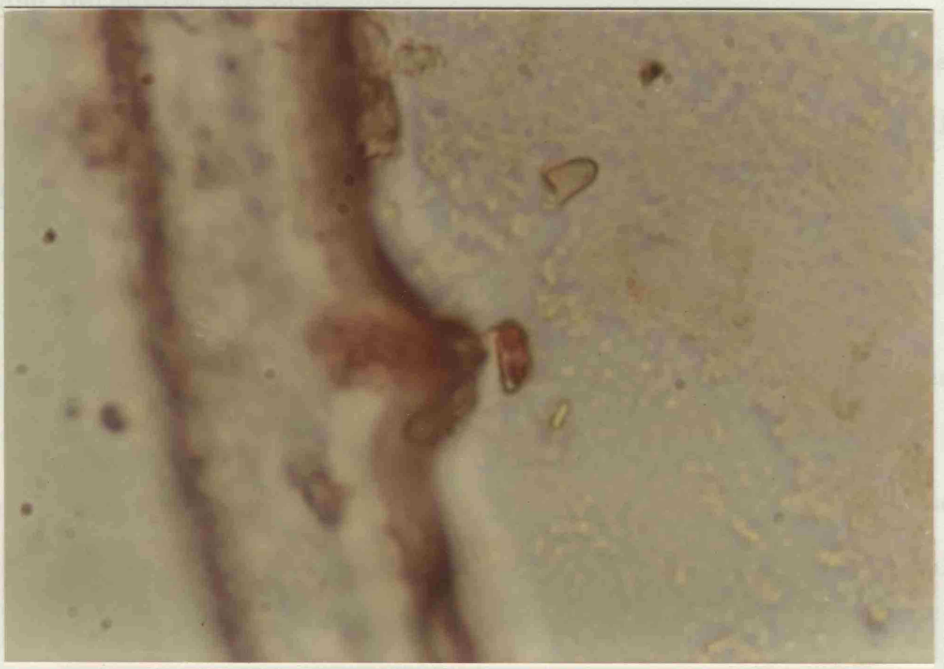

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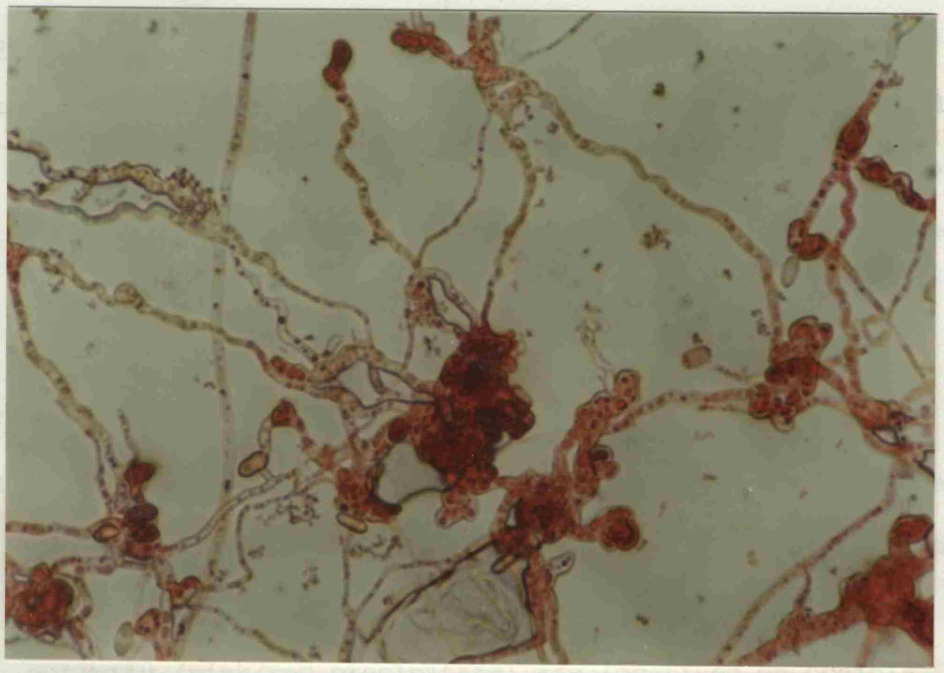

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Esterase detection *in vivo*

Plates 55 to 57. Bright field light micrographs of *M. anisopliae* (ME1) infected blowfly wings, incubated in a medium containing α-naphthol propionate and Fast blue BB to demonstrate non-specific esterase activity. Sites of active enzyme appear as a violet/mauve precipitate.

Plate 55. Conidia 5 h after inoculation. Note strong positive reaction for esterase. Incubation period 5 min.

Plate 56. 32 h after inoculation. Enzyme activity mainly localised on cell walls of appressoria. Note some evidence of extracellular activity. Incubation period 5 min.

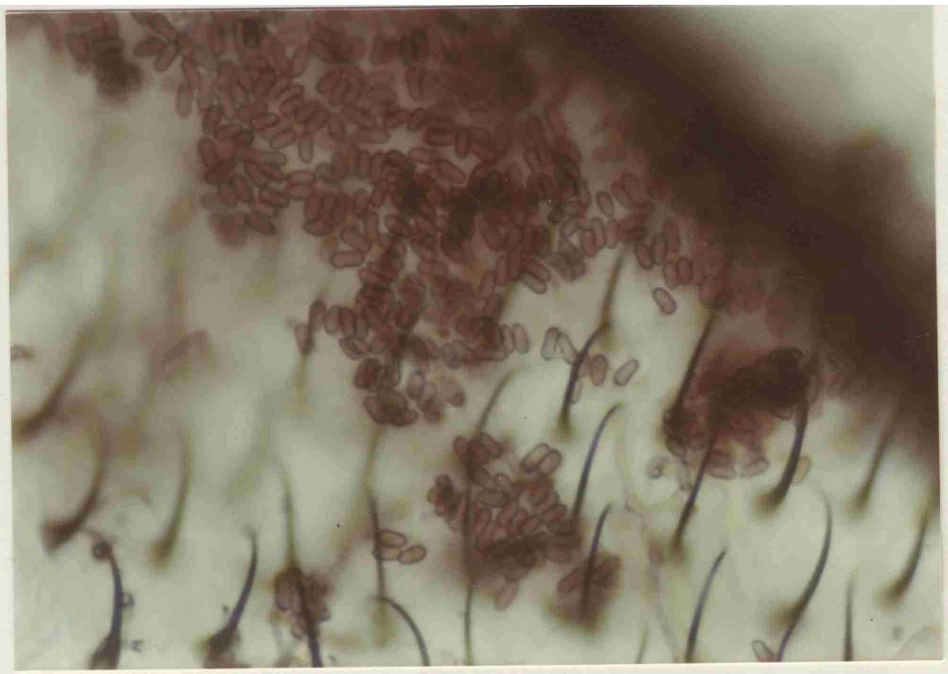
Plate 57. 32 h after inoculation. Activity principally localised on appressorial cells, with additional staining of septa. Incubation period 5 min.

