Peptide Transport in *Candida albicans*

by

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ABSTRACT

Candida albicans has been shown to possess a complex system for the utilization of environmental peptides. The presence of multiple peptide permeases was identified through the creation of a mutant resistant to the effects of nikkomycin, a peptide-nucleoside antibiotic used as an example of a toxic peptide. Peptides transported by the cell are hydrolysed by at least eight peptidases present in the soluble fraction of the cell.

Nikkomycin was a growth inhibitor of *C. albicans* (MIC on malt extract = 25µg/ml). The effect was observed as a profound effect upon the chitin network of the cell wall through inhibition of the enzyme chitin synthase (K_i = 0.16 µM). The alteration of the cell wall in the presence of the drug was followed by phase contrast and fluorescence microscopy in addition to transmission electron microscopy. Three major effects were observed: the inhibition of primary septum formation; a protoplasting effect in growing cells due to the weakening of the cell wall; finally the lysis of the cell wall at a point adjacent to the septum. The effects were analogous to those observed upon treatment of sensitive fungi with polyoxins.

The presence of peptone mixtures or certain defined peptides interfered with the uptake of the drug which shows that nikkomycin enters the cell via the peptide transport system of *C. albicans*. Two important observations were the reversal of nikkomycin activity by serum and the high frequency of spontaneous resistance to the drug. These will have considerable influence upon the development of anticandidal peptide drugs.

The fluorescamine technique was used to follow peptide uptake. Two peptide permeases have been defined for *C. albicans* by comparison of the wild type with a mutant resistant to the effects of nikkomycin through a transport defect. System I has high affinity for dipeptides and a lower affinity for oligopeptides; System II has high affinity for oligopeptides but only a low capacity for a few dipeptides. The specificities of each system are described. It is the loss of System I which confers resistance to nikkomycin in the mutant of *C. albicans* (NIK5). System II has been shown to be inducible when peptides provide the sole nitrogen source for the cell and an equivalent induction appeared to occur in System I.

A new technique for the visualization of peptidase isozymes has been developed based upon the electrophoretic transfer of native proteins from polyacrylamide gels to nitrocellulose sheets. Blotting is followed by an *in situ* stain for enzyme activity. By this method eight peptidases have been identified in the soluble fraction of *C. albicans*, six of which possessed overlapping dipeptidase activity. No differences were found between the peptidases of the wild type and the transport deficient mutant.

Following intracellular hydrolysis of [U^{14}C]alanyl alanine, [U^{14}C]-alanine undergoes rapid metabolism resulting in the release of $^{14}CO_2$ from a cell suspension. This loss of radiolabel has a considerable influence upon the apparent rate of peptide transport when monitoring the uptake of radiolabelled peptides.

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ABBREVIATIONS OF PEPTIDES

All amino acid residues are in the L-configuration unless otherwise stated. This list covers those peptides used in the present study.

ala_2, ala ala	alanyl alanine
ala_3	alanyl alanyl alanine
ala_4	alanyl alanyl alanyl alanine
alafosfalin	alanyl amino ethyl phosphonic acid
ala glu	alanyl glutamic acid
ala his	alanyl histidine
ala leu	alanyl leucine
ala trp	alanyl tryptophan
bacilysin	alanyl anticapsin
gly asp	glycyl aspartic acid
gly_2	glycyl glycine
gly_3	glycyl glycyl glycine
gly leu	glycyl leucine
gly leu tyr	glycyl leucyl tyrosine
gly phe	glycyl phenylalanine
gly pro	glycyl proline
gly try	glycyl tyrosine
glu ala	glutamyl alanine
leu ala	leucyl alanine
leu gly	leucyl glycine
leu_2	leucyl leucine

leu_3	leucyl leucyl leucine
leu pro	leucyl proline
met ala	methionyl alanine
met ala ser	methionyl alanyl serine
met_2	methionyl methionine
m-F-phe ala ala	m-Fluorophenylalanyl alanyl alanine
ser ala	seryl alanine
tyr ala	tyrosyl alanine
tyr gly	tyrosyl glycine
tyr tyr	tyrosyl tyrosine

ABBREVIATIONS

ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bis	bis-acrylamide
CCCP	Carbonylcyanide chlorophenyl hydrazone
DCCI	N,N'-dicyclohexamylcarbodiimide
2,4-DNP	2,4-dinitrophenol
DTNB	5,5,-dithiobis-(-2-nitrobenzoic acid)
dpp	dipeptide permease
EDTA	Ethylene diamine tetracetic acid
met_3-OPEG	methionyl methionyl methionyl-poly-(ethylene glycol)
MIC	minimum inhibitory concentration
MX	malt extract
OPA	o-phthaldialdehyde
opp	oligopeptide permease
PAGE	polyacrylamide disc gel electrophoresis
PCG (buffer)	10 mM Na_2HPO_4-citric acid pH 5.0 supplemented with 0.8% (w/v) D-glucose
PME (buffer)	0.1 M sodium phosphate buffer pH 6.5 containing 10 mM $MgCl_2$ and 1 mM EDTA
TEMED	N,N,N',N' tetramethyl ethylene diamine
TLC	thin layer chromatography
YNB	yeast nitrogen base: $(NH_4)_2SO_4$ nitrogen source
YNBP	yeast nitrogen base: Bactopeptone nitrogen source

Introduction

The transport of substrates into the cell from the extracellular environment is a necessary part of the nutrition of most organisms. In the case of nitrogen metabolism the substrate may be N_2, the oxidized forms NO_2^- and NO_3^-, or the reduced forms: NH_4^+, amines, amides, amino acids, peptides and proteins. Further discussion will be limited to the amino acids and their polymers together with ammonium ions, since these have a considerable effect upon the control mechanisms which regulate nitrogen metabolism.

Both prokaryotic and eukaryotic organisms possess many specific transport systems (permeases) for ammonium ions, amino acids and peptides which overcome the potential impermeability of the cell membrane. When proteins serve as the nitrogen source extracellular proteases may be secreted. The peptides and amino acids which result from this hydrolytic action are then transported into the cell.

The introduction will summarise the current knowledge of the interrelated permease systems for ammonium ions, amino acids and peptides, indicating the structural specificities of each. Emphasis is placed upon the peptide permeases which give the greatest scope for substrate variation and hence the possibility of drug development using the illicit transport concept. (Ames *et al.*, 1973).

The Transport of Ammonium Ions in Bacterial and Fungal Cells

Both bacteria and fungi are able to accumulate ammonium ions from a culture medium against a concentration gradient (reviewed by Brown, 1980) however this field has received far less attention than that of amino acid or peptide transport. Mutants of *Salmonella typhimurium* have been identified with reduced growth rates on ammonium salts (at concentrations below 1 mM). These were unable to maintain a high intracellular ammonium ion concentration, thereby demonstrating the presence of distinct permease systems for this substrate (Broach *et al.*, 1976).

Penicillium chrysogenum was able to grow on methylammonium (methylamine) as the sole nitrogen source (Hackette *et al.*, 1970). In addition, ammonium ions were found to be a potent inhibitor of [^{14}C]-methylammonium transport, indicating that these two metabolites share a common permease. A similar co-transport system was found in *Aspergillus nidulans* (Pateman *et al.*, 1974) by which methylammonium was concentrated 120-fold over the extracellular medium. ^{15}N-enriched ammonium has been used to provide a direct assay of ammonium ion transport however, this was not sufficiently sensitive at concentrations below 0.5 mM to allow kinetic studies (Pateman *et al.*, 1974). [^{14}C]-methylammonium has therefore gained widespread use as an experimental tool and has been used in all of the following studies of ammonium ion transport.

Escherichia coli has been shown to concentrate methylammonium 100-fold intracellularly (Stevenson and Silver, 1977) where it is not metabolized. The accumulation of radiolabel by *E.coli* is therefore a direct measurement of transport. However, due to metabolism this is not the case in all microorganisms (such as *P.chrysogenum* Hackette *et al.*, 1970).

The number of distinguishable ammonium ion permeases varies between bacterial species: A single system has been identified in *Clostridum pasteurianum* (Kleiner and Fitzke, 1981) while two, energy dependent, permeases are operational in *E.coli* each of which transports ammonium and methylammonium. The two systems of *E.coli* differ in their pH optima, one functioned maximally at pH 7.0 , the other at pH 9.0 (Stevenson and Silver, 1977).

At least two ammonium ion transport systems are present in *Saccharomyces cerevisiae* (Dubois and Grenson, 1979). A single system appears to be present in *A.nidulans* (Cook and Anthony, 1978a) however the number of permeases present in other fungal species is unclear.

The control of ammonium ion transport has been shown to occur by two mechanisms: repression of synthesis of the permease and inhibition of the activity of the preformed system. Nitrogen starvation has been shown to have a profound effect upon the rate of ammonium transport in *P.chrysogenum* in which the rate increased 800-fold upon starvation (Hackette *et al.*, 1970). Thus, a decrease in the available nitrogen intracellularly brings about an increase in the activity of the permease for this nitrogen metabolite. In *A.nidulans* the development of the uptake system during nitrogen starvation was inhibited by cycloheximide, thereby indicating the involvement of protein synthesis in the regulation of this permease (Pateman *et al.*, 1974). Derepression of the ammonium transport system did not appear to occur in *S.cerevisiae* (Roon *et al.*, 1975), *Stemphylium botryosum* (Breiman and Barash, 1980) or *E.coli* (Stevenson and Silver, 1977) however specific metabolites have been implicated in the control of the ammonium ion permease: The levels of glutamine and asparagine control

activity in *A.nidulans* (Cook and Anthony, 1978b); glutamine in *S.cerevisiae* (Dubois and Grenson, 1979) and L-amides in *St.botryosum* (Breiman and Barash, 1980).

Hence, even though only limited data are available, ammonium ions enter the cell via kinetically definable systems. These permeases are coupled to an energy source (*E.coli* Stevenson and Silver, 1977; *S.cerevisiae* Dubois and Frenson, 1979) and their activity is regulated by the available intraceullular nitrogen. This pattern is repeated for many substrate permeases: Those for the amino acids will now be considered.

Amino Acid Transport

The transport of amino acids has been studied in many microorganisms. Amino acids have been shown to be incorporated directly into protein and also to serve as a general nitrogen source for the cell via transamination pathways. The transport of amino acids is therefore coupled to an efficient metabolic system to utilize or store the nitrogen provided.

Two methods have been used to study amino acid transport. Firstly, use has been made of amino acid auxotrophs in which the growth response has been shown to be directly related to the transport of the supplied amino acid. This allowed competition studies between the required and non-required amino acids. The technique was limited by the availability of the necessary auxotrophs and did not allow a direct study of amino acid transport. It did, however, give information concerning the multiplicity of permeases.

The second technique available was the use of radiolabelled amino acids. However, problems have been encountered with this method since the various permeases were shown to have differing pH and temperature optima. It was also found that rapid efflux of the radiolabel occurred from bacteria if the buffer used to wash the cells was colder than that of the incubation medium (Payne, 1975).

The transport of any substrate through a membrane requires that the following critera should be met:

1. The recognition of the substrate
2. The translocation of the substrate through the membrane
3. The coupling of the transport process to an energy source
4. The intracellular release of the substrate.

Consideration will now be given to individual cases of amino acid transport indicating how these criteria are achievedby both prokaryotic and eukaryotic cells.

1. Bacteria

Escherichia coli has been shown to possess twelve kinetically defined amino acid transport systems many of which are divisable into high and low affinity components. The transport systems are defined by the amino acids for which they had the highest affinity: glycine, alanine (Robbins and Oxender, 1973); threonine, serine (Templeton *et al.*, 1974); leucine, isoleucine, valine (3-systems. Rahmanian *et al.*, 1973); phenylalanine tyrosine, tryptophan (Brown, 1970); methionine (2 systems. Kadner, 1974);

proline (Morikawa *et al.*, 1974); lysine, arginine, ornithine (Rosen, 1977); cystine (2 systems. Berger and Heppel, 1972); asparagine (2 systems. Willis and Woolfolk, 1975); glutamine (2 systems. Weiner and Heppel, 1971); aspartate (2 systems. Kay, 1971) and glutamate (Halpern *et al.*, 1961; Willis and Furlong, 1975).

There may also be transport systems for cysteine and histidine although these have yet to be kinetically defined for the intact organism (Anraku, 1980). The substrate specificities of the permeases from other bacteria such as *Salmonella typhimurium* appear to be equivalent to those found in *E.coli* (Anraku, 1980).

Amino acid transport systems in bacteria are generally constitutive. However, there are a few exceptions, such as the lysine permease of *Pseudomonas putida*, which was found to be inducible on media containing lysine as the sole nitrogen source (Miller and Rodwell, 1971).

The energetics of amino acid transport in bacteria will be discussed together with those of eukaryotic organisms.

2. Non-Filamentous Fungi

Multiple amino acid transport systems have been defined for *S.cerevisiae*. These include specific permeases for: histidine(Crabeel and Grenson, 1970); methionine (Gits and Grenson, 1967); glutamate (Darte and Grenson, 1975); proline (Seaston *et al.*, 1973); arginine and lysine (Grenson *et al.*, 1966 ; Chan and Cossins, 1976); lysine (Grenson, 1966), and a general amino acid permease (gap) which was shown to have a high capacity for the transport of basic and hydrophobic amino acids (Grenson, *et al.*, 1970). The gap is the only system able to transport D-stereoisomers of the amino acids

which have been found to be toxic to the cell. This has allowed the selection of mutants of *S.cerevisiae* lacking the gap (Rytka, 1975).

Initial studies have been carried out on the amino acid transport systems of *Candida albicans* (Davies, 1979). Competition studies indicated the presence of at least three amino acid permeases: an acidic system; a basic system; and a system specific for the transport of hydrophobic amino acids. Attempts by Davies (1979) to derepress a possible general amino acid permease equivalent to that of *S.cerevisiae* were unsuccessful. However, a proline transport system has been identified which is present at a constitutive level with an additional, inducible level when proline provides the sole nitrogen course (Jayakumar *et al.*, 1979).

Glutathione has been found in the cells of many organisms however, its role in metabolism is unclear. A mechanism of amino acid absorption has been proposed involving the γ-glutamyl cycle (Meister, 1973): if the key enzyme, γ-glutamyl transferase was to be located in the cell membrane one mole of amino acid would be transported per mole of glutathione degraded. This enzyme has indeed been located in the cell membrane of mammalian cells (Meister and Tate, 1976) but there is no direct evidence that it is involved in amino acid transport. The enzymes of the γ-glutamyl cycle are also present in yeasts but their involvement in amino acid transport is unclear. Osuji, *et al.* (1979,1980) reported that the γ-glutamyl cycle played a part in amino acid absorption in *Candida utilis* however, Robins and Davies (1981) found that this was not the case. Due to the contradictions further work will be necessary to show whether the γ-glutamyl cycle plays any part in amino acid transport.

The control of amino acid transport has been shown to occur by two

mechanisms:

i. Transinhibition: If cells were preloaded with histidine it was found that the subsequent rate of histidine transport was reduced (Crabeel and Grenson, 1970). Similarly, preloading with lysine or arginine reduced the activity of the lysine and lysine-arginine permeases (Morrison and Lichstein, 1976). Therefore there appears to be a system of feedback regulation whereby the intracellular concentration of a substrate controls the activity of its own permease without affecting the activity of other transport systems.

ii. Ammonium repression: This phenomenon has been found to be of considerable importance, controlling many aspects of nitrogen metabolism including the transport systems for the nitrogen-containing metabolites. *S.cerevisiae* has been shown to be able to utilize ammonium ions as the sole nitrogen source (Grenson *et al.*, 1970). Under these conditions the activity of the general amino acid permease is reduced due to an inhibition of its activity rather than its synthesis.

Ammonium ions were found to be incorporated into a carbon skeleton via the following scheme:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NAD(P)H \overset{GDH}{\rightleftharpoons} \text{glutamate} + NAD(P)^+ + H_2O$$

The NADP dependent glutamate dehydrogenase (NADP-GDH) was found to be derepressed when the yeasts *Candida utilis* and *S.cerevisiae* were grown

in ammonia limiting cultures (Brown, 1976). Levels of activity were 20-30 times higher than under carbon limitation. Woodward and Cirillo (1977) demonstrated that the rate of uptake of amino acids by *S.cerevisiae* was up to 360 times greater in cells grown on proline as the sole nitrogen source rather than ammonium salts. Mutants lacking the structural gene for NADP-GDH (*gdhA*) were found to grow only slowly on ammonium ions and lacked ammonium repression of the general amino acid permease (Grenson and Hou, 1972). Therefore, NADP-GDH plays a part in the ammonia control of nitrogen metabolism. The precise mechanism for this process in yeasts is unclear. Pateman and Kinghorn (1976) have demonstrated a similar process of ammonium repression in the filamentous fungus *Aspergillus nidulans* and have proposed a mechanism for the ammonia control system:

a. Intracellular and extraceullular ammonia levels are monitored by the cells.

b. NADP-GDH, located in an ammonia monitoring site in the cell membrane, can complex with the extracellular, but not intracellular ammonia. It therefore monitors the extracellular ammonia concentration and determines the levels of the amino acid transport systems in addition to the other ammonia-repressable systems. Further membrane components have also been shown to be involved in monitoring extracellular ammonia and have been defined from mutants deficient in ammonia regulation.

c. A separate site on the NADP-GDH is able to monitor the intracellular ammonia levels and determines the level of ammonia. Cook and Anthony (1978b) found the levels

of asparagine and glutamine to control the uptake of ammonia indicating that ammonia incorporated into glutamine may act as a signal for this process.

d. A control process is proposed which converts intracellular and extracellular ammonia concentrations, in a form of NADP-GDH/NH_4^+ complexes, into signals for the control of ammonia-repressable systems.

Experimental data are still required to show whether this scheme is operational in yeasts. However, it does propose a mechanism to explain the complex interrelationships involved in ammonium-repression.

Amino acids have been found to be able to serve as the sole nitrogen source for fungal cells by undergoing transamination with the release of the carbon skeleton. In the case of the hydrophobic amino acids, fusel oils have been shown to be released (leucine giving *iso*-amyl alcohol; tyrosine giving tyrosol). The nitrogen from these amino acids is incorporated into α-ketoglutarate and forms glutamate which may then be metabolized further (Woodward and Cirillo, 1977). Most amino acids have been found to be rapidly degraded with little being incorporated directly into proteins.