"Lipase" detection *in vivo*

Plate 58. *M. anisopliae* (ME1) infected blowfly wings, incubated in a medium containing Tween 80 and $CaCl_2$ to demonstrate lipase activity. Sites of enzyme activity (black precipitate) are localised in appressoria. Incubation period 8 h.

NAG'ase detection *in vivo*

Plate 59. *M. anisopliae* (ME1)-infected blowfly wing, incubated in a medium containing α-naphthol-AS-β1-N-acetyl-β-D-glucosamine and GBC to demonstrate NAG'ase activity. Sites of enzyme activity (pink/red precipitate) are localised on conidia. Incubation period 1 h.



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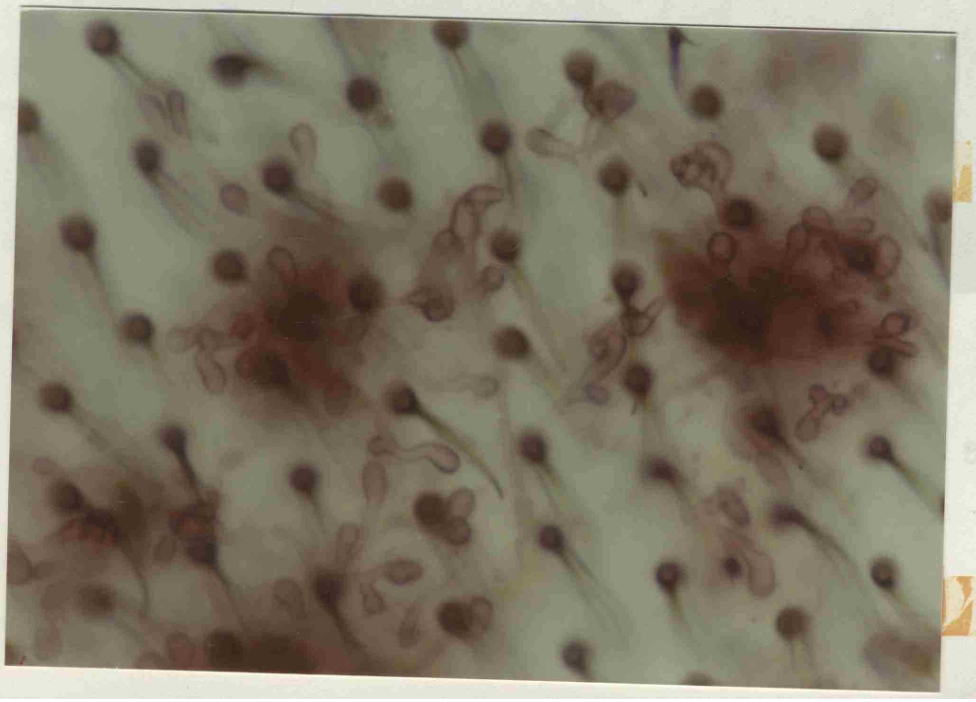

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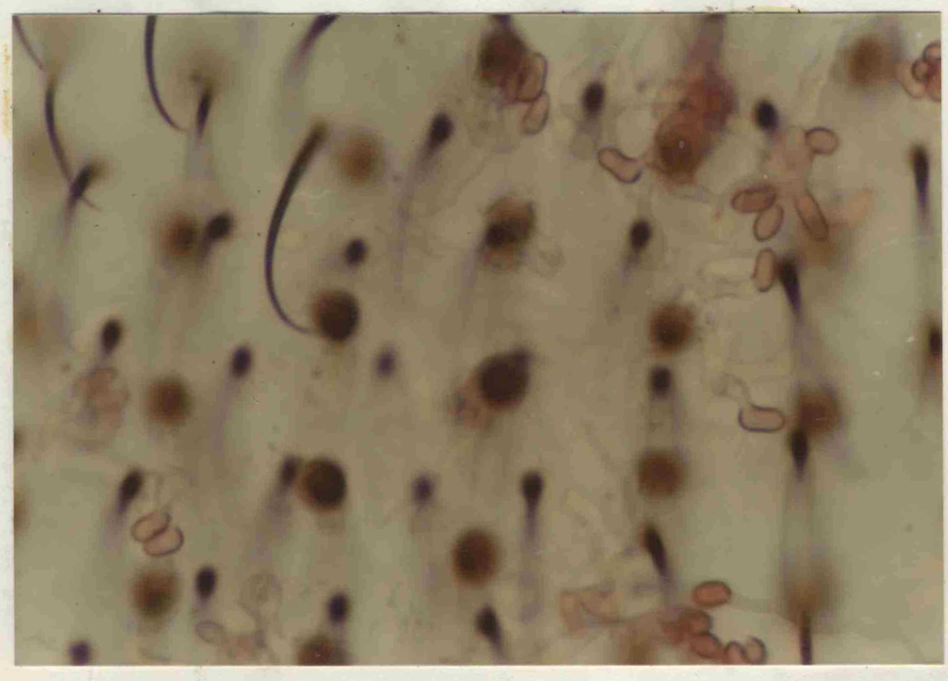

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Table 39. Histochemical localisation of cuticle-degrading enzymes produced *in situ* during the early stages of MEl infection on blowfly wings.

Activity	Substrate	Pre-germinating conidia	Germinating conidia	germ-tubes	Developing appressoria	Mature appressoria	Appressorial mucilage
Protease	BGGAMN	+	++	+	++	+++	-
Aminopeptidase	L-Alanine MN	-	+	-	-	+++	+++
"Lipase"	Tween 40	-	-	-	+	+	-
	Tween 60	-	-	-	+	++	-
	Tween 80	-	+	-	++	+++	-
	α-naphthol-AS-nonanoate	+	+	-	-	-	-
Esterase	α-naphthyl-propionate	++	++	+[a]	++	+++	-
NAG'ase	Naphthol-AS-βl-N-Acetyl-β-D-glucosaminide	+	+	-	-	-	-

a) localised on septa, weak (+), moderate (++) and strong (+++) positive reactions.

6. Fluorescent staining of fungal structures

Results from histochemical and SEM studies suggested that the production of enzymes and mucus occurred principally at the hyphal tips and appressoria. It is possible therefore that these structures may have walls with an increased permeability to macromolecules.

In all filamentous fungi investigated the wall is made up of several layers, the inner most of which is composed predominantly of chitin or β(1-3) glucans. Outer layers appear to contain little or no chitin (Burnett, 1979). It follows therefore that calcofluor M2R New which binds to β-glucans and chitin (Kritzman *et al.*, 1978) or wheat germ agglutinin (WGA) which is specific to chitin oligomers (Pistole, 1981) would preferentially bind to areas where the outer wall polymers are incomplete and which as a consequence may be more permeable.

Regions of intense fluorescence were found on hyphal tips and septa of young fungal colonies which had been stained with either calcofluor or FITC-WGA (Plates 60 &61). It is noteworthy that binding of FITC-WGA even occurred on hyphae from 1% chitin/basal salts media (Plate 61) which had high extracellular chitinase activities (0.72 - 1.32 μmoles NAG $ml^{-1}h^{-1}$). This suggests that some aspect of the organisation of the apex polymers protects the chitin from enzymolysis.

Colonies from older cultures in which growth had ceased showed far less overall binding of either stain and no localised regions of intense fluorescence. Ungerminated conidia also failed to stain.

Appressoria produced terminally to germ tubes on cuticle or laterally to hyphae on slide cultures (Materials and Methods) fluoresced with both calcofluor and FITC-WGA (Plates 62 & 63). This indicates that the cell walls of these structures like unswollen hyphal tips possess different properties to ordinary lateral walls. No labelling with FITC-WGA was observed when the colonies were preincubated with non-fluorescent WGA or when treated with FITC-WGA preincubated (30 min at 35^{o}C) with chitotriose (2 mM); a specific inhibitor of the lectin (Mirelman *et al.*, 1975).

Plates 60 to 63. Microscopic appearance of *M. anisopliae* (ME1) incubated with calcofluor M2R New or WGA-FITC.

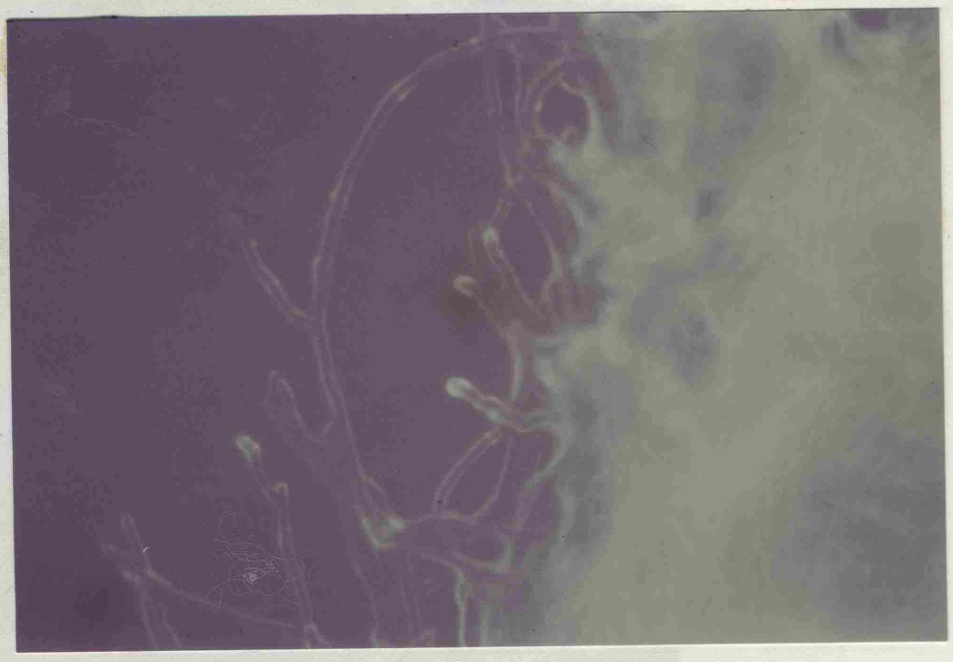
Plate 60. Mycelium from 1% cuticle culture (3 d) treated with calcofluor. Note calcofluor binds almost exclusively to hyphal tips and septa.

Plate 61. Mycelium from 1% chitin culture (5d) stained with WGA-FITC. Note binding to hyphal tips and septa.

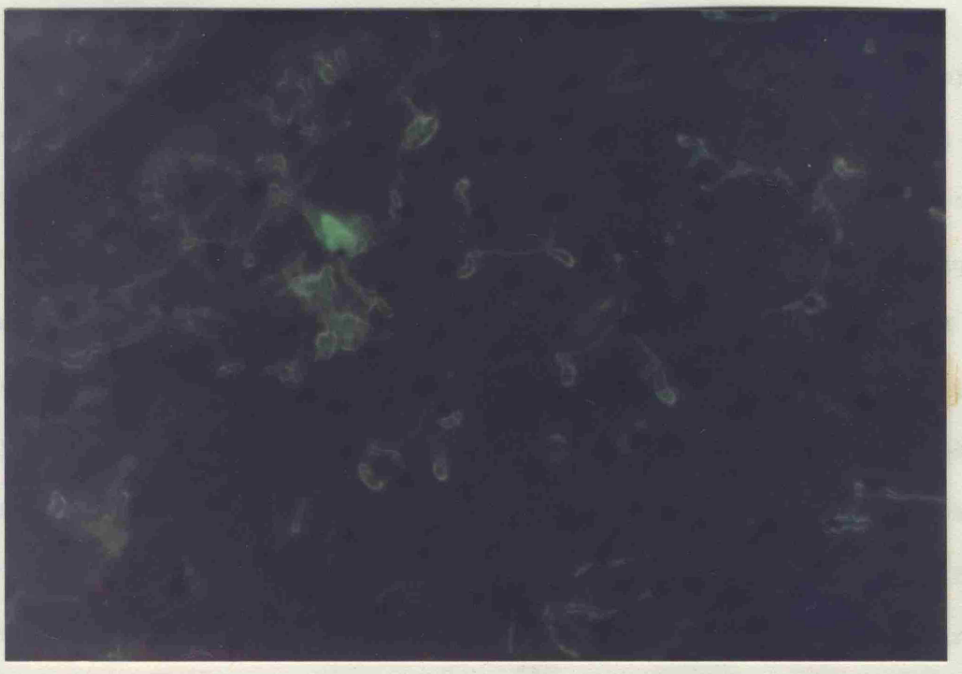
Plate 62. Fluorescence micrograph (FITC-WGA) of *M. anisopliae* (ME1) incubated at 27° for 24 h on an excised wing of *C. vomitaria*. Note. binding to appressoria.