An important difference between the utilization of amino acids by bacteria and by yeasts has been found to be the presence of the yeast vacuole. Work by Wiemken and Durr (1974) showed that 60% of the free amino acids were present in the vacuole of *S.cerevisiae*. It would appear that the vacuole was acting as a store while the metabolically active amino

acids, such as glutamate, were located in the cytoplasm. A similar situation was found in *Candida utilis* (Wiemken and Nurse, 1973). A specific vacuolar arginine permease has been reported (Boller *et al.*,1975) It was found to be saturable and differed in specificity from that located in the plasma membrane. If the cytoplasmic pool is not expandable, the rate of amino acid uptake by whole cells may be affected by the vacuolar permease. Arginine was found to be retained within the vacuole by binding to polyphosphate (Durr *et al.*,1979). Whether a similar situation exists with other amino acids has not been established.

3. Filamentous Fungi

The amino acid transport systems of the filamentous fungi have been studied and, as found for the yeasts, multiple transport systems are present. *Neurospora crassa*, which can be taken as representative of this group, possesses five defined amino acid transport systems: a neutral and aromatic system (Wiley and Matchett, 1966); a basic system (Pall, 1970a); a general amino acid transport system (Pall,1969); an acidic system (Pall, 1970b); and a methionine-specific transport system (Pall, 1971).

The methionine permease was found to be derepressable under conditions of sulphur limitation (Pall, 1971). The general and acidic permeases are ammonium repressable while the remaining two systems are constitutive.

The mechanism of ammonia-repression has been described previously. However, a further effect of ammonium ions on the plasma membrane of

N.crassa has been reported (Slayman and Goodman, 1975): extracellularly supplied ammonium ions have been shown to bring about a partial depolarization of the membrane and hence a decrease in the proton-motive force. This results in a decreased ability to transport metabolities. The second mechanism of control has been shown to be equivalent to that of trans-inhibition in *S.cerevisiae:* Pall (1971) showed that the intracellular methionine concentration controlled the activity of the methionine-specific permease.

Substrate-specific binding proteins have been found to be associated with the amino acid transport systems of *N.crassa*. The most highly characterised of these is the tryptophan-binding protein (Wiley, 1970), which was obtained by cold osmotic-shock of germinated conidia. It was found to be a glycoprotein (mw 200,000 daltons) and is a component of the neutral amino acid transport system. The function of fungal binding proteins is unclear. However, it is possible that they serve to increase the local concentration of the substate for a permease, thereby increasing the rate of transport at low extracellular substrate concentrations (Wolfinbarger, 1980).

The Energetics of Amino Acid Transport

1. Bacteria

The amino acid permeases of *E.coli* can be divided into two classes: those which are senitive to osmotic shock (shockable) and those which are

insensitive. The shockable systems have been found to possess associated binding proteins which are only loosely bound to the cell membrane and include the permeases for glutamine, arginine, histidine and the leucine-isoleucine-valine (LIV) system. Osmotic shock was found to reduce the activity of the LIV system. However, the activity returned when the binding protein was restored to the cells (Anraku, 1968). The binding proteins appeared to increase the availability of the substrate for the transport system by side-chain specific and stereospecific binding (Oxender and Quay, 1975). They are therefore equivalent to those previously described for fungal cells.

The mechanisms of energy coupling have been found to differ between the two classes of bacterial amino acid permeases (Berger, 1973; Berger and Heppel, 1974). Shockable systems appear to be dependent upon energy derived from phosphate-bonds and, to a lesser extent, upon a membrane potential. They are therefore highly sensitive to arsenate (Lieberman and Hong, 1976; Lieberman *et al.*, 1977). The shock-resistant systems are dependent upon a proton-notive force derived from either respiration or from the action of an ATPase and are therefore highly sensitive to dinitrophenol.

The chemiosmotic theory of Mitchell (1966) proposed that active transport of metabolites was linked to transmembrane gradients of ions and of charge. Oxidative electron transport and a proton-translocating ATPase were considered to be organized within the cell membrane such that they created a gradient of protons and of charge (Harold, 1976). It was this gradient (composed of a membrane potential and a pH gradient) which was termed the proton-motive force (p.m.f.). For *E.coli* the pH gradient

was found to be such that the internal pH was approximately 7.8 over the range of external pH from 5.5-8.0 (Padan *et al.*, 1976). The transport of neutral and acidic amino acids was shown to be proton-linked such that symport of the substrate and of at least one proton occurred. Evidence has been produced which indicates that the proton-linked systems have K_M values which vary with pH (Booth and Hamilton, 1980 . Alanine uptake by *E.coli* through system *dag* A :K_M=6 μM at pH 5.5, K_M=22 μM at pH 7.8). If the internal pH was higher than the external pH this would contribute to the accumulation of the substrate within the cell. Although the data are very limited this may possibly account for one aspect of energy coupling.

Sodium ion gradients have been shown to be involved in glutamate transport by *E.coli* (MacDonald *et al.*, 1977). The gradients were generated by a Na^+/H^+ antiport system energized by the p.m.f. . Glutamate has been shown to enter the cell in symport with Na^+. The latter is then removed from the cell by the Na^+/H^+ antiport system thereby removing the coupling ion from the cytoplasm. The absence of this ion was found to prevent leakage of glutamate from the cell, in contrast to the proton-linked systems where the coupling ion (H^+) was present in the cytoplasm thereby allowing some degree of efflux.

2. Fungi

Amino acid transport in fungi was found to be accompanied by an influx of protons. The stoichiometry of this symport system has been

studied for *S.cerevisiae* and other yeasts (Eddy and Nowacki, 1971); Seaston *et al.*, 1973,1976; Cockburn *et al.*, 1975; Eddy, *et al.*,1977). The general amino acid permease of *S.cerevisiae* showed a stoichiometry of about $2H^+$ equivalents per mole of amino acid transported while the transport of lysine, proline, and methionine was accompanied by an influx of only one proton. Glutamate transport in *S.cerevisiae* has been reported to be accompanied by a maximum of 3 protons. *Candida utilis* showed a similar proton influx with 1 mole of H^+ per mole of glycine, lysine or arginine and 2 moles of H^+ per mole of glutamate (Eddy *et al.*, 1977).

Protons were found to be effluxed fromthe cells by an ATP-driven proton pump (Bowman *et al.*, 1978). However, when energy metabolism was inhibited by the presence of antimycin and 2-deoxy-D-glucose the charge influx was neutralised by an efflux of K^+ ions (Eddy, *et al.*, 1977).

Amino acid transport therefore appears to be driven by a proton-motive force generated by a membrane-bound ATPase. The pH gradient in *S.cerevisiae* was found to be such that the intracellular pH was near 6 when the extracellular pH was 4.5 (Salhony *et al.*, 1975). Initial work with membrane vesicles of *S.cerevisiae* confirms the involvement of the p.m.f. since the pronophore CCCP was found to eliminate transport of glycine and proline (Merkel *et al.*, 1980).

The transport of glutamate, lysine and leucine into *C.albicans*

was shown to be inhibited by the presence of azide, DNP , CCCP , DCCI and arsenate. This suggests that energization of transport in this yeast is similar to that previously described (Davies, 1979.)

Thus amino acid transport generally occurs via many permeases specific for either a single amino acid or a group of closely related amino acids. Binding proteins may be present, with high affinity for individual amino acids, which increase the availability of the substrate for the permease. The rate of amino acid uptake is controlled by both the intracellular concentration of specific amino acids which control their own permease (transinhibition), and by the levels of metabolic nitrogen within the cell (ammonium repression). The permeases are linked to metabolic energy (ATP) through the generation of a proton-motive force (except in the case of the shock-sensitive bacterial permeases). The pH difference across the cell membrane may play a role in the intracellular release of the amino acids.

Hence, all of the criteria for substrate transport across the cell membrane are achieved. A comparison with ammonium ion transport reveals similarities in specificity, control and probably energization. Equally the pH difference may play a role in the intracellular release of

ammonium ions. The transport of peptides also has to meet these requirements. however, in contrast to the preceeding examples of transport, the substrate released intracellularly is available for rapid hydrolysis. Thus, the control mechanisms may be more complex. Equally the number of individual substrates available precludes individual permeases for each peptide, as has been found for certain amino acid transport systems. Therefore differences in determinants of specificty between amino acid and peptide permeases are to be expected. The peptide permeases of prokaryotes and eukaryotes will now be discussed.

Peptide Transport

Peptides have been found to an important nitrogen source for many microorganisms. Media supplemented with peptones have provided an excellent growth medium for most bacteria and fungi. To serve as nutrients it is necessary that the peptides should interact with the cell surface and enter the cell. Therefore they must either be hydrolyzed to their constituent amino acids (which can then be transported through the previously described systems), or enter the cell intact through specific peptide permease systems. Intracellular hydrolysis would then result in the release of amino acids.

There is evidence for the existence of peptide permeases in bacteria, fungi and mammals although data are restricted to either a few species in each class, or in the case of mammals, to a limited number of tissues. The data available will now be assessed.

1. Bacteria

Amino acid transport has been separated from peptide transport by the use of auxotrophs and confirmed by the use of mutants. Those lacking amino acid transport systems maintained their ability to transport peptides and peptide transport deficient mutants retained their capacity for amino acid transport. The intracellular accumulation of intact peptides has been demonstrated in *E.coli* (Payne and Bell, 1979), and *Streptococcus faecalis* (Nisbet and Payne, 1982). This provided absolute proof for the existence of peptide-specific transport systems.

Some bacterial speces such as *Clostridium histolyticum* (Kessler and Yaron, 1976) have been shown to produce extracellular peptidases and so interpretation of transport in these cases would become more complex. Most work has been carried out on *E.coli*, *S.faecalis* and *Pseudomonas aeruginosa* which do not produce such enzymes.

Peptide transport in *E.coli* was shown to be accompanied by a rapid efflux of the constituent amino acids of the peptide (Payne and Bell, 1977). The rates of exodus of various amino acids were not identical, certain residues such as glutamine, aspartic acid, glutamic acid, asparagine and serine were not effluxed. These residues did not enter the amino acid pool and were therefore subjected to rapid metabolism following hydrolysis of the peptide within the cell. The use of radiolabels has therefore been found to produce considerable underestimates of the rate of transport arising from the efflux and metabolism of the amino acid constituents (Payne and Nisbet, 1980).

Table 1.1 The structural specificities of the peptide permeases

The references to the data are contained within the text

ND = not determined

	E.coli	*S.cerevisiae*	*C.albicans*	*N.crassa*	Mammalian Small Intestine
N-terminal	Positive charge on nitrogen required	Acetylation tolerated although such peptides may utilize a separate system	Acetylation tolerated although such peptides may enter through a separate system	Free N-terminal required	Free N-terminal required
C-terminal	Esterification tolerated. Substitution (as in alafosfalin) also tolerated	Esterification tolerated	Esterification not tolerated. Substitution (as in alafosfalin) tolerated	ND	Free C-terminal required
Stereo-chemistry	dpp requires L-L-peptide, opp tolerates C-terminal D-residue.	Only L-residues tolerated	D-residue at the N-terminal of oligopeptide tolerated. Dipeptides must be L-L	ND	D-residues may be tolerated.
Chain length	Size limit occurs at 650 daltons	$>met_5$ (Naider, *et al.*, 1974) $<ala_4$ (Nisbet and Payne, 1979b)	ND	Optimal chain length 3-5 residues	Rate decreases with increasing chain length, 4 residues hydrolysis occurs before transport.
Peptide-bond	α-N methyl tolerated β-ala tolerated at C-terminal Does not tolerate γ- and ε-linked peptides	α-N can be methylated	β- and γ-linked peptides have low affinity	ND	α-N can be methylated. β-alanyl residues tolerated. β-asp and γ-glu residues not tolerated
Multiplicity of permeases	2 systems: opp + dpp Active	One general system Active	One general system (possibly two) Apparently active	One oligopeptide permease Apparently active	ND Apparently passive

The use of toxic peptides will be discussed later, however the production of mutants resistant to such drugs showed that *E.coli* possesses two peptide transport systems: one specific for dipeptides (the dpp), the other for oligopeptides (the opp) (Payne, 1968; Gilvarg and Levin, 1972). These have also been described in other bacterial species including *S.faecalis* (Nisbet and Payne, 1982). Recently Payne *et al.* (1982) have described a third peptide transport system in *S.faecalis*, the App, highly specific for anionic peptides with N-terminal glutamyl or aspartyl residues. There is no evidence for such a system in *E.coli*

The specificites of the two systems present in *E.coli* will now be discussed, with reference to other bacterial systems, and are summarized in Table 1.1.

a. N-terminal α-amino group

α-N-acyl peptides were cleaved by *E.coli* . Auxotrophs were, however, unable to utilize such peptides, indicating that they were not transported (Gilvarg and Katchalski, 1965). The requirement was not for a free amino group but for a positive charge on the nitrogen atom since N-alkyl but not N,N-dialkyl-peptides were transported (Payne, 1971). This specificity applies to both the dpp and opp systems and also to other bacterial species, for example *Pseudomonas aeruginosa* (Miller and Becker, 1978).

b. C-terminal carboxyl group

Derivatives of peptides either devoid of the carboxyl group or with a derivatized carboxyl terminal were transported by both the opp and dpp . Most dipeptide derivatives such as lysyl cadaverine (lys_2 without a carboxyl group) entered through the opp of *E.coli* (Payne and

Gilvarg, 1968a). However, alafosfalin (alanyl-aminoethylphosphonic acid) was inactive against a dpp deficient mutant of *E.coli* and was therefore transported through the dpp (Atherton *et al.*, 1979a). The C-terminal specificity of the opp was also found to be less strict than the dpp in *S.faecalis* (Nisbet and Payne, 1982).

C. The peptide bond

Gly-β-ala was transported, at a low rate, by the opp and was unable to use the dpp due to the incorrect conformational relationship between the amino and carboxyl groups (Payne, 1973). The opp also demonstrated some degree of specificity since gly gly β-ala but not β-ala-gly gly was transported. In addition γ- and ε-linked peptides failed to compete with α-linked peptides acting as growth substrates for auxotrophs of *E.coli* (Payne, 1972a). There is therefore a general requirement for α-linkages in transported peptides.

If the peptide-bond was methylated at the α-nitrogen, as in glycyl sarcosine, the peptide was still transported at a low rate (Payne, 1972b). Such peptides were resistant to hydrolysis and therefore accumulated within the cell. This clearly demonstrated the separation of peptide transport and hydrolysis (Payne and Bell, 1979).

The hydrogen of an α-carbon in a peptide bond was not involved in the mechanism of oligopeptide transport in the *Lactobacilli*. *L.casei* rapidly transported gly-AIB-ala (AIB=α-aminoisobutyrate) through the opp system. This was also true for peptides containing cycloleucine (Young, *et al.*, 1964).

d. Sterospecificity

In all bacteria studied the dpp showed a requirement for dipeptides in the L-L-configuration. The opp of *E.coli* transported oligopeptides with a D-residue at the C-terminus (Payne, 1980). However, there were considerable differences in the tolerance of various bacterial species.

e. Side-chain specificity

The side-chain specificities of both the dpp and opp have been shown to be very broad (Payne and Bell, 1979), both systems were capable of transporting derivatized peptides and those with non-naturally occuring side-chains. This has led to the development of illicit transport (Ames *et al.*, 1973) which will be discussed in detail at a later point.

f. Size-limit for peptide transport

Work following the growth of *E.coli* auxotrophs showed a cut-off point in chain length above which growth on that peptide could not occur (Payne and Gilvarg, 1968b). The size-limit was found to be dependent upon the hydrodynamic volume of the molecule and occurred at approximately 650 daltons. Peptides larger than the size limit were unable to interact with the transport system. Therefore the size limit would appear to be due to a sieving effect from the cell wall. The structure responsible for sieving has been shown to be a protein, porin, which can be released from the cells by osmotic shock (reviewed by Payne, 1980). Whether porin-deficient mutants of *E.coli* are altered in their capacity to transport peptides has not been determined.

2. Non-Filamentous Fungi

The peptide transport systems of two non-filamentous fungi has been studied. *Saccharomyces cerevisiae* and *Candida albicans*. The structural specificities of these will now be discussed and are summarized in Table 1.1.

i. *Saccharomyces cerevisiae*

Studies of peptide transport in *S.cerevisiae* parallel those carried out on bacteria. Peptide uptake has been shown to occur independently of amino acid transport (Becker *et al.*, 1973; Naider *et al.*, 1974) and the absence of extracellular peptidases has permitted studies using auxotrophs. However, these have been superceded by the use of radiotracers and, more recently, by the continuous assay system using fluorescamine (Payne and Nisbet, 1981).

a. N-terminal

Auxotrophic growth tests have indicated a tolerance of acylated methionine-containing dipeptides and oligopeptides (Naider *et al.*, 1974). N-acetyl gly leu and N-acetyl leu_3 were not, however, utilized by a leucine auxotroph (Marder *et al.*, 1977). Both auxotrophic competition studies and radiotracer studies have indicated that N-acetyl met_3 is either a weak competitor for a general peptide transport system or, alternatively, enters the cell via a separate system (Marder *et al.*, 1977; Becker and Naider, 1977). This point will be discussed later.

b. C-terminal

The carboxyl-terminal of a peptide may be esterified and still transported. Considerable lag periods were, however, observed in the

growth of auxotrophs using methyl esters of met_3 , met_4 or met_5 (Naider *et al.*, 1974). Furthermore it was found that the benzyl ester of leu_2 served as a growth substrate for a leucine auxotroph (Marder *et al.*, 1977). The methyl ester of met_3 was found to compete with uptake of $[^{14}C]met_3$ indicating that these peptides share a common permease (Becker and Naider, 1977).