Plate 63. Fluorescence micrograph (FITC-WGA) of hyphal growth on a glass surface, 72 h after inoculation. Note binding on appressoria.

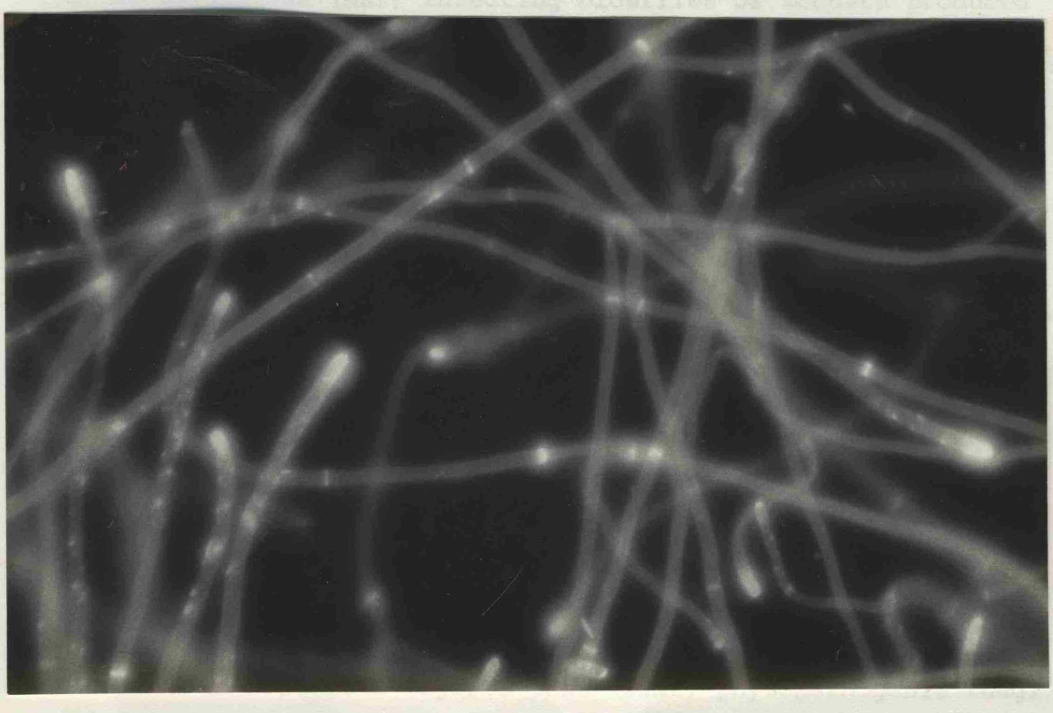
60



61



62



63

DISCUSSION III

The progress of disease in adult blowflies *Calliphora vomitaria* resembles previous reports of pathogenesis by *M. anisopliae* in a variety of insects. Symptoms were displayed in the absence of substantial mycelial growth which suggests the involvement of fungal toxins. Recently Samuels (personal communication) demonstrated that destruxins (low molecular weight cyclic peptide toxins; Roberts, 1980) are present in culture fluids of ME1. Destruxins have been established as the probable cause of death of silkworms infected with *M. anisopliae* (Roberts, 1980). Several of the fungal CDE found in this study are also potentially toxic as their substrates are important components of insect tissues.

M. anisopliae (ME1) infecting blowflies or locusts produced appressorium like structures viz. swellings at hyphal apices formed on the cessation of germ tube elongation (Emett and Parbery, 1975). Appressoria are also produced by *M. anisopliae* growing on *Hylobius pales* (Schabel, 1976a) and wireworm larvae (Zacharuk , 1970b). Penetration *via* an apparently unmodified germ tube occurred occasionally on soft *Manduca* cuticle. This mechanism of entry appears to be unusual for *M. anisopliae* although McCauley *et al.* (1968) reported non-appressorial penetration through spiracles of wireworm larvae.

There are numerous reports that (some) phytopathogenic fungi form appressoria preferentially on host surfaces (qv Emmett & Parbery, 1975). That *M. anisopliae* ME1 did not specifically require an

invertebrate surface was demonstrated by the production of appressorial-like structures against glass or hardened agar. As appressoria were never found in liquid cultures it is probable that a contact stimulus is a pre-requisite for appressorial formation. The correlation between agar concentration and appressorial production shows that hardness is one component in the thigmotropic response. Similar, a different strain of *M. anisopliae* formed appressoria against glass but not 1.5% water agar (Schabel, 1978). These observations may partly explain occasional direct penetration of soft *Manduca* cuticle whereas appressoria were always formed against the hard cuticles of blowflies and locusts.

To the author's knowledge, there is no available information on the sensing mechanisms of entomopathogens to contact or other stimuli from insect cuticles. However, in the rust *Uromyces phaseoli* contact stimulus is the trigger for the "typical pattern" of fluctuation of cAMP levels which modulate the changes leading up to appressoria formation (Staples and Hock, 1984).

It seems unlikely however, that contact stimuli are the only factors governing appressorium formation by *M. anisopliae*. In some cases germ tubes grew for 100 - 300 µm before producing appressoria, during which time they must have frequently contacted the host surface. By analogy with plant pathogens other factors may be implicated such as exhaustion of nutrients or the presence of cuticular waxes inhibitory to growth (qv. Emmett and Parbery, 1975). Although extensive pre-appressorial growth may delay penetration it could, by allowing aggregation of fungal structures, enhance

invasiveness through mass action. Several soil-borne plant pathogens such as *Rhizoctonia solani* (Christou, 1962) seem to employ a similar strategy. Proliferation of appressorial cells and mucus production would ensure a firm anchorage so assisting the application of mechanical force (Schabel, 1978). Such appressorial plates were also associated with high levels of histochemically-detected enzymes which may facilitate hydrolysis of cuticle components.

Studies on the distribution of enzyme activities in successive developmental stages suggested that protease, lipase/esterase, aminopeptidase and NAG'ase activities were present on or in spores prior to germination. It has already been demonstrated (Sect. II, 6A) that protease and NAG'ase can be extracted from dormant spores in varying amounts by washing in water, salt solutions or detergents. Binding of enzymes to spore walls could have considerable adaptive significance allowing modification of the substrate and release of nutrients from the time the spore is deposited on the cuticle. The function of the internal particulate protease in pregerminating and germinating spores is not known. However the endocellular protease of *Neurospora crassa* may function to weaken spore wall layers thus facilitating the emergence of germ tubes (Leighton *et al.*, 1970). This presumably would require localisation of enzymes at the site of germination and there have been reports of vesicle accumulation at points of wall rupture (e.g. Bracker, 1971). Nicholson *et al.* (1972) demonstrated that esterase activity was present at the spore apex of *Venturia inaequalis* prior to germination. It should be realised that the substrate they used, indoxyl acetate can be degraded by several serine proteases (Pearse, 1972).

At least part of the low enzyme levels on pre-appressorial germ tubes may be residual from germination. Nearly all appressoria irrespective of their size and development showed strong positive reactions for lipase/esterase and protease suggesting the reappearance of activity at the initiation of appressorium formation. However, small, apparently developing, appressoria and some of the larger ones, which had failed to produce penetration filaments, possessed only trace levels of aminopeptidase. It seems therefore that initiation of appressoria precedes production of this enzyme which may only become active in mature appressoria at the onset of the penetration process. The sequence of enzyme production presumably reflects the order of gene expression during differentiation of the appressoria. To the author's knowledge there have been no studies on the biochemical events relating to germination and differentiation in entomopathogens. However, work on *U. phaseoli* has suggested that DNA and RNA replication are involved in construction of the appressorium (Ramakrishnan and Staples, 1970; Staples, 1984), and the synthesis of many new proteins which may appear sequentially (Staples, 1984). It appears therefore that initiation of appressoria may be the trigger for a sequence of pathogenicity factors which, in MEl, includes production of cuticle-degrading enzymes.

Aminopeptidase activity differed from that of the other enzymes in extending beyond the appressorium *per se* into the surrounding mucus. This suggests that the mucilage has a greater attraction for aminopeptidase than either the cuticle or fungal wall. Whether this attraction involves electrostatic forces or a more specific association with mucus constituents can only be surmised at present. It is

noteworthy however that the aminopeptidase has a far lower isoelectric point (pI 4.5) than the protease (pI 9.5) or major esterase activity (pI 9.5). One would expect therefore the aminopeptidase to be especially vulnerable to binding if the mucus possessed a net positive charge. Such a mechanism might allow free diffusion of aminopeptidase through the mucus (depending on how structured the mucus was) while retaining the enzyme in the vicinity of the infective structures. Regrettably to date relatively little is known about the charged groups of mucus,fungal and host surfaces which would help in determining the validity of this theory (see Fargues, 1984).

Appressoria or penetration filaments were the only structures produced by ME1 on insect cuticle which possessed large amounts of cell-bound lipase/esterase and aminopeptidase, they were also the principal sites of bound protease activity. In addition, extracellular enzyme activities (protease and aminopeptidase) were exclusively associated with appressoria or appressorial plates. Staining of ME1 for wall β-glucans with calcofluor M2R new or chitin with FITC-WGA revealed that mucus and enzyme-producing regions such as apical tips and appressoria possessed much higher amounts of amenable glucans. Apical tips of *Botrytis cinerea* also stain with calcofluor and it has been suggested that this reflects a local cell wall lysis in areas of cell expansion (Gull and Trinci, 1974). If the same is true for *M. anisopliae* then the incomplete appressorial cell wall may allow enzymes and mucus polymers to diffuse through the wall to become extracellular. Chang and Trevithick (1974) have suggested that apical walls act like sieves *viz.* contain large pores that permit unimpeded passage of macromolecules such as enzymes into the

environment. During growth deposition of secondary wall material reduces the pore size preventing egress of macro-molecules (Chang *et al.*, 1974). If this hypothesis is correct then large molecules such as NAG'ase should be more easily trapped during wall rigidification than a smaller species like protease. As a consequence the former enzyme would be more difficult to extract, as was observed in Sect. II, 7A. A corollary of this is that enzyme-releasing agents such as mercaptoethanol may not act directly on bonds between enzyme and wall components but cause disruption of wall polymers thereby creating openings for the release of enzymes.

The potential role in pathogenesis of the enzymes identified *in vivo* can be predicted from their properties (assuming that forms produced in culture and on cuticle are identical) and the known structure of cuticle. Proteins are the major constituents of pro-cuticle (Sect. I.). Results obtained previously concerning the consistently high protease production by all virulent isolates (Table 5), the rapid production of protease, *cf.* chitinase on cuticle medium (Sect. I) and the masking of cuticular chitin by protein (Sect. II, 5D) suggest that protease may have a major role in cuticle degradation. This possibility is lent weight by the histochemical detection of protease during infection by MEl - to the author's knowledge this is the first demonstration of protease production by a pathogenic fungus during infection. Previously Michel (1981, unpublished, reported in Fargues, 1984) was unsuccessful in demonstrating protease (using the chymotrypsin substrate N-benzoyl-DL-phenylalanine-β-naphthylamide (BPAN)). Negative results were also obtained here with MEl using either BPAN or the trypsin substrate N-benzoyl-L-arginine-β-naphthyl-amide. BGGAN and BGGAMN however were readily hydrolysed, indicating

that chain length is an important factor determining the susceptibility of aryl peptides to hydrolysis by fungal endo-peptidases. Similarly in spectrophotometric assays Nachlas *et al.* (1964) found that mammalian trypsin hydrolysed N-CB^{Z}-diglycyl-L-arginine-β-naphthylamide 220 times faster than benzoyl-L-arginine-β-naphthylamide. To date, most histochemical determinations of protease have utilised the simpler amides, with long chain synthetic peptides being employed for kinetic studies (reviewed in Gossrau, 1984). Recently however, long chain peptides have been used successfully to demonstrate endopeptidases in mammalian cells (Gossrau, 1984). It is evident that they are to be preferred over amides in histochemical studies.