The presence of a poly-(ethylene glycol) group at the C-terminus of met_3 did not prevent the peptide from interacting with the transport system even though the molecule could not be transported (Naider *et al.*, 1980). The K_i for this process (5.1×10^{-5} M) was close to the K_M for met_3 transport (7.7×10^{-5} M , Becker and Naider, 1977). It was also found that Boc-met_3-OPEG , in which the N-terminal was also blcoked, was unable to compete with met_3 transport and indicated that the permease showed the same specificity for the conjugate as for the free peptide (Naider *et al.*, 1980). For met_3-OPEG to interact with the permease either the transport system is located external to the barrier for poly-(ethylene glycol) or specific pores exist in the cell envelope through which various substrates can interact with the cell membrane. The latter possibility is consistent with the observation that the yeast α-mating factor (a dodecapeptide) can interact with its receptor in the plasma membrane of *S.cerevisiae* even though tetrapeptides do not serve as substrates for the peptide permease (Ciejek *et al.*, 1977; Nisbet and Payne, 1979b).

c. Side-chain specificity

This appears to be very broad. Early indications of specificity were, however, demonstrated by the absence of growth of a lysine auxotroph of *S.cerevisiae* on any lysine-containing peptides even though these were

hydrolyzed by a cell extract (Becker *et al.*, 1973). The first systematic study was carried out by Nisbet and Payne (1979b). This showed that basic peptides were transported at a greater rate than acidic peptides with some lysine-containing peptides being transported in contradiction of earlier results. It was also found that the presence of an N- or C-terminal glycine residues significantly reduced the rate of peptide transport. Dipeptides of the sequence ala-x were generally transported at a greater rate than those of the sequence x-ala.

d. The peptide bond

Only limited data are available however sarcosine-containing peptides were rapidly accumulated by *S.cerevisiae* (Nisbet and Payne, 1979a). These peptides were resistant to hydrolysis and clearly demonstrated that peptide transport was independent of the hydrolytic system.

e. The size limit for peptide transport

A methionine auxotroph was able to grow on met_5 which indicated a higher size limit than that found for bacteria (Naider *et al.*, 1974). The systematic study of Nisbet and Payne (1979b) showed the upper size limit to occur at the tripeptide ala_3 with the transport of ala_4 not being detected. Therefore there is the possibility of considerable strain differences which should always be considered in studies of this nature.

f. Stereospecificity

Auxotrophs requiring leucine were shown to grow on L-leu-L-leu but not on D-leu-L-leu or L-leu-D-leu. This was not directly attributable to the stereospecificity of the transport system because L-leu-D-leu and D-leu-L-leu were hydrolyzed at only a low rate by a cell extract (Marder *et al.*, 1977). Nisbet and Payne (1979b) have shown the transport of both dipeptides and tripeptides to be highly stereospecific: L-ala-D-ala, D-ala-L-ala , L-ala-L-ala-D-ala , L-ala-D-ala-L-ala and D-ala-D-ala-D-ala were not transported.

g. Multiplicity of permeases

Initial studies indicated that there was no competition between [^{3}H]acetyl met$_3$ and met met [^{14}C]met even though both were transported into cells. This could be interpreted in two ways: firstly there may be two transport systems in operation, one for peptides and their esters, the other highly specific for acylated peptides. Secondly N-acetyl met$_3$ may have only a low affinity for a single peptide permease. A study of the kinetics of uptake indicated that N-acetyl met$_3$ (K_M=16.0×10^{-5} M) was only a weak competitor with met$_3$ (K_M=7.7×10^{-5} M). Competition for a single system would not therefore be observed (Becker and Naider, 1977). Two concurrent studies confirmed the presence of a single peptide permease in *S.cerevisiae* (Marder *et al.*, 1978; Nisbet and Payne, 1979b). Marder *et al.*, (1978) produced mutants resistant to L-ethionyl-L-alanine which were shown to be cross-resistant with L-leu-L-leu-L-eth. The toxic

effect of these peptides was brought about by intracellular hydrolysis and release of the methionine analogue L-ethionine. No significant differences were found in the peptidase activities of the wild type and the mutant. Therefore the transport capacity for both dipeptides and tripeptides was lost in a single-step mutation. This was confirmed by the inability of the mutant to transport [^{14}C] gly leu while [^{14}C] methionine uptake was unaffected.

Nisbet and Payne (1979b) produced a bacilysin resistant mutant of *S.cerevisiae* and also demonsrated a simultaneous loss of dipeptide and tripeptide transport capacity. No data are available concerning the transport of N-acylated peptides by the mutant and so the possibility remains that these are transported via a separate system.

ii. *Candida albicans*

The peptide transport system of *C.albicans* has been studied in some detail with the particular objective of producing antifungal agents selectively toxic for this fungal pathogen.

Studies using *C.albicans* WD18-4, a methionine and lysine double auxotroph, showed that no extracellular peptidases were produced (Lichliter *et al.*, 1976). Growth studies could therefore be related to peptide transport. The essential points of this work were:

1. Peptides could be N-acetylated and still transported

Table 1.2 The effect of peptides on the uptake of met met [^{14}C]met by *C.albicans* WD18-4

from Logan *et al.* (1979)

Competitors	Non-competitors
met ala met	met_2
leu_3	L-methionine
gly met gly	lys_3
ala_3	met met-D-met
met ile met	D-met-D-met-D-met
D-met-L-met-L-met	met_3-OMe
N-acetyl met_3	met_3-ONH_2

Table 1.3 The effect of competitors on the incorporation of radiolabelled peptides into *C.albicans* 6406

Results are expressed as percentage inhibition of incorporation. The initial concentrations of the radiolabelled peptides were 0.3 mM

From Davies (1980)

Competitor (3 mM)	gly [^{14}C] phe	ala [^{14}C] ala	ala ala [^{14}C] ala
gly phe	(83)	21	38
gly leu	69	54	36
gly_2	26	6	0
ala_2	85	(83)	86
ala leu	-	91	100
leu_2	-	100	-
gly_2	52	18	-
ala_3	81	59	(85)
gly_3	6	0	0
leu_3	-	100	100
gly	0	-	-
phe	0	-	-
ala	-	0	0
alafosfalin	-	48	39
ala-alatetrazole	-	100	100
N-acetyl ala_3	-	-	0
N-acetyl ala gly ala	-	0	0
L-ala-D-ala	-	0	-
D-ala-L-ala	-	0	-
D-ala-D-ala	-	0	-
α-gly ala	-	65	59
γ-glu ala	-	4	11
α-glu ala ala	-	52	66
γ-glu ala ala	-	0	10
β-ala ala	-	0	-

2. Derivitization of the C-terminal prevented uptake.
3. All lysine-containing peptides, except lys gly , were found to be non-growth substrates. This was not found to be due to a lack of intracellular hydrolysis.
4. Some amino acids such as L-ethionine and L-norleucine, both methionine analogues, could be incorporated into methionine-containing peptides without altering the growth response. This indicated that the peptide permease showed a certain degree of side-chain tolerance; however the peptides were not toxic to the cells.
5. D-stereoisomers were tolerated at a limited number of positions within a peptide. L-met-L-met-L-met and D-met-L-met-L-met were utilized by the auxotroph whereas L-met-L-met-D-met , D-met-D-met-D-met and L-val-L-met-D-met were not utilized.
6. All stereoisomers and esters, except D-met-D-met-D-met were found to be hydrolyzed by a cell extract.

The uptake of radiolabelled peptides has been followed by two groups: Logan *et al.* (1979) used met met [^{14}C] met (*C.albicans* WD18-4) and Davies (1979,1980) used gly [^{14}C] phe, ala [^{14}C] ala and ala ala [^{14}C] ala (*C.albicans* 6406). The results are summarised in Tables 1.2 and 1.3.

The optimum pH for transport has been descirbed as pH 3.5-4.5 (Logan *et al.*, 1979) and pH 4.5 (Davies, 1980) with a temperature optimum of 37^{o}C in both caes. Kinetic studies were carried out in both

cases, the K_M values determined being:

met_3	$K_m \doteq 33\ \mu M$
gly phe	$K_m \doteq 350\ \mu M$
ala_2	$K_m = 250\ \mu M$
ala_3	$K_m \doteq 115\ \mu M$

However, it should be noted that the above kinetic values may be affected by the rate of intracellular hydrolysis and the subsequent metabolism of the amino acid residues.

Both Logan *et al.* (1979) and Davies (1980) showed that amino acids had no competitive effect for peptide uptake and confirmed the separation of amino acid and peptide transport in *C. albicans*.

a. Multiplicity of permeases and the N-terminal

Competition studies between radiolabelled and non-labelled peptides showed contradictory results. Logan *et al.* (1979) found that met_2 showed little competition for the uptake of met_3 whereas tripeptides were effective competitors. N-acetyl met_3 was a competitor confirming the earlier auxotrophic response studies (Lichliter *et al.*, 1976). Davies (1980) found that both dipeptides and tripeptides showed mutual competition for transport and concluded the presence of a single peptide permease in this organism. This study found N-acetylated peptides to be ineffective competitors for transport in contradiction of all previous work.

Studies on the mode of action of bacilysin (Figure 1.1) have complemented the studies of the peptide transport system in *C.albicans*. Bacilysin is a dipeptide containing the toxic residue anticapsin, an inhibitor of amidotransferase. This antibiotic has been found to be highly active against bacteria and *C.albicans*. The activity against *C.albicans* was found to be higher on minimal agar than on Sabouraud's agar (Kenig and Abraham, 1976). Ala phe (15 mM) completely inhibited the action of bacilysin (0.37 μM). No amino acids had such an effect implying that bacilysin enters the cell via the peptide permease. No data are available concerning the peptide transport capacity of the spontaneously resistant mutants found within the zone of inhibition. The transport of bacilysin (=tetaine), through the peptide transport system was also studied by Chmara *et al.*, (198o) who synthesized derivatives of the related epoxypeptides (Borowski *et al.*, 1979). The results showed that N-acylated derivatives of the epoxypeptides were active against *C.albicans* indicating that the transport of N-terminal blocked peptides does occur. This was in agreement with the results of both Lichliter *et al.*, (1976) and Logan *et al.*, (1979).

Steinfield *et al.* (1979) and Ti *et al.* (1980) described the synthesis of peptides linked via the N-terminal residue to 5-fluorocytosine (5-FC) and 5-fluoroorotic acid. 5-FC is a potent anticandidal agent. These studies were carried out to test the use of the peptide transport system as a method of drug delivery as had been proposed by Becker *et al.* (1977). However they also show the effect of N-terminal blocks on peptide transport. Steinfield *et al.* (1979) found that the conjugates which were produced had half lives of only 0.3-17.6 hours and gave MIC values equivalent to that of free 5-FC. This indicated the

breakdown of the conjugates in the growth medium. Ti *et al.* (1980) produced conjugates with longer half lives; however these were highly pH dependent. The half life for N^4(succinyl ala leu) 5FC was 1.7 hours at pH 7.2 increasing to 49 hours at pH 5.0. This was reflected in the MIC values obtained against *S.cerevisiae* which is highly sensitive to 5-FC: at pH 7.2 the activity of the conjugate was equivalent to that of free 5-FC implying that hydrolysis and hence release of 5-FC into the growth medium was occuring. However, at pH 5.0 the MIC for 5-Fc was far lower than that obtained for the conjugate; this reflected the low transport rate of N-acylated peptides by *S.cerevisiae*. *C.albicans* showed equivalent sensitivities to the conjugate at both pH values implying that transport of the intact drug occurred at pH 5.0 followed by the release of 5-FC upon intracellular hydrolysis.

This evidence indicates that N-terminal blocked peptides can enter *C.albicans* via the peptide permease. Davies (1980) did not find N-acetylated peptides to inhibit the uptake of radiolabelled peptides. This may possibly indicate the presence of strain variations in *C.albicans* equivalent to those found in *S.cerevisiae* which have been described previously.

b. C-terminal

The methyl ester and amide of met_3 showed little competition for met_3 transport (Logan *et al.*, 1979). This indicated a requirement for a free carboxyl terminus. Davies (1980) showed that the requirement

was for a free acidic function in dipeptides: both alafosfalin and ala ala tetrazole competed for uptake of ala [^{14}C] ala and ala ala-[^{14}C] ala . Furthermore, Davies (1980) demonstrated the intracellular accumulation of alafosfalin indicating that this molecule enters the cell via the peptide permease.

c. Stereospecificity

Logan *et al.* (1979) found that L-met-L-met-D-met and D-met-D-met-D-met did not compete for transport of met_3 while D-met-L-met-L-met was a competitor. This confirmed the earlier auxotrophic growth studies and indicated a tolerance of D-resides at the N-terminal of an oligopeptide. Davies (1980) showed that none of the stereoisomers of ala_2 other than L-ala-L-ala could compete for ala [^{14}C] ala incorporation and demonstrated that there was no tolerance of D-residues in dipeptide transport.

The antibiotic L-arg-D-allothreonine-L-phe , produced by *Keratinophyllum terreum*, has been shown to be active against a number of fungi including *C. albicans*. Synthetic derivatives were produced with the structure L-arg-D-X-L-phe (X=ala, tyr, val, phe, leu. Eisele, 1975). All showed antibiotic activity with the effect being neutralized by the addition of the central L-amino acid. These linear peptides have proved to be resistant to hydrolysis and so, although the mode of action is not clearly understood, it suggests that thepermease tolerates D-residues in the central position of a tripeptide.

d. The peptide bond

There appears to be a general requirement for α-linkages. γ-gly ala , γ-glu ala ala and β ala ala had only low affinity for the peptide permeases (Davies, 1980). There is no information available concerning the transport of sarcosine-containing peptides by this organism.

The peptide permease of *C.albicans* shows a number of important differences from both the systems of *E.coli* and that of *S.cerevisiae*. These include the toleration of D-isomers and the effect of N- and C-terminal blocks (Table 1.1). Consideration will now be given to the filamentous fungi and mammalian systems.

3. Filamentous Fungi

The peptide transport system of only one filamentous fungus, *Neurospora crassa*, has been studied. This was a limitedstudy but it does delineate the system.

Peptide transport by a leucine auxotroph was shown to be separate from amino acid transport (Wolfinbarger and Marzluf, 1974). Dipeptides could not be utilized and the upper size limit occurred at a hydrodynamic volume equivalent to leu_3 . The optimal size of peptides for transport occurred overthe range 3-5 amino acid resiudes (Wolfinbarger and Marzluf, 1975a).

For tyrosine-sensitive strains of *N.crassa* gly leu tyr has proved to be toxic. It was therefore possible to select a gly leu tyr resistant (*glt-r*) strain of the leucine auxotroph. Uptake studies using

gly leu [^{3}H] tyr showed that *glt-r* had lost over 90% of the transport capacity for this peptide. This indicated that there was probably only one (oligopeptide) transport system present in this organism (Wolfinbarger and Marzluf, 1975b and 1976). It is constitutive the growth medium having no effect upon peptide transport. Peptide transport was totally lacking in conidiospores of this organism.

N-acetyl gly leu tyr was unable to compete with gly leu [^{3}H] tyr for transport. This indicated that N-terminal blocked peptides are not transported by *N. crassa* (Wolfinbarger and Marzluf, 1975b).

4. Mammalian peptide transport

Peptide transport in the small intestine has been studied in depth, the renal and brain systems to a lesser extent. The characteristics of the intestinal uptake system are outlined below and have been the subject of two recent reviews (Matthews and Payne, 1980; Ganapathy and Leibach, 1982).

1. Substitution of the amino terminal reduced or abolished peptide transport. N-acetyl gly_2 did not inhibit gly pro transport (Rubino *et al.*, 1971) and N-Boc gly leu only weakly inhibited gly leu transport (Das and Radhakrishnan, 1975).
2. Amidation of the C-terminal reduced the affinity of the peptide for the transport system. Gly gly-ONH_2 did not inhibit β-ala his or gly sar sar transport (Addis *et al.*, 1974 and 1975a).

3. Some β-alanyl peptides were transported as where sarcosine-containing peptides (Addison *et al.*, 1975a and b). Peptides containing β-aspartyl and γ-glutamyl linkages were not transported. However, the rates of hydrolysis of β-alanyl and sarcosine-containing peptides were very low (Addison *et al.*, 1975b; Matthews, *et al.*, 1974).
4. D-amino acid residues were tolerated; however the rates were diminished when compared with the L-steroisomers (Asatoor *et al.*, 1973).
5. Hydrophobic side-chain residues appeared to increase the affinity of the peptide for the transport system (Das and Radhakrishran, 1975).
6. The rate of peptide transport decreased with chain length. Peptides larger than four amino acid residues underwent brush-border hydrolysis before transport (Adibi and Morse, 1977).
7. Peptide transport was independent of amino acid transport. Many peptides were mutually competitive for uptake. However the presence of multiple permeases could not be excluded (Addison *et al.*, 1974).
8. Two mechanisms of peptide transport appeared to be in operation (Adibi, 1971):
   i) hydrolysis by brush-border peptidases followed by transport of the constituent amino acids through the established systems.
   ii) the transport of intact peptides followed by intracellular hydrolysis.

Mechanisms of Peptide Transport

Many mechanisms have been proposed for the transmembrane transport of simple peptides into all of the organisms so far discussed. In contrast to amino acid transport, for which only the uptake of the unaltered molecule is possible, peptide transport can be accompanied by hydrolysis at any point during the translocation process.

Five mechanisms have been proposed for the transport of peptides (Payne and Gilvarg, 1978; Payne, 1980). These were originally described for bacterial systems; however they share many similarities with the transport of peptides into eukaryotic cells. These proposals will now be discussed with an indication of the characteristics which would be expected from such mechanisms.

1. Extracellular hydrolysis of peptides followed by the transport of the constituent amino acids through their respective permeases. This would require the presence of peptidases either into the extracellular medium, in the periplasmic space or within the cell wall. It would be characterized by competition for uptake between peptides and exogenously supplied amino acids. Extracellular peptidases have been identified in certain bacteria (for example *Clostridium histolyticum*. Kessler and Yaron, 1976) . Therefore this mechanism may play a role in the uptake of peptides in such cases.

2. Peptide transport and hydrolysis associated with the cell membrane: This would be as in (1) except that the peptidases would be bound to the cell membrane. Therefore free peptidases would not be demonstrated.
3. Membrane-bound peptidases generate cleavage products, without outward release: In this case the amino acid constituents would be transported via carriers inaccessable to externally supplied amino acids. Therefore the specificity of this model would reside with the peptidase. Oligopeptides may be either sequentially cleaved or alternatively be partially cleaved and transported as amino acids and a peptide. The partial peptide would then be cleaved intracellularly. This does not explain the accumulation of intact peptides observed in *E.coli* (Payne and Bell, 1979) and *S.cerevisiae* (Nisbet and Payne, 1979a). However, this is a mechanism proposed for intestinal peptide transport (Ugolev, *et al.*, 1977).
4. Group translocation: A membrane peptidase binds peptides extracellularly. These would then be hydrolyzed and released intracellularly as free amino acids. Again this mechanism does not explain the intracellular accumulation of certain peptides.
5. Peptide transport: This model suggests that dipeptide and oligopeptide carriers exist in the cell membrane and that these are distinct from amino acid carriers. These deliver peptides to the intracellular environment where they are cleaved

by the intracellular peptidases. Therefore this is true peptide transport which has been identified in most of the microorganisms previously described. However, in certain instances the data are insufficient to preclude the operation of systems (3) and (4) since the accumulation of intact peptides has not been demonstrated.