Although it was possible to show inhibition of protease *in vivo* by PMSF, the histochemical techniques did not allow any further characterisation. The protease(s) was therefore extracted from infected cuticle; its alkaline pH optimum (against Ride Hide azure) and reactions with TAME and PMSF, were consistent with the extracellular alkaline serine protease produced *in vitro* (Sect. II, 2A). Although this enzyme cannot solubilise sclerotized cuticle (Sect. II, 5A-D) it degrades locust endo-cuticle to peptides with mean residue length of 5. Probably therefore the action of exo-peptide hydrolases, like aminopeptidase, will be required to further degrade the peptides and render them amenable for uptake and metabolism. The aminopeptidase may also have a more direct role in penetration if it is capable of hydrolysing links inaccessible to the protease, though to date there is no evidence for this.

The protease and aminopeptidase were clearly distinguishable by, amongst other things, their reaction with PMSF. It was far more difficult

to distinguish between various other activities of CDE where the possibility of overlap of substrate specificies occurred. As demonstrated in Sect. II, 2H, the alkaline serine protease possesses esterase activity, and it is reported to be difficult to distinguish histochemically between carboxylic esterases and serine proteases (reviewed in Pearse, 1972). As all the non-specific esterase iso-enzymes produced *in vitro* by MEl appeared to be serine hydrolases (Plate 7) the use of PMSF did not allow histochemical differentiation of any individual enzyme. However activity was far greater against α-naphthylpropionate compared with the acetate derivative which indicates a preponderance of the protease and/or the esterase iso-enzymes with pI 5-6 (Fig. 49 ;. Table 27).

The distinction between esterases and lipase activity is also one of considerable debate and there is doubt about whether lipase can be demonstrated histochemically (Lake, 1972 a,b). Several workers believe that a categorical demonstration of lipases would require the use of a triglyceride emulsion (e.g. Darnton and Barrowmann, 1969; Lake, 1972 a,b) but even for externally-bound activities good enzyme substrate contact would be very hard to achieve. To date almost all attempts to localise lipase histochemically have utilised soluble Tween esters as substrates. However this method can be criticised on a number of grounds, including the slow penetration of Tween substrate into tissues, the poor localisation of precipitate and lack of specificity. For these reasons, Pearse (1972) discouraged its use. As in the present study Tween 80 appeared to give fairly good localisation, the major criticism concerns its lack of specificity. Tweens including Tween 80 may be degraded by non-specific esterases (e.g. Buno and Marino, 1952; Darnton *et al.*, 1969). It

is not valid, therefore, to identify a lipase solely on the basis of activity against a Tween, although many workers have done so, e.g. Pekrul *et al.*, 1979). Michel (1981, PhD thesis). At one time lipase and esterase activities were distinguished by activation of the former with low concentrations of the salts of bile acids. However, not all lipases are so activated (Brokerhoff and Jensen, 1974) while several non-specific esterases are (Pearse, 1972). Most microbial lipases are serine enzymes (Brokerhoff and Jensen, 1974) and in that respect would not be distinguished from the non-specific esterases produced by MEl.

On all these grounds the Tween hydrolysing enzyme produced by *M. anisopliae in vivo* cannot be categorically identified with the lipase produced *in vitro*. Further doubts must be raised by the inability to extract true lipase (assayed against olive oil) from infected cuticle. Obviously however non-detection may have reflected numerous factors such as binding of lipase to cell walls and cuticle components. The different sites of activities against Tween 80 (localised in appressoria) and naphthol AS nononoate (localised in conidia) suggest that the enzyme degrading Tween is distinguishable from at least one medium chain non-specific esterase. In this context it is interesting that Michel (according to a table reproduced in Fargue, 1984) found esterase activity (hydrolysis of α-naphthyl acetate) on conidia and appressoria of *B. bassiana* while "lipase activity" (hydrolysis of Tween 80) was only associated with conidia.

The possible biological role of lipase (i.e. triglyceride hydrolysis) in pathogenesis is not as obvious as many workers seem

to believe. An ultrastructural study of *M. anisopliae* development on wireworm demonstrated the early histolysis of the "wax layer" followed by the rest of the epicuticle (Zacharuk, 1970c) which might implicate lipase/esterase activity. However, in most insects including locusts (Lockey, 1976) and flies (Louloudes *et al.*, 1962) the "extractable" cuticular lipids are composed mainly of a complex mixture of alkanes and alkenes with triacylglycerols and wax esters as comparatively minor components. Lipase/esterase would probably therefore only have a major role if the "bound" surface lipid and "non extractable" cuticular lipid fraction contained esters. Unfortunately however very little is known of the chemistry of bound cuticular lipids or their esters (Blomquist, 1984).Wigglesworth (1970) has tentatively suggested that cuticulin (outermost layer of the epicuticle) is a multiple polyester cross-linked by ester bonds. If this is correct, then an esterase with characteristics somewhat similar to cutinase (see Kolattukudy, 1984) would probably be required for its hydrolysis.

If such an enzyme was capable of softening or changing the nature of the surface epicuticle, it might not only provide nutrients and assist penetration but also increase the ability of pathogenic fungi to adhere. Similarly Nicholson (1984) has suggested that adhesion to plant surfaces by appressoria may involve alteration by esterase(s) of the cuticle's wettability. It is noteworthy that in insects cuticular lipids are thought to offer protection from micro-organisms by, amongst other things, the non-wetting properties they imp art to the surface (Blomquist, 1984).