Energetics of peptide transport

The rapid intracellular hyrolysis of peptides generally precludes the demonstration of intracellular accumulation of peptides. In *S.cerevisiae* hydrolysis was observed within 30 secs of transport (Becker and Naider, 1977), certain peptides are however resistant to hydrolysis. *E.coli* has been shown to accumulate orn_3 (Payne and Bell, 1979) while sarcosine-containing peptides were demonstrated in the cytoplasm of *S.cerevisiae* (Nisbet and Payne, 1979a). More evidence for the accumulation of peptides came from studies of peptidase mutants of *Salmonella typhimurium* which were shown to accumulate both [^{14}C] gly pro and pro [^{14}C] gly (Yang *et al.*, 1977; Jackson, *et al.*, 1976).

A metabolizable energy source has been shown to be necessary for peptide accumulation in *E.coli* (Payne, 1972a; Payne and Bell, 1979), *S.cerevisiae* (Nisbet and Payne, 1979a), *C.albicans* (Logan *et al.*, 1979), and *N.crassa* (Wolfinbarger and Marzluf, 1976). This evidence, in conjunction with the accumulation of certain intact peptides, indicates that peptide transport is an active process in microorganisms. Individual

cases will now be considered.

E.coli

Payne and Bell (1979) demonstrated that inhibitors of energy production prevented transport through either the dpp or the opp . Arsenate depleted the intracellular ATP pool of *E.coli* and decreased the rate of uptake of both dipeptides and oligopeptides by more than 80%. 2,4-DNP and CCCP had little effect when glucose served as the energy source but showed a greater inhibitory effect when lactate was used. This suggested that the permeases were dependent upon phosphate-bond energy rather than the maintenance of a proton gradient. Therefore, these permeases resemble the shock-sensitive amino acid transport systems. However, no binding proteins for [^{14}C] gly gly have been detected following osmotic shock of the cells. Membrane vesicles of *E.coli* failed to transport [^{14}C] gly gly and this suggested that binding proteins may be involved in transport (Cowell, 1974). The binding of other peptides to proteins present in the shock-fluid has not been assayed. This may explain the apparent absence of binding proteins and confirm that the energization of peptide transport in *E.coli* is equivalent to that of the shock-sensitive amino acid permeases.

S.cerevisiae

Several initial studies have been carried out on the energization of peptide transport in *S.cerevisiae*. It has been found that the system

is sensitive to both pH and temperature and that it is inhibited by azide, 2,4-DNP and cyanide (Becker and Naider, 1977). Cycloheximide was found to be ineffective indicating no short-term dependence upon protein synthesis. Arsenate was found to have no effect upon peptide transport; however this was probably due to the presence of phosphate in the buffer system. Payne and Nisbet (1981) have shown aresenate to be an effective inhibitor when using a phosphate-free buffer. In addition Payne and Nisbet (1981) have shown the process to be inhibited by DCCI , CCCP and diethylstibestrone. The energization of peptide transport in *S.cerevisiae* therefore resembles that of amino acid transport in that it requires the maintenance of a proton-motive force with rapid inhibition being brought about by the protonophore CCCP and the ATPase inhibitor diethylstilbestrone.

C.albicans

The use of metabolic inhibitors has shown that peptide uptake is inhibited by 2,4-DNP , N-ethyl maleimide and DCCI (Logan *et al.*, 1979) and, in addition to these, CCCP and arsenate (Davies, 1980). Therefore, the system appears to be dependent upon an ATP-generated proton-motive force as described for *S.cerevisiae*.

N.crassa

Very limited data are available. No intracellular accumulation of peptides has been demonstrated but the system is active in the sense that it required metabolic energy and was inhibited by azide (Wolfinbarger and Marzluf, 1975b).

Mammalian systems

Peptide transport into intestinal brush border vesicles has been sbown to be independent of Na^+ ions. It appears to be a passive process acting down a concentration gradient. The intracellular peptide concentration never exceeded that of the medium (Berteloot *et al.*, 1981). Therefore this is in contrast to all microorganisms which have been studied.

The uptake of peptides achieves the criteria required for any transport system: Recognition, translocation, energization and intracellular release. When considering true peptide transport the latter three processes are very similar to those of the amino acid permeases. However, the recognition of the substrate differs. Amino acid permeases have high affinity for substrates with either a specific side-chain residue or a group of closely related side-chains (such as the leucine, isoleucine, valine system of *E.coli*. Rahmanian *et al.*, 1973). Therefore, this high degree of specficity does not allow the transport of unusual amino acids or D-isomers (in *S.cerevisiae* only the gap will transport D-isomers. Rytka, 1975). In contrast, the peptide permeases recognize peptides of a certain chain length (*E.coli* has two permeases: The dpp and the opp. *N.crassa* transports only oligopeptides) while the side-chain specificity is generally very broad. Therefore the transport of peptides containing unusual amino acid residues, such as the anti-capsin in bacilysin, or D-isomers is permitted. Equally the N - and C-terminals appear to be recognized by the permease; however the specificity of this process differs between the organisms studied: C-terminal esterification is tolerated by *E.coli* and *S.cerevisiae* but not by *C.albicans* or the mammalian small intestine (Table 1.1).

The toleration of unusual amino acid residues within a peptide has allowed the development of the illicit transport concept of drug development (Ames, *et al.*, 1973) which will be discussed later. The differences in the specificty of various organisms is being used to develop drugs with high activity for specific organisms but with low toxicity for the host. For some drugs which enter the cell by illicit transport (such as bacilysin) to be active they must be hydrolyzed, following transport, to release the toxic moiety. Therefore, the fungal peptide hydrolytic system will be considered before a discussion of peptide drugs.

The fungal peptide hydrolytic system

S.cerevisiae

Many peptidases and proteases have been isolated from *S.cerevisiae*. The following discussion will be restricted to the hydrolysis of peptides in relation to their transport.

Early work (Becker *et al.*, 1973) showed that many peptides are rapidly hydrolyzed by a cell extract of *S.cerevisiae*. These included many which were not utilized by auxotrophs of this organism. Marder *et al.*,(1977) demonstrated that some non-growth substrates were hydrolyzed at only a low rate including pro pro leu , L-leu-D-leu , and D-leu-L-leu while Nisbet and Payne (1979a) showed that sarcosine - containing peptides were accumulated within the cell. Therefore this is an indication of both side-chain and stereospecificity amongst the peptidases. The rates of intracellular hydrolysis are extremely

rapid, some hydrolysis occurred after 30 secs. of met_3 transport (Becker and Naider, 1977). The same report showed that acetylation of met_3 reduced the rate of hydrolysis.

Loss of peptide transport capacity by mutation brought about only a negligable reduction in peptidase activity (Marder *et al.*, 1978). Therefore, there is no interdependence between the two systems.

Rose *et al.*, (1979) separated the peptidases of the soluble fraction of *S.cerevisiae* by polyacrylamide gel electrophoresis. Four bands of activity against dipeptides and tripeptides were demonstrated which corresponded with the four aminopeptidases described by Matile *et al.*, (1971). A single dipeptidase was later separated from one of the aminopeptidases with which it co-ran on electrophoreses (Rose, *et al.*, 1979). Carboxypeptidase activity was not found by the polyacrylamide gel technique although the presence of carboxypeptidase Y is well documented (Hayashi, 1976). The inactivity was probably because carboxypeptidase Y requires activation before hydrolytic activity is achieved.

There has been a number of reports concerning the localization of peptidase activity in *S.cerevisiae*. Aminopeptidase I , proteases A and B and carboxypeptidase Y are found within the vacuole while their respective inhibitors are located in the cytoplasm together with a single dipeptidase (Lenney *etal.*, 1974; Matern *et al.*, 1974; Frey and Rohm, 1978; Wiemken *et al.*,1979). One half of the aminopeptidase II activity was shown to be external to the cell membrane. However, as this

enzyme exhibits only limited activity against peptides and greater activity against amino acid-p-nitroanilides its function is uncertain (Frey and Rohm, 1979). There have also been reports of a highly specific cell surface endopeptidase involved in the cleavage of the yeast mating pheromone, α-factor (Ciejek and Thorner, 1979). The enzyme appears to be a highly specific endopeptidase and does not appear to affect the uptake of peptides. Parker, *et al.*, (1980) concluded that there is no significant extracellular peptidase activity related to the utilization of peptides. Peptidase activity concerned with peptide utilization is apparently restricted to the soluble fraction of this yeast.

It is interesting to note that, if peptides are hydrolyzed within the vacuole of the cell, peptide transport must occur across the vacuolar membrane in addition to the plasma membrane. Peptide transport systems in the vacuolar membrane may differ in their specifities when compared with those of the cytoplasmic membrane. This would reflect the arginine transport system found within the vacuolar membrane (Boller *et al.*, 1975).

Candida albicans

There have been relatively few reports concerning the peptidase and protease systems of *C. albicans*. All of the studies to date have shown the absence of extracellular hydrolytic activity against small transported peptides (Lichliter *et al.*,1976; Logan *et al.*, 1979).

Two intracellular peptidases have been identified. A single leucine aminopeptidase was shown to be located intracellularly (Kim, *et al.*, 1962). The small amount of extracellular activity was attributed to lysed cells in the growth medium. The second peptidase to be identified was present as a subunit of a highly unusual protein, Canditoxin (Iwata, 1981). This protein was shown to be located in the cytoplasm and consisted of four subunits two of which had carboxypeptidase activity (the third had phosphomonoesterase activity while the fourth was not identified). Canditoxin was shown to exert a highly cytotoxic effect on mammalian cells; however it would appear to be produced by only one strain of *C.albicans*. Other workers have been unable to identify this protein in their strains (Chattaway *et al.*, 1971).

Staib (1965) found that *C.albicans* was able to utilize serum proteins as the sole nitrogen source by the production of an extracellular protease. Under these conditions growth of *Candida* was found to be dependent upon pH , the optimum being pH 4.6-5.5. Remold *et al.*, (1968) described the purification of this enzyme (pH opt. 3.2, molecular weight 40,000 daltons), it was found to exhibit low side-chain specificity but showed preferential attack towards hydrophobic residues. A protease with low specificity would produce a large number of small peptides and amino acids which could then be transported into the cell through the established transport systems.

Recent work (Germaine and Tellefson, 1981) has demonstrated that the protease was active in cultures below pH 5.0, above this pH it was inactivated. This explained the pH dependence for growth on serum proteins described by Staib (1965). Germaine and Tellefson (1981) also showed that human saliva was inhibitory to the production of protease by *C.albicans* suggesting the proteolytic activity may possibly be of low importance in the human oral cavity. However, MacDonald and Odds (1983) have found that a mutant of *C.albicans* lacking protease secretion showed lower pathogenicity for mice than did the wild type. Therefore this enzyme appears to be of importance in pathogenicity.

N.crassa

The peptide hydrolytic system of *N.crassa* has been studied in some depth and, together with research concerning peptide transport in this organism, clearly demonstrates the mechanism of peptide utilization.

Eleven intracellular peptidases have been identified in *N.crassa* (Tan and Marzluf, 1979). They are constitutive and show a considerable overlap in their specificities. Peptides larger than leu_3 (the size limit for the peptide transport system) induced the production of extracellular peptidehydrolytic activity (Wolfinbarger, 1980). The oligopeptide permease in conjunction with the five amino acid permeases thus produce an effective mechanism for utilizing the hydrolysis products of proteins. The constitutive nature of the peptide transport system provides a means by which the organism can capture any environmental

peptides. The regulation of the production of peptide hydrolytic activity is not clearly understood: Although a leucine auxotroph of *N.crassa* produced the enzyme in response to peptides larger than those which could be transported, the *glt-r* strain (which lacked the peptide transport systems) produced the enzyme in response to any added peptide (Wolfinbarger, 1980).

Peptide drugs

There are two broad classes of peptide drugs: those which are transported into the cell where they are resistant to hydrolysis and act intact, and those which are transported and the cleaved to release a toxic moiety.

1. Drugs which are hydrolyzed

This class includes many of the recently developed "smugglin" compounds. The principle of illicit transport was first described as a means for impermeant molecules to enter the cell "disguised" as peptides (Ames *et al.*,1973). Ames *et al.*(1973) found that histidinol phosphate ester was unable to enter *Salmonella typhimurium* unless incorporated into the peptide gly gly-histidinol phosphate. This peptide was found to support growth of a histidine auxotroph of *S.typhimurium*, transport and subsequent intracellular cleavage of the peptide was therefore occuring which resulted in the release of histidinol phosphate within the cell. Ames *et al.*(1973) also demonstrated that amino acid analogues are far more inhibitory towards

growth of *S. typhimurium* when presented in the form of tripeptides rather than amino acids.

Many naturally-occuring compounds have now been recognized as acting by illicit transport and, in addition, the principle has been used to develop synthetic peptide-drugs acting in a similar way. The properties of a few natural and synthetic drugs will now be discussed.

Bacilysin

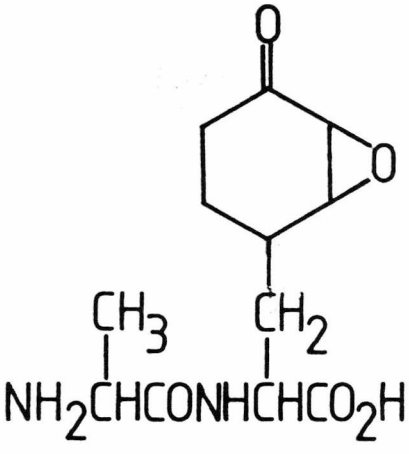
Bacilysin (Figure 1.1) is a dipeptide produced by *Bacillus subtilis* A14 (Walker and Abraham, 1970a and b). It has a broad antibiotic spectrum being active against both gram positive and gram negative bacteria as well as certain yeasts including *C. albicans* (Kenig and Abraham, 1976). The drug was found to be transported intact, intracellular hydrolysis results in the production of anticapsin a potent inhibitor of amidotransferase (E.C.2.6.1.16). Anticapsin is not itself transported via the amino acid permeases of either bacteria or *C. albicans*. Antibiotic activity is therefore dependent upon the intact peptide which can enter the cells.

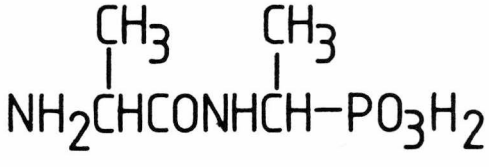
Phosphinothricyl alanyl alanine

This tripeptide was isolated by Bayer *et al.* (1972), from the culture filtrate of *Streptomyces viridochromogenes*. The active moiety, a γ-phosphinate analogue of glutamic acid, has been found to be a potent inhibitor of *E. coli* glutamine synthase. The transport of this

Figure 1.1 Bacilysin

FIgure 1.2 Alafosfalin





drug has been studied (Diddens *et al.*,1976) and has been shown to occur via the opp of *E.coli*.

Phaseolatoxin (N^{δ}-phosphosulphamyl)ornithyl alanyl homoarginine

Phaseolotoxin, produced by *Pseudomonas syringae* pv. *phaseolica*, has been shown to be an inhibitor of carbamoyl transferase. It is transported via the opp of both *E.coli* and *S.typhimurium* and is hydrolyzed by the intracellular peptidases. Resistance to phaseolotoxin produced mutants of both orgnaisms deficient in the oligopeptide transport system (Staskowicz and Panopoulos, 1980).

Phosphonopeptides

This was the first group of antibacterial agents to utilize the illicit transport principle. In these peptides the carboxyl terminus has been replaced by a phosphonyl group. The C-terminal amino acid, L-1-aminoethylphosphonic acid [L-ala(P)] , is an inhibitor of L-alanine racemase. Therefore it prevents formation of D-alanine which is required for bacterial cell wall biosynthesis. A series of the peptides with various N-terminal amino acid residues was produced. The activity of these reflected the current knowledge of the bacterial peptide transport system (Allen *et al.*, 1979; Atherton *et al.*, 1979 a and b).

1. The dipeptides showed a stereochemical requirement for the LL configuration.

2. Alteration of the N-terminal amino acid residue reflected those rates of transport obtained for the x-ala peptides with a normal carboxyl terminal.
3. The optimal chain length varied between bacterial species.
4. The stereospecificity of the higher chain length phosphono-peptides varied greatly between bacterial species. This was also found for the normal oligopeptides (Payne, 1980).

This research resulted in the production of the antibacterial agent alafosfalin (ala-ala(P), Figure 1.2), which is rapidly transported and hydrolyzed by many bacteria. Therefore, ala(P) is released intracellularly while it is not itself transported via the bacterial amino acid permease.

2. Peptide drugs which act without hydrolysis

This class includes both antibacterial and antifungal agents which have been used to a great extent in the study of peptide transport.

Tri-L-ornithine (orn_3), which inhibits protein synthesis (Gilvarg and Levin, 1972), has been used extensively in the isolation of bacterial opp-deficient mutants. Since the drug acts without hydrolysis the only mutation likely to give total resistance is in the peptide permease. Mutation at the target site is also possible; however such an instance has not been reported. Other drugs in this category include gly-L-leu and L-val-L-val-D-val both of which have been found to be threonine deaminase inhibitors (Wasmuth and Umbarger, 1974; Shankman, *et al.*, 1963).

A major class of antifungal agents have also been shown to enter the cell via the peptide permease: the polyoxins.

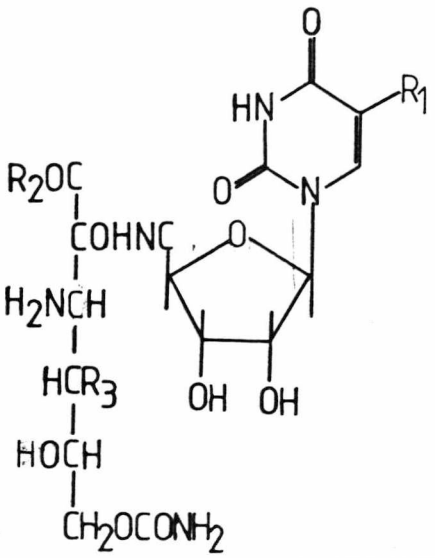
The polyoxins

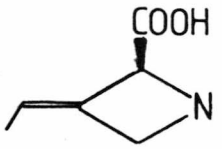
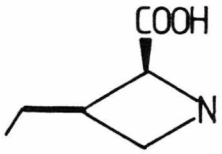
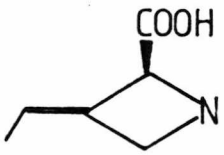
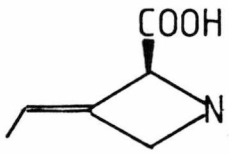
The polyoxins, analogues of UDP-N-acetyl-D-glucosamine are inhibitors of chitin synthase (chitin:UDPacetyl-glucosaminyl transferase E.C.2.4.1.16). (Endo *et al.*, 1970). Chitin has been found to be important in the maintenance of fungal cell wall rigidity. Polyoxins therefore have a profound effect upon fungal morphology.

Thirteen polyoxins have been isolated from culture filtrates of *Streptomyces cacaoi* and have been designated polyoxins A-M (Isono and Suzuki, 1979). The structures of the polyoxins are shown (Figure 1.3). All of the drugs, except polyoxin C, are peptide-nucleosides and have been shown to enter the cell via the peptide permease: the presence of peptides in the growth medium antagonized the action of polyoxins while free bases or amino acids showed no such effect (Mitani and Inoue, 1968; Hori *et al.*, 1977).

The effects of the polyoxins on the growth of many fungi have been described. Polyoxin D inhibited elongation of the fruit body stipes of *Coprinus cinereus* and resulted in total lysis of the tissue (Gooday *et al.*, 1976). In *Trichoderma viride* the mycelium was found to bulge following loss of rigidity. However, lysis did not occur until the addition of a potent lytic system (Benitez *et al.*, 1976). Weakened cell walls were also noted in *Mucor rouxii* (Bartnicki-Garcia and

Figure 1.3 The structures of the polyoxins (from Isono and Suzuki, 1979)



	R_1	R_2	R_3
A	CH_2OH		OH
B	CH_2OH	OH	OH
D	COOH	OH	OH
E	COOH	OH	H
F	COOH		OH
G	CH_2OH	OH	H
H	CH_3		OH
J	CH_3	OH	OH
K	H		OH
L	H	OH	OH

C

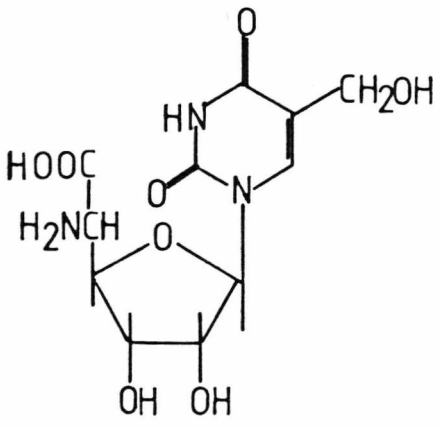
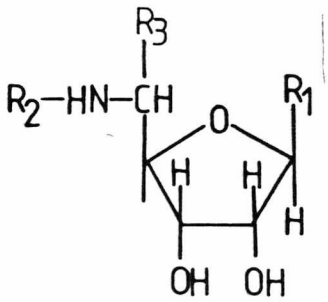
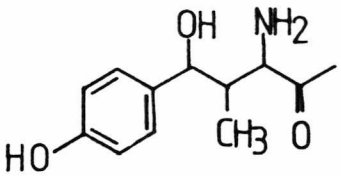
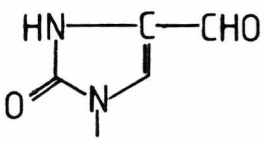
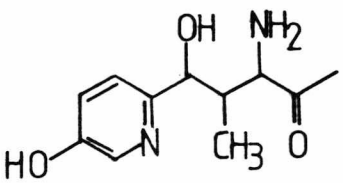
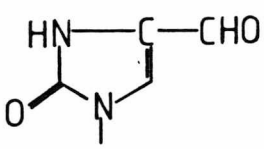
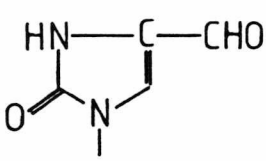
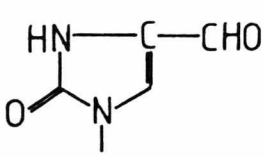
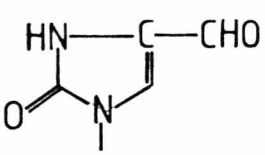


Figure 1.4 The structures of the nikkomycins (from Zähner *et al.*, 1982)



	R_1	R_2	R_3
B	Uracil		OH
B_x		〃	OH
Z	Uracil		OH
X		〃	OH
J	Uracil	〃	Glu
I		〃	Glu
M	Uracil	H	Glu
N		H	Glu
C	Uracil	H	OH
C_x		H	OH

Lippmann, 1972) and *Schizophyllum commune* (MacGruder, 1979) after treatment of the fungi with polyoxins.

Keller and Cabib (1971) found that polyoxin A was inactive against *Saccharomyces carlsbergensis* either as intact cells or spheroplasts. However, it was a potent inhibitor of the cell-free chitin synthase. Therefore chitin synthase is located intracellularly and the lack of inhibition of growth was due to a lack of penetration of the cell membrane. A similar effect was found in *C.albicans* (Chiew *et al.*, 1980) in which the cell-free chitin synthase was highly sensitive to polyoxin D (K_i=1.2 μM) while the drug had no effect upon the production of germ tubes by this organism. Polyoxin D was found to be active against *S.cerevisiae* (Bowers *et al.*, 1974) and produced abnormal morphological forms. The site of action of the drug was in the septal region with the production of the chitinous primary septum being prevented by the inhibition of chitin synthase.

Cases of resistance to polyoxins have been reported (Gooday, 1979). However, there is only one report of resistance being due to an altered chitin synthase (*Schizophyllum commune*, MacGruder, 1979). A mutant of *Alternaria kikuchiana* resistant to polyoxin A was found to have a deficient uptake system for both polyoxin A and peptides (Hori *et al.*, 1976) Polyoxins can be considered to be peptides with highly modified side-chain residues. Therefore they could be of use in the selection of fungal peptide transport deficient mutants.

The nikkomycins

The nikkomycins (Figure 1.4) are a group of peptide-nucleosides produced by *Streptomyces tendae* Tu 901 (Dahn *et al.*, 1976). Structurally they are closely related to the polyoxins and also inhibit the chitin synthase of many fungi and insects. Partial inhibition of protein synthesis by nikkomycins has also been noted (Dahn *et al.*, 1976).

The structures of all of the nikkomycins have been determined. The chiral centres were all found to be in the L-configuration (Konig 1980). Variations in the inhibitory action have been attributed to the alteration in the base moiety (Muller *et al.*, 1981) however no data are available concerning the effect of alteration of the peptide chain.

The mode of uptake of the nikkomycins has not been elucidated; however it is assumed to be similar to that of the polyoxins.

A third class of peptide-nucleoside drugs has been described: The neopolyoxins (Kobinata *et al.*, 1980; Uramoto *et al.*, 1980). The planar structures of neopolyoxins A and C are equivalent to nikkomycins X and Z respectively. However, there is still some doubt about whether they are identical compounds.

Drugs which enter the cell via the peptide permease are therefore efficient antifungal and antibacterial agents. There is considerable scope for the development of synthetic peptide-drugs active against fungal pathogens. Consideration will now be given to *Candida albicans* a possible target organism for such drugs and which was the object of the present study.

Candida albicans

The pathogenic fungus *Candida albicans* has been shown to replicate primarily by the production of buds from blastospores (yeast cells). The formation of pseudohyphae and true hyphae has also been demonstrated. There have been many reports concerning the regulation of morphology. It has been found that the blastospores or hyphal forms could be synchronously produced by the release of stationary phase cells (blocked early in the cell cycle) with fresh medium. Buds were formed at 25-30°C while filamentous growth followed at 35-40°C (Evans, *et al.*, 1975; Mitchell and and Soll, 1979). Other factors have been defined as inducing filamentous growth and a comprehensive list was given by Odds (1979). These factors include a pH ≥7.0 , low oxygen tension, a polysaccharide carbon source, the presence of N-acetylglucosamine and a low sulphydryl concentration in the growth medium.

Serum at 37°C has been found in induce filamentous growth. Chattaway *et al.*, (1980) isolated a peptide and a glycopeptide from seminal plasma which could induce the hyphal form of growth. However, when hydrolyzed to their constituent amino acids these were still able to induce the transition. Thus the meaning of these results remains unclear. Germination also appears to be accompanied by an increase in the level of intracellular adenosine 3':5'-cyclic monophosphate.

No precise mechanism for the yeast-mycelial transition has been demonstrated. Mitchell and Soll (1979) have shown that in the yeast phase the septum is deposited at the intersection between the mother cell and bud and formation began at a time point equivalent to the start of evagination. Mycelium formation commenced with an initial outgrowth from the mother cell (the germ tube), the septum was produced at a variable

point but seldom at the intersection between the two cells. In this case septum formation commenced 30 mins after initial evagination. No major differences in the cytoplasmic proteins have been identified (Brummel and Soll, 1982). However, it is possible that minor proteins (not visualized by the techniques used) may play a regulatory role in phenotypic expression.

The major differences between the blastospore and hyphal forms of growth is in the shape of the cells. Shape is determined by the cell wall and therefore this structure has attracted much attention. Transmission electron microscopy has shown it to be composed of as many as five distinguishable layers (Djaczenko and Cassone, 1971). Biochemical studies have shown the cell wall to contain $\alpha(1,6)$- and $\alpha(1,2)$-linked mannan, $\beta(1,6)$- and $\beta(1,3)$-linked glucan with lesser amounts of chitin, protein and lipid (Chattaway, *et al.*, 1968). Fluorescent labelling has shown the chitin to be restricted to the inner regions of the cell wall in addition to the primary septa and bud scars (Tronchin *et al.*, 1981). Three times as much chitin has been shown to be present in the mycelial form when compared with the blastospore from (Chattaway, *et al.*, 1968). Independent studies have both shown the specific activity of chitin synthase to be greater in hyphae than blastospores (Braun and Calderone, 1978; Chiew, *et al.*, 198.). There was no corresponding increase in the specific activity of the $\beta(1,3)$-glucan synthase (Orlean, 1982). No reports are available concerning the activity of mannan synthase in the two forms. The increased activity of chitin synthase may account for the observation that N-acetyl-glucosamine induced the blastospore to mycelial transition (Simonetti, *et al.*, 1974).

The ultrastructure of the intracellular organelles of both forms appears to be equivalent to that observed in other eukaryotes (Odds, 1979). Studies concerning the metabolic pathways of *C.albicans* are limtied however; they suggest that the pathways are similar to those found in other yeasts. Both ultrastructure and metabolism have been comprehensively reviewed (Odds, 1979).

As a member of the *fungi imperfecti* no sexual cycle has been demonstrated for *C.albicans*. There have been many attempts to determine the ploidy of the organism which, in various reports, has been described as haploid (Poulter, *et al.*,1981; Sarachek, *et al.*, 1981) or diploid (Olaiya and Sogin, 1979; Whelan, *et al.*, 1980; Whelan and Magee, 1981). Recently a detailed study has been carried out providing conclusive evidence that *C.albicans* is diploid (Riggsby, *et al.*, 1982). This will have considerable importance in interpreting the spontaneous drug resistance shown by this organism (Whelan, *et al.*, 1981).

The genus *Candida* has been found to contain a number of pathogens of medical importance including *C.albicans*, *C.guilliermondii*, *C.krusei*, *C.parapsilosis*, *C.stellatoidea* and *C.tropicalis*. Of these *C.albicans* is themost frequently encountered and the most pathogenic (Odds, 1981). For many years *C.albicans* has been known to be a normal commensal of the human gut. However, as an opportunistic pathogen it is able to colonize other tissues and hence produce the disease condition known as candidosis. As has been mentioned previously, *C.albicans* produces hyphae at 35-40^{o}C and pH 7 , conditions normally found within the

human body. The involvement of the dimorphic transition in pathogenesis is unclear since both blastospores and hyphae have been found at a site of infection (Odds, 1981). However, the ability of the organism to produce mycelia may increase its resistance to phagocytosis by the host's defence mechanism or aid in tissue invasion and hence increase its pathogenicity.

The ability of *C.albicans* to produce an extracellular protease (Staib, 1965) may be of significance in pathogenicity. Staib (1969) demonstrated that a non-protoeolytic strain could not produce an extensive infection in all organs in contrast to a proteolytic strain. Equally MacDonald and Odds (1983) showed a mutant lacking the protease to be of lower pathogenicity than the wild type. Both protease production and the yeast to mycelial transition are therefore of importance in the establishment and maintenance of an infection.

Certain conditions predispose humans to candidosis. These have been shown to include disorders such as diabetes mellitus, cancers and mechanical factors such as burns, the wearing of dentures and prolonged immersion of the hands in water. The use of broad-spectrum antibiotics has been shown to alter the host's microflora and enable opportunistic yeasts such as *C.albicans* to gain prevalence. However, it is uncertain whether the drugs bring about the invasion by *C.albicans* since the patient is often debilitated before administration and is therefore predisposed to candidosis (Odds, 1979).

Superficial candidoses are common, 4-5% of women suffer Candidal vaginitis (Odds, 1979) while as many as 60% of elderly denture wearers were found to suffer from denture stomatitis caused by *C.albicans* (Budtz-Jorgensen *et al.*, 1975). System candidoses are less common but more serious. They can involve either a single organ or multiple organs in which the organism has spread through the bloodstream (*Candida* septicaemia). Amongst the organs which have been identified as sites of systemic candidosis are the heart, central nervous system, the eye and the bone-joints. An example of this form of invasion is *Candida* endocarditis which has gained prevalence since the introduction of open heart surgery (Odds, 1979). Mortality from this disease is high with antifungal treatment rarely succeeding. Recovery has been achieved by surgical removal of the site of infection in conjunction with the use of antifungal agents.

There are a limited number of antifungal agents available for the treatment of candidoses. Prevalent amongst these are the polyene macrolides, the imidazoles and 5-fluorocytosine.

Polyenes

The polyenes, secondary metabolites of the genus *Streptomyces*, are all macrolides with β-hydroxylated portions and a conjugated double-bond system within a lactone ring. The ring sizes vary between 12 and 37 carbon atoms. The most important members of this group, in relation to the treatment of candidoses, are nystatin and amphotericin B. The mode of action of each can be considered to be the same in that they bind irreversibly to the sterol components of the cell membrane. The specificity

of binding to fungal or mammalian cells resides in the composition of the cell membrane. Fungal cells contain ergosterol whereas mammalian cells contain cholesterol. The greater the affinity of the polyene for ergosterol the greater will be the antifungal activity with reduced toxicity for the host tissues (Archer and Gale, 1975). Binding of polyenes to ergosterol has been shown to result in the destruction of the membrane's integrity and hence permeability changes. This produced leakage of K^+ ions (Gale, 1974) and other important constituents (Lampen, 1966). Membrane alterations result in metabolic deterioration and cell death.

Imidazoles

This large group of synthetic imidazole-containing drugs includes two antifungal agents useful in the treatment of candidoses: Miconazole and ketoconazole. Swamy, *et al.*, (1974) found miconazole to cause leakage of 260 nm absorbing material, amino acids, proteins and inorganic cations from *C. albicans* and concluded the presence of an altered membrane permeability. Van den Bossche, *et al.*, (1978,1979) revealed that both miconazole and ketoconazole inhibited the incorporation of $[^{14}C]$-acetate into ergosterol. However, the precise antibiotic activity remains unclear: Certain gram positive bacteria such as *Staphylococcus aureus* are sensitive to miconazole even though their membranes contain no sterol (Holt, 1972). A recent report has suggested that the principle site of action of ketoconazole is the respiratory chain of *C. albicans* (Uno, *et al.*, 1982). Further work showed that this occurs through inhibition of cytochrome C oxidase (Shigematsu, *et al.*, 1982).

Ketoconazole was found to be highly active against *C.albicans* (inhibition of growth at 0.01 μg ml^{-1}) and shows only low toxicity to human fibroblasts (toxic at concentrations ≥100 μg ml^{-1}. Aerts *et al.*, 1980). Miconazole is however more toxic to fibroblasts (and other cell types) and less active against *C.albicans*, the use of this drug has therefore been largely superceded by ketoconazole.

5-Fluorocytosine (5-FC)

Polak and Scholar (1975) have shown that 5-FC inhibits both DNA and RNA synthesis whereas production of proteins (with some abnormal composition) was less sensitive. A marked increase in carbohydrate synthesis was also observed. 5-FC is transported into cells and deaminated to 5-fluorouridylic acid by cytosine deaminase. The incorporation into RNA results in its fungistatic effect. The absence, or low activity, of cytosine deaminase in mammalian cells results in the low toxicity of 5-FC and allows its use as an antifungal agent (Heeres and Van den Bossche, 1980). The inhibition of thymidylate synthetase has been reported (Diasio, *et al.*, 1978). This occurs following conversion of 5-Fc to 5-fluoro-2-deoxy-uridylic acid and is therefore a second site of action of 5-FC. The use of 5-FC is limited by both the number of resistant isolates and the frequent spontaneous mutations, which give resistant variants of *C.albicans* (Whelan, *et al.*, 1981).

Synergism

It has been demonstrated that many yeasts, including *C.albicans*, are more susceptible to a combination of amphotericin B (AmB) and 5-FC than to either drug alone. This synergistic action does not appear to involve the enhanced uptake of 5-FC in the presence of AmB since AmB inhibited the transport of 5-FC into cells (Beggs, *et al.*, 1981). This was in contradiction of previous reports (Medoff, *et al.*, 1971 and 1972). Beggs, *et al.*, (1981) suggested that the synergistic action came from the exclusion of 5-FC from the cells until the AmB level fell (due to its instability) at which point 5-FC was able to exert its own antifungal effect. The use of AmB and 5-FC in combination allows the use of lower AmB concentrations thus reducing toxicity without reducing the antifungal activity. In addition the combination reduced the number of spontaneously resistant mutants which arise from the use of 5-FC alone. This combination has become of some clinical importance in the treatment of *C.albicans* and other systemic infections.

Candida albicans is therefore a pathogen of considerable medical importance. Incidences of serious systemic candidosis appear to be increasing and there is the need for the development of new drugs. Peptide drugs such as alafosfalin have proved to be successful as antibacterial agents and demonstrate the possibility of drug development by the principle of illicit transport. Many fungal species, including *C.albicans*, have been shown to possess peptide transport systems and it may therefore be possible to develop peptide drugs with good selectivity.