In vitro, production of enzymes on locust cuticle occurred sequentially over several days (Sect.1) with chitinase among the

last enzymes produced. It seems likely that the order of enzyme synthesis in cultures could also occur during cuticle penetration. Indeed production of proteolytic enzymes always preceded that of NAG'ase during infection of fly wings. There was no evidence for the production of an active chitinase in the 40 h following inoculation. *In vitro* the late appearance of inducible chitinase is most probably due to the delayed exposure of chitin and release of inducers following prior action of protease (Sect. II, 5D). However the apparent absence of chitinase from infected fly wings < 40 h after inoculation could additionally be due to inhibitors in the cuticle or inadequate extraction procedures. Nevertheless, failure to detect the products of chitin hydrolysis in comminuted infected cuticle indicates that the activity of chitinase, if present, is negligible.

The apparent absence of chitinase during the first critical hours after inoculation suggests that this enzyme is not involved in cuticular penetration. Perhaps therefore, chitinase functions solely to provide nutrients during the saprophytic phase of fungal growth.

In vitro, high levels of chitinase were present in actively growing cultures. It has been suggested that to preserve their integrity, fungal walls may lack chitin when growing in a medium which allows extracellular chitinase production (Huber, 1958). The binding of FITC-WGA to chitinase producing hyphae of MEl seriously questions this claim. It was previously shown that only where the integrity of the mycelium is disrupted does cell wall chitin become subject to degradation by fungal chitinase (Sec. I). Presumably therefore,

chitin is not the sole component of hyphal tips or appressoria of *M. anisopliae*; it is likely that other polymers may protect the chitin. In the apex of *Neurospora crassa*, β-glucan overlies the chitin microfibrils, and some glycoprotein is also present (Hunsley and Kay, 1976).

GENERAL DISCUSSION

The results obtained from this work suggest the following as a plausible sequence for enzymatic degradation during penetration of unsclerotized cuticle:

a) Cuticle-degrading enzymes (CDE) secreted before penetration by conidia and appressoria provide nutrients and modify the cuticle surface; possibly assisting adhesion of fungal structures. Esterases may be particularly relevant in this context.

b) Enzymes secreted by fungal infection structures disperse through the cuticle (assisted by fungal growth) *via* epicuticular filaments, pore canals and dermal gland ducts.The apparently localised action of CDE (qv. Charnley, 1984) may result from molecular sieving, binding to fungal walls or binding to cuticle.

c) Constitutively produced proteolytic enzymes weaken the cuticle matrix, facilitate mechanical penetration and unmask cuticular chitin.

d) Chitinase, produced at low basal levels by the penetrating fungi, binds on to exposed chitin and releases NAG to initiate induced synthesis as an autocatalytic process.

The general involvement of CDE in infection is suggested by their presence in germinating conidia, penetration structures and infected cuticle. The constitutive nature of endo- and exo-proteases and NAG'ase supports the likelihood of their production *in vivo*. Although most of the CDE of *M. anisopliae* are subject to CR, as chitin and many of the cuticular proteins are insoluble (Neville, 1975) the effects of CR on CDE synthesis during infection are likely to be minimal.

The major structural component of insect cuticle is protein (*c*. 75% w/w in *S. gregaria*) and a number of findings suggest a primary role for proteases in infection. Proteases were consistently produced in high amounts by all entomopathogens examined and were among the first enzymes to appear during growth in cultures on ground locust cuticle or on fly wings *in vivo*. Chitinases, when they occurred, always appeared substantially later. It is conceivable that this sequence reflects the physiochemical susceptibility of cuticular components and relates to the significance of these enzymes in parasitism, as has been established with host-wall-degrading enzymes of plant pathogenic fungi. For this group, endo-pectinases which degrade the host cell wall matrix are the first enzymes produced and are key determinants in the pathogenicity of many facultative parasites (*cf*. endo-proteases); the last enzymes of the sequence correspond to cellulose, the structural framework of walls (*cf*. the chemically and structurally related chitin which may fulfil a similar function in cuticle), and usually play a minor role in parasitism (Cooper and Wood, 1975; Cooper, 1977, 1983).

Substrates in comminuted cuticles were amenable to degradation by enzymes in culture filtrates and by purified CDE. However, protease substantially enhanced subsequent degradation by chitinase which implies that cuticle chitin is shielded by protein. Similarly hemicellulases and cellulases produced by *C. lindemuthianum* are effective in degrading plant cell wall polysaccharides only when the surrounding polygalacturonide has been degraded by endo-pectic enzymes (Karr and Albersheim, 1970). An interesting and analogous system for unsclerotised cuticle is the degradation of insect cuticle by endogenous enzymes at the moult, when only a proportion

of the old cuticle is shed (the sclerotized exuviae). The remaining cuticle is digested and recycled (Charnley, 1984). It is suggested that synthesis of a 'trypsin-like' protease precedes that of chitinase, and its action is necessary to unmask cuticular chitin which is then degraded by chitinase and chitobiase to NAG (Bade and Stinson, 1978). Sclerotized cuticle is relatively resistant to enzymolysis by both endogenous and fungal enzymes. As previously discussed, this resistance could limit those regions that fungi can penetrate. It does not however prevent penetration of sclerotized locust cuticle by *M. anisopliae* (MEl); presumably mechanical pressure plays a major role in this situation. A critical ultrastructural study may help reveal the relative contributions of enzymic hydrolysis and mechanical pressure to penetration of hard and soft cuticles. In contrast to the numerous ultrastructural studies of infection by plant pathogenic fungi only one investigation has been conducted with an entomopathogen (e.g. Zacharuk, 1970 a,b,c). Cytochemical techniques applicable to TEM could be used to identify putative lipid degrading enzymes (Duddridge and Sargent, 1978), NAG'ase (Pugh, 1973), aminopeptidase (Seligmann, 1970) and protease (Gossrau, 1984). The location of a fungal cutinase during fungal penetration of a plant cuticle with ferritin-conjugated antibody (Shaykh *et al.*,1977) also demonstrates the potential of an immunocytological approach if applied to CDE of entomopathogens. A different approach would be to prevent by surgical means the release of the hormone bursicon (e.g. Abboud, Charnley, Reynolds and Williams Wynn, 1983) which controls sclerotisation and melanisation or selectively prevent melanisation with α-methyl-α-hydrazine DOPA (Reynolds, 1976). The use of such procedures may reveal the contribution of these changes

(sclerotization and melanisation) in resistance to infection.