The previous studies of the peptide transport system in *C.albicans* have relied upon the use of radiolabelled substrates. The recent development of alternative assay methods has allowed more detailed studies to be carried out. The object of this study was therefore to define the peptide transport system of *C.albicans* and to investigate any potential for the development of peptide-drugs.

CHAPTER 2: MATERIALS AND METHODS

MATERIALS

L-[U^{14}C]-alanine
UDP-N-acetyl-[^{14}C]-D-glucosamine
} The Radiochemical Centre, Amersham.

Primulin: Hopkins and Williams, Technical Dye.

Nikkomycin: (80% nikkomycin containing nikkomycins X and Z as major components). Gift from Bayer A. G.

Polyoxin D: Gift from Dr. P. Jeffries, University of Kent.

Polyoxin (crude): (10% polyoxins, A,B,K and L)
Gift from the Sandoz Forschungsinstitut, Vienna.

Fluorescamine and alafosfalin: Gifts from Dr. P. S.Ringrose, Pfizer (U.K.) Ltd.

Bacilysin: Gift from Professor E. P. Abraham, Oxford.

Blasticidin S: Gift from the Kaken Chemical Co., Japan.

L-ala-D-ala
D-ala-L-ala
D-ala-D-ala
N-acetyl ala ala
ala ala-ONH_2

} Cambridge Research Biochemicals

Reagents for electron microscopy: TAAB Laboratories

Media: Oxoid except where otherwise stated.

All other chemicals were from Sigma.

All amino acid residues are in the L-configuration unless otherwise stated.

METHODS

Organisms and Culture

Candida albicans strains 113 , 124 , 125 , 495 , and 539 were obtained from the Sandoz Forschungstinstitut, Vienna. Strain STM-1 was from Dr. F. C. Odds, Leicester. *C.albicans* 124 was used routinely. *Saccharomyces cerevisiae* S7 (*a* mating type) was obtained from Dr. S. Oliver, University of Manchester Institute of Science and Technology.

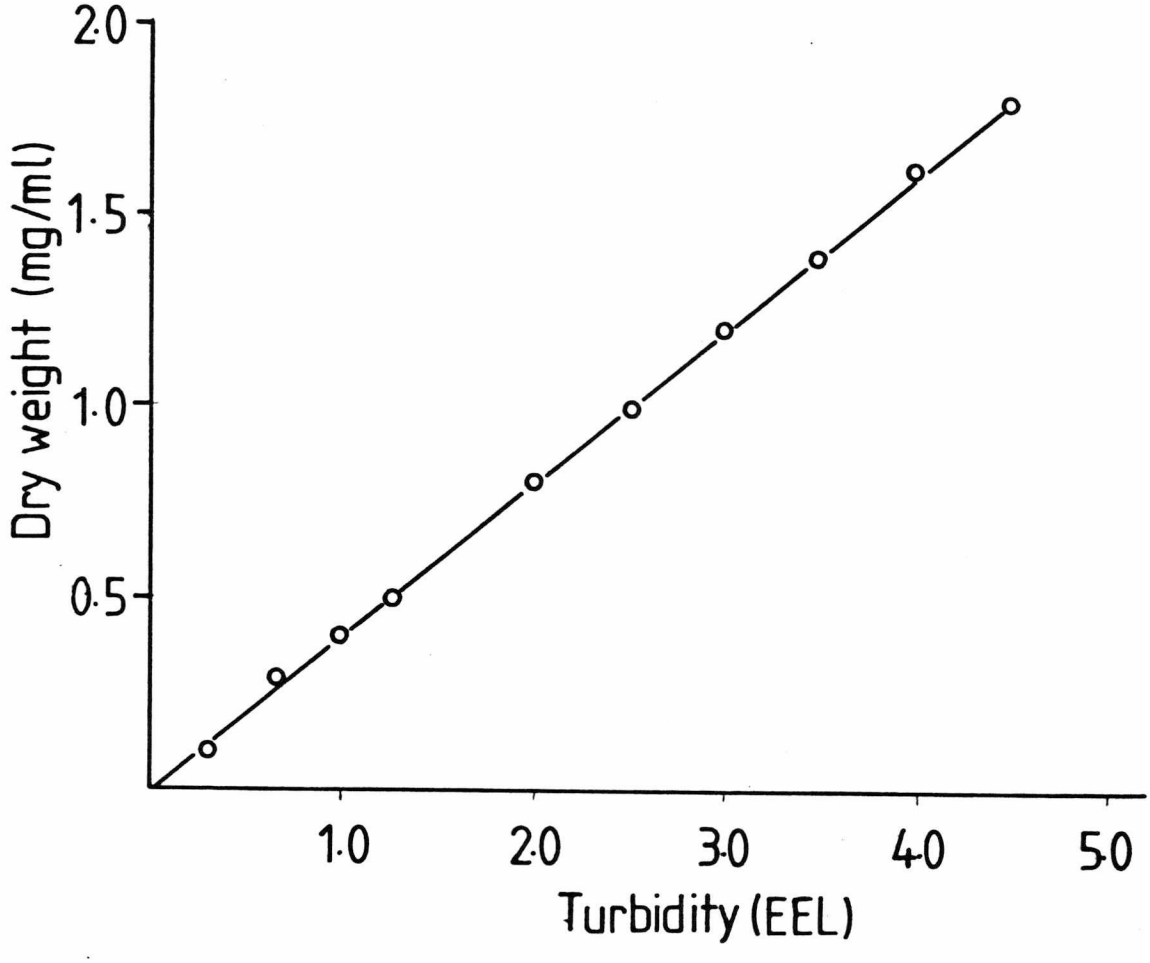
C.albicans was maintained on 2% (w/v) malt extract plates at 37°C , the organism being subcultured every two weeks onto fresh medium. Culture purity was checked microscopically. *S.cerevisiae* was maintained on MYPG (0.3%(w/v) malt extract; 0.3%(w/v) yeast extract; 0.5% (w/v) mycological peptone; 1% (w/v) glucose) plates at 30°C and subcultured every two weeks.

The growth of cultures in liquid media was followed turbidometrically using an EEL colorimeter with green filter. Calibration of turbidity to dry weight was achieved by filtering a culture of known turbidity through tared sintered glass discs and drying to constant weight at 120°C . The calibration curve is shown (Figure2.1).

Batch growth of cells was performed routinely on three media:

Figure 2.1 Calibration curve of turbidity (EEL) *versus* dry weight of cells

Cells were grown on YNBP at 30°C. Samples (10 ml) were filtered through tared sintered glass filters and dried to constant weight at 120°C. Turbidity was measured using an EEL colorimeter with green filter.


2·0
1·5
1·0
0·5
Dry weight (mg/ml)
1·0
2·0
3·0
4·0
5·0
Turbidity (EEL)

1. YNB: Yeast Nitrogen Base with ammonium sulphate (0.5% w/v) and amino acids (Difco) supplemented with 1% (w/v) D-glucose. Sterilized by filtration.
2. YNBP: Yeast Nitrogen Base without ammonium salts or amino acids (Difco) supplemented with 2% (w/v) Bacto-peptone and 2% (w/v) D-glucose. Yeast Nitrogen Base was filter sterilized, Bacto-peptone and D-glucose were autoclaved (116^{o}C for 20 mins).
3. MX: Malt extract (2% (w/v)) sterilized by autoclaving at 116^{o}C for 20 mins.

Growth was followed at 30^{o}C and 120 r.p.m. on a Gallenkamp orbital incubator. In each case the inoculum was 10^{5} cells/ml. 25 ml cultures in 250 ml flasks were used routinely.

S.cerevisiae was grown at 30^{o}C and 120 rpm on a defined minimal medium of the following composition (per litre), it was sterilized by filtration.

D-glucose	2 g
$(NH_4)_2SO_4$	6 g
Na_2HPO_4	5.75 g
KH_2PO_4	4.65 g
EDTA	0.60 g
$CaCl_2$	50 mg
$MgSO_4.7H_2O$	0.25 mg

$ZnSO_4.7H_2O$	0.20 mg
$MnSO_4.4H_2O$	20 mg
$CuSO_4.5H_2O$	5 mg
$FeSO_4.5H_2O$	0.1 mg
$NaMoO_4.2H_2O$	5 mg
myo-Inositol	1.25 mg
Thiamine	0.25 mg
Calcium panthothenate	0.25 mg
p-aminobenzoic acid	0.125 mg
pyridoxin	0.25 mg
D-biotin	0.025 mg

Induction of the mycelial phase

The mycelial phase of *C.albicans* was induced using two methods.

1. Cells were grown to late-log phase on YNB at 30°C and then inoculated at 10^6/ml into 20% (v/v) newborn calf serum (Gibco) in distilled water prewarmed to 37°C. After incubation at 37°C and 60 rpm germination occured in >95% of cells within 2 hours.
2. The method of Schwartz and Larsh (1980) provides a means of induction on a defined medium. Cells were washed from MX plates with phosphate-buffered saline (PBS, 10^{-3}M K_2HPO_4-NaH_2PO_4/0.85% (w/v) NaCl pH 8.5) , washed twice with PBS by centrifugation at 1500x g and then resuspended in YNB . The suspension was stored at 25°C for 6 hours and then held overnight at 4°C. This resulted

in a culture containing >95% singlet cells. Flasks of AP+ medium (see below) were inoculated with 10^6 cells/ml and incubated at $40^{o}C$ and 100 rpm. Germination of >90% of cells was observed within 2 hours.

AP+ medium

Autopow MEM (Flow Laboratories Inc. cat.no.11-110-24) , was modified as follows (per litre):

Autopow MEM	10 g
1M K_2HPO_4-Na_2HPO_4 pH 6.8	10 ml
100 × Trace element stock (see below)	10 ml
D-glucose	9 g
glycine	1 g
D-biotin	250 g

The final pH of the solution was adjusted to 6.0 with NaOH. Sterilization was by filtration.

Trace elements for AP+ (per 100 ml , 100× stock solution)

Boric acid	5 mg
$CuSO_4.5H_2O$	0.4 mg
$FeCl_3.6H_2O$	2 mg
$MnSO_4.4H_2O$	4 mg

$ZnSO_4.7H_2O$	4 mg
$NaMoO_4.2H_2O$	2 mg
$CoCl_2.6H_2O$	4 mg

Light microscopy

Cells were observed using a Zeiss Universal Research microscope under phase contrast illumination. 40x and 100x phase objectives were used routinely.

Fluorescence microscopy

Cells were harvested by centrigugation and washed twice with 0.1 M NaH_2PO_4-Na_2HPO_4 pH 7.5 containing 0.8% (w/v) mannitol. They were then resuspended in this buffer supplemented with 0.01% (w/v) primulin for 1 min, harvested and washed twice with the buffer before observation. A Zeiss Universal microscope was used equipped with epifluorescent optics (filter set 487706) and 63x and 100x objectives.

Photography

In both cases an Olympus OM-2 camera was used with Ilford film: HP5 (400ASA) for fluorescence or PAN F (50 ASA) for phase contrast. Iford microphen was used to develop the film in both cases.

Electron Microscopy

Cells were fixed for 10 mins with 2.5% (w/v) glutaraldehyde in 0.085 M Cacodylate buffer pH 7.4, pelleted by centrifugation and re-suspended in the same solution overnight. The pellet was postfixed with 1% (w/v) OsO_4 in veronal/acetate buffer pH 6.0 for 90 mins. The cells were dehydrated through an ethanol series and embedded in Spurr's resin (Spurr, 1969). Sections were cut using a Reichert OMU-3 ultramicrotome. These were stained with 5% (w/v) uranyl acetate in 1% (v/v) acetic acid and lead citrate (Reynolds, 1963) and observed using an AE1 801A electron microscope with an accelerating voltage of 60 kV.

Production of mutants

Blastospores were grown to mid-log phase in YNB. These were harvested by centrifugation (1500xg), washed and resuspended in 65 mM KH_2PO_4 (0.5 ml packed cell volume in 10 ml KH_2PO_4). 0.7 ml of this suspension was distributed on a 20 ml YNB plate and incubated at 37°C for 24 hours. When washed from plates these cells were observed to be >95% singlet blastospores. These were diluted to 10^6/ml in YNB and 15 ml of this suspension was irradiated for 50 secs with an Hanovia bactericidal ultraviolet lamp from a distance of 30 cm. This gave a 0.5% survival rate (Figure 2.2). The irradiated culture was then stored at 37°C for 3 hours. Drug resistant mutants were selected by plating irradiated cells onto 2% (w/v) MX plates containing 50 $\mu g\ ml^{-1}$ nikkomycin. After incubation at 37°C any viable colonies were picked out and plated onto further drug containing MX.

Figure 2.2 The effect of ultraviolet light on a suspension of blastospores of *C.albicans* 124.

A suspension of 10^6 cells/ml was irradiated with an Hanovia bactericidal ultraviolet lamp from a distance of 30 cm. Viable cells were determined by growth on MX plates.

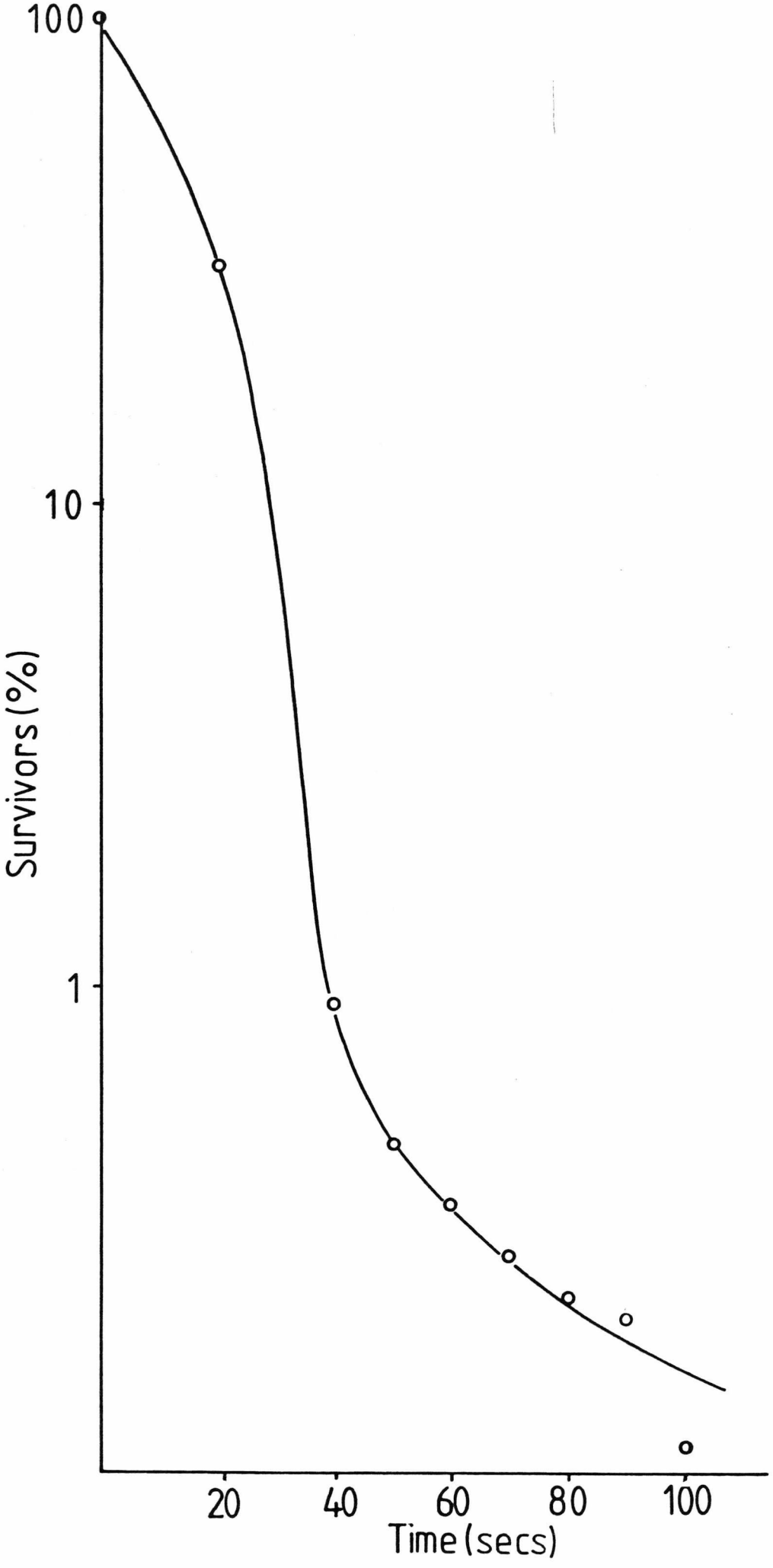

100
10
1
Survivors (%)
20
40
60
80
100
Time (secs)

Figure 2.3 Emission spectra of the reaction products of (a) alanine and (b) ala ala with fluorescamine at pH 6.2. The peptide product fluoresced maximally at 480 nm whereas there was low fluorescence from the amino acid product (Excitation 390 nm)

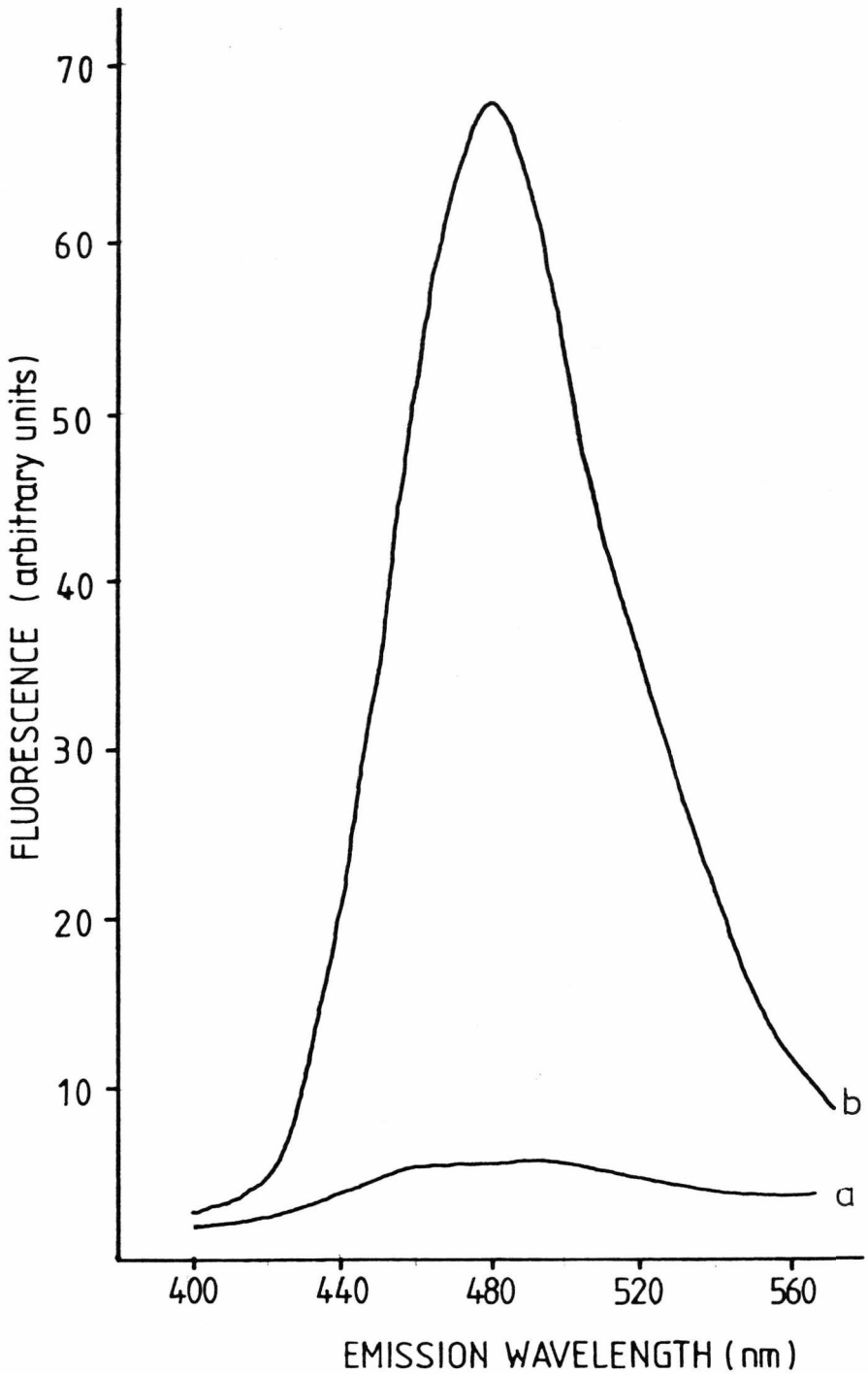
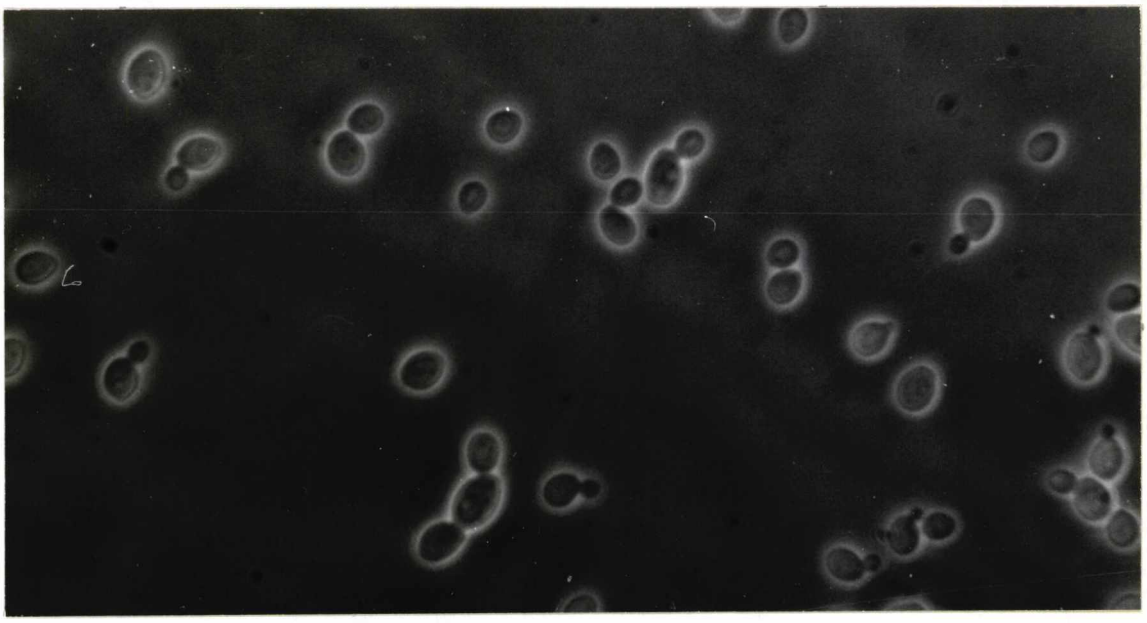
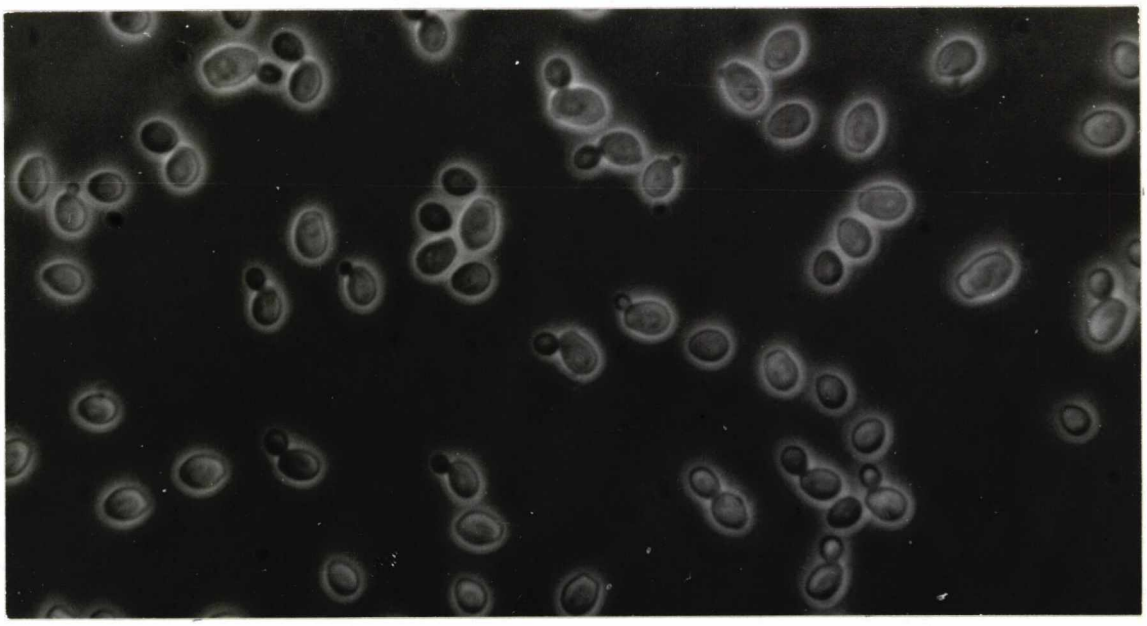

70
60
50
40
30
20
10
FLUORESCENCE (arbitrary units)
b
a
400
440
480
520
560
EMISSION WAVELENGTH (nm)

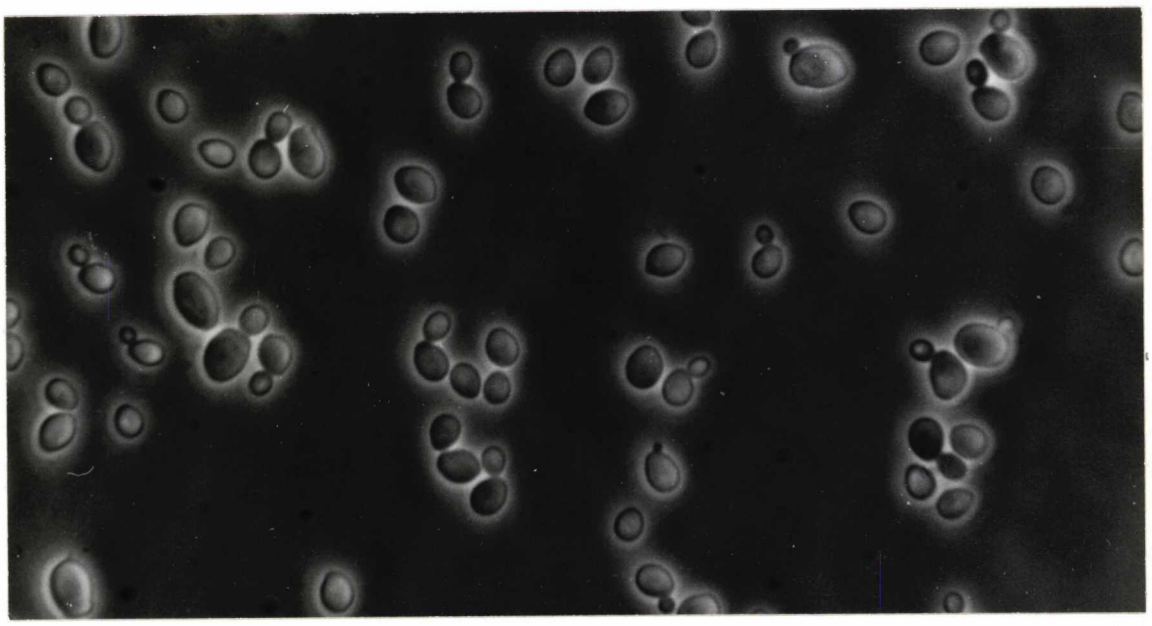
Figure 2.4 Phase micrograph of mid-log phase blastospores of *C.albicans* 124 grown on YNBP at 30°C and 120 rpm (×1100)

Figure 2.5 Phase micrograph of cells as in Figure 2.4 harvested, washed three times and resuspended in PCG

Figure 2.6 Cells as in Figure 2.5 after 2 hours storage at 4°C







Peptide transport

(a) Fluorescamine method

Fluorescamine (4 phenylspiro furan-2(3H),1'-phthalan 3,3' dione) reacts extremely rapidly with both amino acids and peptides possessing a free amine terminus. The fluorescent yield of the product varies with pH such that at pH 6.2 the fluorescence is due to peptides with only a very low yield from amino acids (Figure 2.3), (Weigele, *et al.*, 1972; Udenfriend, *et al.*, 1972; Perrett, *et al.*, 1975). This reaction can therefore be used to give a direct measurement of the reduction in the peptide concentration in the extracellular medium (Nisbet and Payne, 1979a) and has recently been modified to provide a continuous assay technique for peptide transport (Payne and Nisbet, 1981).

Unless otherwise stated cells were grown to mid-log phase on YNBP. They were then harvested by centrifguation at 1500xg and the pellet was washed three times with PCG (10 mM Na_2HPO_4-citric acid pH 5.0 supplemented with 0.8% (w/v) D-glucose). The pellet was finally resuspended in a small volume of PCG and stored for a maximum of 2 hours at $4^{o}C$. No changes in the morphology of the cells were apparent upon harvesting or storage (Figures 2.4-2.6).

Peptide stocks were prepared in PCG and preincubated at $37^{o}C$. The inoculum was diluted and also incubated at $37^{o}C$ for 10 mins. If metabolic inhibitors were used these were added followed by a further

10 mins incubation. Finally peptide(s) were added together with any competitors, giving a working volume of 10 ml and a cell density equivalent to 1.0 mg dry wt./ml. Incubation was at 37°C with shaking (80 strokes/min). Samples (0.5 ml) were removed at timed intervals and filtered through Whatman GF/A filters using a Hoeffer 10-place filtration unit. The filtrate was collected and assayed for peptide concentration.

The peptide solution (50 µl) containing up to 250 nmoles peptide was diluted with 2.5 ml sodium tetraborate (adjusted to pH 6.2 with HCl). Fluorescamine solution (0.5 ml, 0.15 mg ml^{-1} in acetone) was added and the mixture was vortexed rapidly. The fluorescence of the solution was measured within 30 mins using a Perkin-Elmer MPF-3 spectrofluorimeter (Excitation 390 nm, Emission 480 nm). Calibration curves were determined for each peptide used.

(b) Transport of [$U^{14}C$] ala ala

The method used was essentially the same as that previously described. Samples of the cell suspension (1 ml) containing the radiolabelled peptide were filtered through Whatman GF/A filters which were then washed with 10 ml PCG at 25°C. The filters were dried under infra-red illumination and placed in small scintillation vials with 3 ml PCS scintillation cocktail. Radiolabel was assayed using a Packard Tricarb 3375 scintillation counter. Corrections for quenching were by an internal standardization method.

For both methods rates of uptake were determined in triplicate in, at least, two separate experiments.

Release of $^{14}CO_2$ from [U ^{14}C] ala ala

A cell suspension was prepared as for the peptide uptake experiments. The concentration of [U ^{14}C] ala ala was increased to 2 mM and the incubation was carried out in 50 ml centre-well flasks. 1 ml ethanolamine (2:1 (v/v) dilution in ethanol (95%)) was added to the centre well. The peptide solution was preincubated in the flask for 10 mins at 37^{o}C . The assay was initiated by the addition of a pre-warmed cell suspension to give a density of 1.0 mg dry wt./ml. The vessel was sealed, shaken at 80 strokes/min and incubated at 37^{o}C . The experiment was terminated by the removal of the seal followed by immediate sampling of the ethanolamine solution. Three flasks were used for each time-point required. The entrapped ^{14}C was assayed by the method previously described and correlated with ^{14}C present in [U ^{14}C] ala ala . Chemiluminescence was not observed.

Thin layer chromatography (TLC)

A 10 µl sample of a peptide solution was spotted onto a cellulose chromatography plate (Eastman 13254) and run with butan-1-ol:acetic acid: distilled water (60:15:25(v/v)) as solvent. Plates were developed with ninhydrin spray (Merck) and photographed within 1 hour.

Synthesis of [U^{14}C] ala ala

The synthesis of alanine benzyl ester was achieved by the method of Zervas *et al.* (1957). Remaining syntheses were essentially the same as those described by Fruton (1949).

(a) Synthesis of alanine benzyl ester (ala-OBz)

Alanine (0.11 moles), p-toluenesulphonic acid (0.14 moles) and excess benzyl alcohol were refluxed for 5 hours with benzene as solvent. Water was collected azeotropically. The solution was allowed to cool to 0°C . Under these conditions ala-OBz p-toluenesulphonate precipitated and was subsequently washed with diethyl ether and dried *in vacuo*.

(b) Synthesis of carbobenzoxyalanine (cbo-ala)

L-alanine (6 mmoles) was added to a solution of [U^{14}C]-L-alanine (250 μ Ci: 171 mCi/mmol). 2 M NaOH (2 ml) was added and the solution was cooled to 0°C . Subsequently a 2-fold excess of benzoyl chloride was added as a 50% (w/v) solution in toluene. A pH of 10 was maintained by the addition of 2 N NaOH until the reaction was found to be complete by TLC . The solution was extracted three times with diethyl ether. Ether in the aqueous layer was removed under reduced pressure and the pH of the remaining solution was reduced to pH 2.0 by the addition of 2 N HCl. The cbo-L-alanine was washed with water by filtration and dried *in vacuo*.

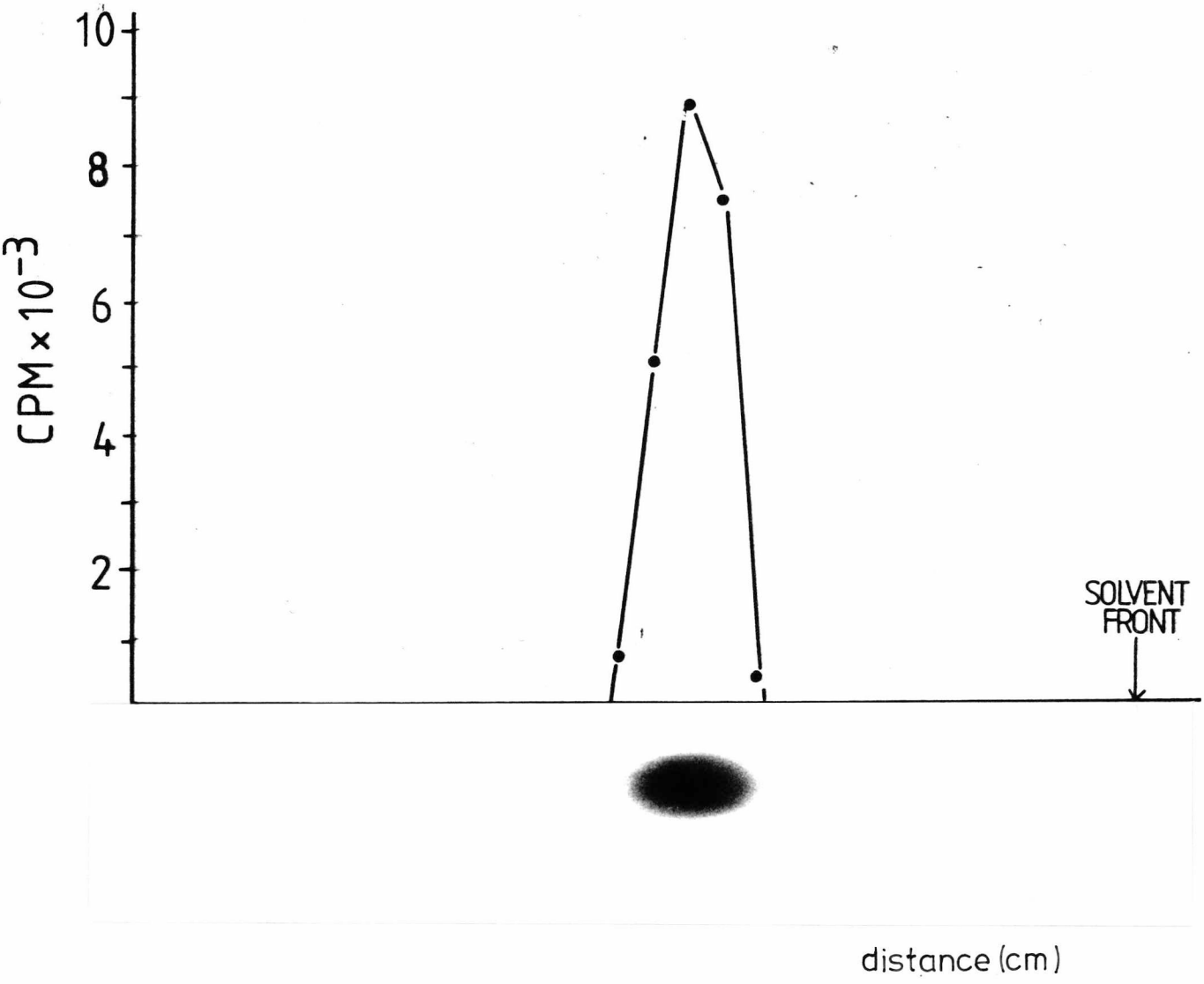
(c) Coupling of cbo-L-alanine and alanine-OBz

The p-toluenesulphonic acid was liberated from the benzyl ester by the addition of 1 equivalent of N-methylmorpholine in tetrahydrofuran. One equivalent of cbo-alanine was added to the solution which was then cooled to $0^{o}C$. One equivalent of DCCI (N,N'-dicyclohexamyl carbodiimide) was added and the reaction was allowed to proceed overnight in a stoppered vessel. The crystalline dicyclohexyl urea was removed by filtration, the solvent was removed from the filtrate by evaporation and the residue was dissolved in ethyl ethanoate. This solution was then washed three times with 1 N HCl , three times with 4: (w/v) $NaHCO_3$, and twice with saturated NaCl . The organic layer was dried over $MgSO_4$. After filtration the solvent was removed under reduced pressure leaving cbo-ala-ala-OBz . TLC revealed the presence of impurities which were removed by chromatography using a silica column (Merck) and ethyl ethanoate:chloroform (3:1 (v/v)) as solvent. The blocked peptide was produced as a single recoverable peak.