Physiochemical changes could occur during infection which may weaken cuticle. Thus Lipke and Geoghegan 1971a) suggested that sclerotized cuticle obtains generalised protection as a result of H bond formation during the dehydration that is a feature of sclerotization. In the light of this Charnley (1984) speculated that a reduction of the pH of the cuticle by a fungal metabolite could loosen the bonds between protein molecules and further the action of hydrolytic enzymes. This hypothesis is supported by Kodaira's (1961) observation of oxalic acid crystals on the surface of *Bombyx mori* killed by *B. bassiana*. However, *in vitro* the pH of unbuffered cuticle cultures (*B. bassiana, V. lecanii* and *M. anisopliae*) rose over 5 d from 6.0 to *c.* 8.5 suggesting that during penetration, metabolism of cuticle components is unlikely to result in a weakening of H bonds.

The mode of action of proteolytic enzymes on cuticle remains to be established. A preliminary study showed that pure endoprotease released peptides(mean residue length DP = 5) of which the gross amino acid composition was determined. The next step would be to determine the specificity of peptide bonds cleaved. This may be possible by techniques such as dansylation which reveal terminal amino acids of peptides. Separation of peptides by ion exchange chromatography and TLC or HPLC could then allow sequencing of amino acids. The knowledge gained would have wide implications because to date no primary amino acid sequence has been determined for any cuticular proteins (Neville, 1975; Jeuniaux, 1984).

For the complete degradation of insect cuticle, the action of chitinase(s) as well as protease is necessary (Samsinakova *et al.*, 1971).However, in spite of several studies on enzyme production (e.g. Samsinakova *et al.*, 1971; Pekrul and Grula, 1979; Gabriel, 1968), the isolation, purification and characterisation of enzymes of the chitinase complex has yet to be achieved for any pathogenic fungi. This contrasts with the wealth of information on fungal cellulases, which exist in distinct synergistic forms, sometimes as bound complexes (Wood and McCrae, 1978; Lamed *et al.*, 1983). An analogous enzyme system could be predicted from the chemical and physical similarities between the two polymers. No evidence was found in this study on *M. anisopliae* for production of separate forms specific for colloidal and crystalline chitin. However, distinct activities against chitin and chitosan, as well as an NAG'ase were distinguished. Further purification and separation should clarify whether any of these enzymes exist as multiple forms. This is an important consideration as assay of total activity may overshadow the contribution of one key enzyme or isoenzyme (e.g. Sexton *et al.*, 1980).

No active chitinase was evident during germination and penetration. Some reasons for this have already been suggested.This does not necessarily preclude the production of chitinase in infected cuticle, although presumably quantities of enzymes must be very small and may also exist at least partly in bound forms. Even low levels of bound enzyme could potentially affect wall structure sufficiently to assist penetration (*cf.* production of bound cell-wall degrading enzymes by obligately biotrophic plant parasites, Cooper (1984); alternatively it may be that significant levels do not appear until

after penetration has been achieved. As with cellulose in plant cell walls (Cooper, 1983, 1984) chitin degradation may not be required for cuticle penetration. It could be sufficient as suggested by Gabriel (1968b)for digestion of protein alone to enable mechanical penetration following loosening of cuticular lamellae.

These and other observations warrant a fundamental study to investigate the contribution of individual enzymes to penetration. Examination of the pathogenicity of induced mutants with altered capacity for enzyme production would be a powerful approach. The knowledge of enzyme regulation obtained in this study allows choice of media for selection of mutants deficient, super-productive, constitutive, or non-repressible for several enzymes. The application of specific enzyme inhibitors during infection may also elucidate the role of individual activities in pathogenesis, e,g. NAG'ase by 2-acetamido-2-deoxy-D-gluconolactone, or carboxypeptidase by β-phenylpropionate. Additionally, attempts could be made to use antisera specific to single enzymes, in view of the success of this technique with *F. solani* cutinase (Shaykh *et al.*,1977).

An alternative approach would be to determine the ability of *M. anisopliae* to invade, and its enzymes to degrade cuticle of modified composition. Mention has already been made of producing unsclerotised cuticle. Also, diflubenzuron (dimilin) could be used to produce cuticle either devoid of chitin or with a proportion of dimilin-affected daily growth layers. Ultrastructural localisation of fungal enzymes in dimilin-affected cuticle should reveal whether chitinase is inducible *in vivo* as it is *in vitro*. The ability of enzymes to degrade cuticle of modified composition could provide

valuable information not only on the significance of CDE in parasitism, but also on the physiochemical nature of insect cuticle.

It remains to be determined what effect, if any, cuticle composition could have on host susceptibility. *V. lecanii* and *B. bassiana* which possess low virulence against *S. gregaria* (Heibronn, unpublished) showed substantial growth and enzyme production on locust cuticle. This suggests that host resistance was not due to an inability to degrade or utilise the major cuticle constituents. However the present evidence suggests that regions of the cuticle would provide either specific chemical barriers (e.g. epicuticle, Smith *et al.*, 1982) or a general physical barrier (e.g. exocuticle) to penetration. By delaying and perhaps staggering the arrival of penetrant hyphae such barriers could optimise the efficiency of the haemocytic defences.

APPENDIX I

Isotonic Locust Saline, pH 6.8 (Evans and O'shea, 1978)

Chemical	mM	g l^{-1}
NaCl	140	8.180
KCl	10	0.747
$CaCl_2$	4	0.588
$NaHCO_3$	4	0.336
NaH_2PO_4	6	0.936
Glucose	90	16.210

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. A.K. Charnley and Dr. R.M. Cooper for their advice, help and encouragement during the course of this study.

Special thanks are due to crop protection and electron microscopy technicians for their assistance, especially Mr Neil Weston for his help with the iso-electric focussing.

The author was in receipt of a post-graduate studentship from Bath University Research Fund. I am indebted to Mrs. J Harbutt for the excellent typing of this thesis.

Finally I would like to acknowledge the Great God *Ganesh* who, being the gentleman he is, has always stood by me.