The blocks were removed simultaneously by hydrogenation using H_2 (gas) and a PdO_2 catalyst with methanol as solvent. $[U^{14}C]$ ala ala was produced as a precipitate. TLC revealed that this peptide co-ran with an ala ala standard (Sigma) and that radioactivity was located in the single ninhydrin positive spot (Figure 2.7). The specific activity of the peptide was 36.8 μ Ci /m mol .

Figure 2.7 Purity of [U^{14}C]ala ala

[U^{14}C]ala ala was subjected to thin layer chromatography. A 1 cm strip along the path of the peptide was marked out. The cellulose was scraped from the surface along the pathway at 0.5 cm intervals and placed in small scintillation vials. PCS scintillant (3 ml) was added and the quantity of ^{14}C determined using a Packard Tricarb scintillation counter.


10
8
6
4
2
CPM x 10⁻³
SOLVENT FRONT
distance (cm)

Assay of peptidases

(a) Preparation of cell extracts

A mid-log culture was harvested by centriguation at 1500xg and washed twice in 20 mM Tris-HCl pH 7.5. The cells were resuspended in the buffer (0.5 g wet wt. per ml), mixed with 3 ml of 0.45 mm glass beads per ml of suspension and broken in a Braun homogenizer for a total of 90 secs with liquid CO_2 cooling. Breakage was checked microscopically. Beads were removed by centrifugation at 1500xg while the supernatant was cleared of cell debris by centriguation at 125,000xg . Protein concentrations were determined by the method of Lowry, *et al.* (1951) using bovine serum albumin as standard.

(b) Electrophoresis

The cell extract was diluted (3:1) with sample buffer (20 mM Tris-HCl pH 7.5, 10% (v/v) glycerol with bromophenol blue as tracker dye), and submitted to electrophoresis under non-denaturing conditions. A 6% polyacrylamide slab gel at pH 8.9 with a 3% stacking gel at pH 6.7 was used as described by Davies (1964). The electrode buffer was 5 mM Tris - 38 mM glycine pH 8.3. The slab gels had the following dimensions: 7 cm x 7 cm x 1 mm and were run without combs, the sample being applied as a continuous line across the stacking gel at a loading of 800 µg/gel. They were run at a current of 5 mA until the dye front was 0.5 cm above the end of the gel.

6% gel :	24% (w/v) acrylamide, 0.6% (w/v) bis	4.9 ml
	0.2% (w/v) ammonium persulphate	5.0 ml
	0.37 M Tris-HCl pH 8.9	2.5 ml
	Distilled water	7.31 ml
	TEMED	25 µl
3% gel:	28% (w/v) acrylamide, 0.735% (w/v) bis	2.55 ml
	0.2% (w/v) ammonium persulphate	5.0 ml
	62 mM Tris-HCl pH 6.7	3.0 ml
	Distilled water	13.45 ml
	TEMED	25 µl

(c) Blotting

A sheet of nitrocellulose paper (0.22 µm pore size, millipore) was washed for one hour in distilled water and placed onto a "Scotch Brite" scouring pad supported by a perforated perspex plate. The slab gel to be blotted was washed briefly in distilled water and placed onto the nitrocellulose sheet, a second scouring pad and perspex plate was then added. The assembly was placed in the apparatus shown in Figures 2.8 and 2.9 with electrodes on opposing faces such that the nitrocellulose faced the anode (Towbin, *et al.*, 1979). The tank was filled with 25 mM Tris-192 mM glycine buffer pH 8.3 and a voltage gradient of 6 V/cm was applied for 10 mins. The assembly was then immediately separated and the nitrocellulose sheet was cut into strips of 3 mm width for either protein or peptidase staining.

Figure 2.8 The apparatus used for the electrophoretic blotting of proteins from polyacrylamide gels to nitrocellulose

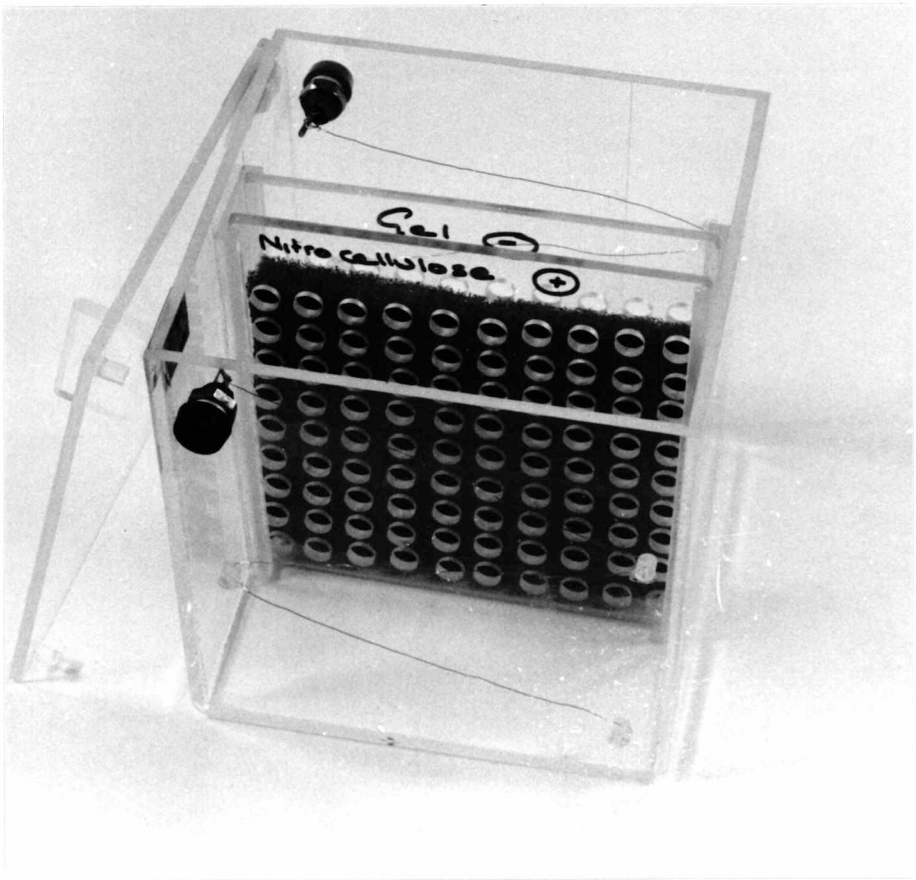
Figure 2.9 Diagram of the apparatus shown in Figure 2.8. A perspex box with electrodes on opposing faces held a sandwich consisting of:

(a) perforated perspex sheet

(b) 'Scotch;Brite" scouring pad

(c) Polyacrylamide gel

(d) Nitrocellulose sheet


Gel
Nitro cellulose

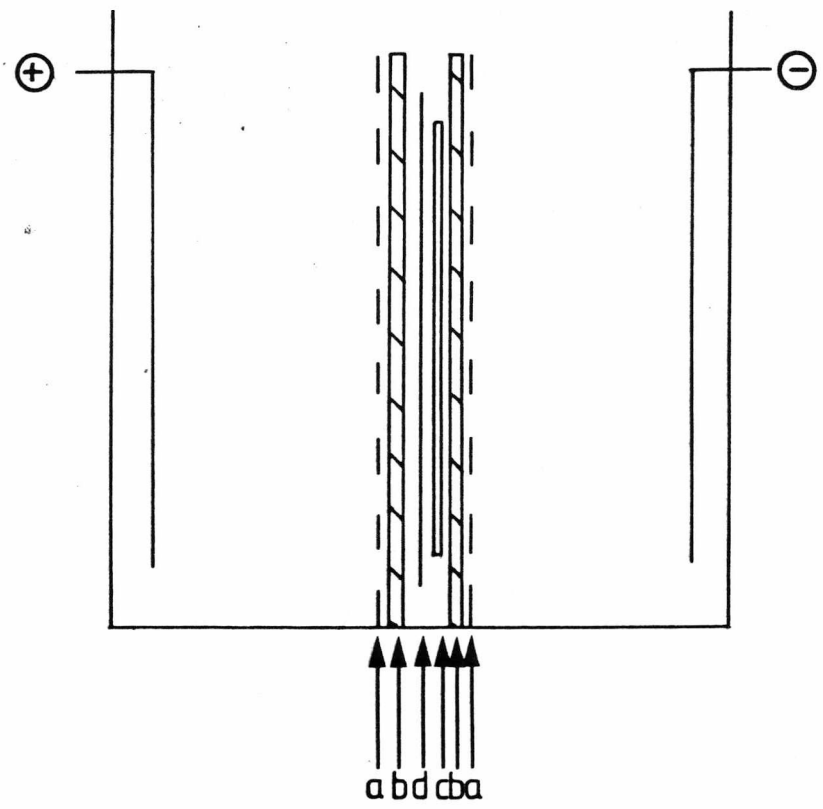
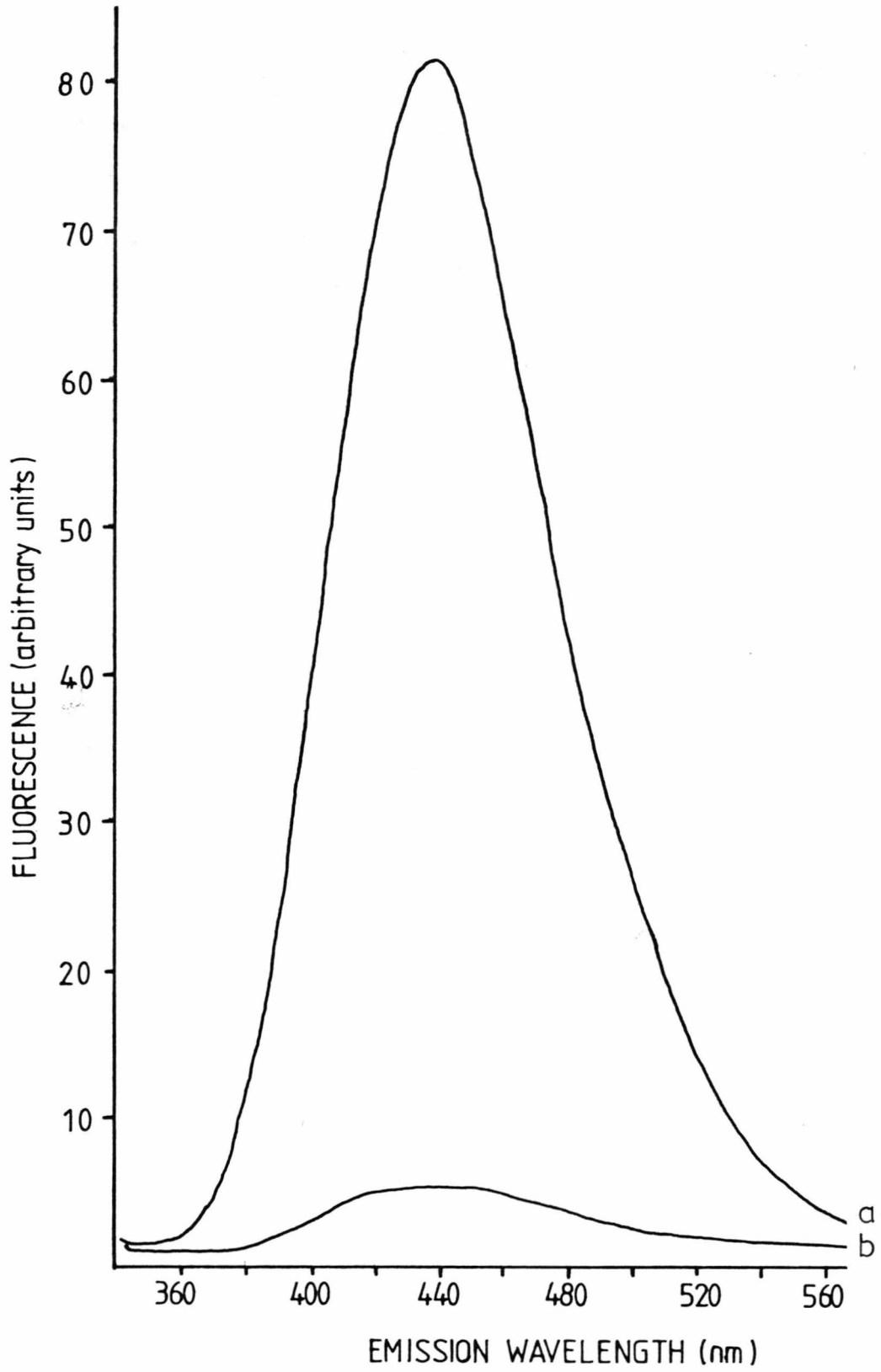

a b d c b a

Figure 2.10 Emission spectra of the reaction products of (a) alanine and (b) ala ala with o-phthaldialdehyde at pH 9.0. The amino acid product fluoresced maximally at 455 nm (Excitation 345 nm) whereas the peptide product showed only low fluorescence.


80
70
60
50
40
30
20
10
FLUORESCENCE (arbitrary units)
a
b
360
400
440
480
520
560
EMISSION WAVELENGTH (nm)

(d) Protein staining

Nitrocellulose blots were stained with amido black (0.1% (w/v) in 45% (v/v) methanol and 10% (v/v) acetic acid) and destained with a solution containing 90% (v/v) methanol and 2% (v/v) acetic acid.

(e) Peptidase staining

The method of Tan and Marzluf (1979) was modified and applied to nitrocellulose sheets. The incubation medium contained (per ml 0.1 M Na_2HPO_4-HCl pH 7.5): 100 µg L-amino acid oxidase; 170 µg nitroblue tetrazolium chloride; 17 µg phenazine methosulphate and 400 µg peptide. Each strip of nitrocellulose was incubated in 5 ml of this solution at 37°C in the dark until peptidase bands were observed. This generally occured within 3 hours.

Peptidase activity measurements

The amino terminals of both amino acids and peptides react with o-phthaldialdehyde. At pH ≥9.0 the fluorescent yield of the product with amino acids is far greater than that of peptides (Figure 2.10). The method used is that described by Porter *et al.* (1982) .

A 10 µl sample of a cell extract was added to a peptide solution (in 0.1 M Na_2HPO_4-NaH_2PO_4 pH 7.5 containing 0.5 M sucrose) at 37°C . Samples (50 µl) were removed at 2 minute intervals and

immediately added to 3 ml of the buffered OPA reagent with rapid vortexing.

OPA reagent: 50 ml 0.1 M sodium tetraborate
80 mg o-phthaldialdehyde in 2 ml ethanol (95%)
200 µl 2-mercaptoethanol
5 ml 20% (w/v) sodium dodecyl sulphate
} To a total volume of 100 ml with water

The fluorescence of the solution was measured after 2 mins (Excitation 345 nm; Emission 455 nm). Standard curves were determined for the constituent amino acids of each peptide.

Drug activity assays

(a) Plate method

An overnight culture grown on YNB was diluted to 10^6 cells/ml. A 500 µl portion of this suspension was spread over the surface of a 20 ml MX plate (2% (w/v) agar) and incubated at 37°C for 30 mins. After this period a 6 mm diameter disc of chromatography paper (Whatman 17 chroma) containing the drug, dried into the filter from solution, was placed in the centre of the plate. Plates were incubated at 37°C for 48 hours and the diameter of the zone of growth inhibition was determined. Solvent controls were carried out in each case.

(b) minimum inhibitory concentration (MIC)

An overnight culture was used to inoculate serial dilutions of a drug in either YNB or MX at 10^3 cells/ml. Tubes were incubated at $37^{o}C$ for 48 hours at which point both total cell numbers and viable cell numbers were determined.

Chitin Synthase Assay

The method used was based on that of Ryder and Peberdy (1977). An overnight culture grown on Brain Heart Infusion (Difco) at $37^{o}C$ was harvested at 1500xg and washed twice with 0.1 M sodium phosphate buffer pH 6.5 containing 10 mM $MgCl_2$ and 1 mM EDTA (PME buffer). Cells were resuspended in PME (1 ml packed cell volume/0.5 ml buffer) and were broken with an equal volume of 0.45 mm glass beads in a Braun homogenizer with liquid CO_2 cooling until >95% cell breakage was observed (about 90 secs). The beads were removed by filtration and the whole cells and cell walls sedimented by centrifugation at 1500xg for 5 mins. The supernatant was centrifuged at 125,000 g for 30 mins to sediment the crude membrane fraction. This pellet was resuspended in 3 mls PME . Chitin synthase has been shown to be present in the cell as a zymogen (Cabib and Farkas, 1971; Cabib, 1974; Braun and Calderone, 1978) it was therefore necessary to proteolytically activate the enzyme. The membrane fraction was incubated with 50 $\mu g\ ml^{-1}$ trypsin (Bovine pancreas) at $25^{o}C$ for 10 mins. After this time a 2-fold excess of soybean trypsin inhibitor was added to terminate proteolysis. This suspension was used as a crude chitin synthase preparation.

The assay was carried out in a total volume of 100 µl comprising:

6 mM uridine diphopho-N-acetyl-D-glucosamine	10 µl
UDP-N-acetyl-D-[^{14}C]-glucosamine (1µCi/ml)	10 µl
400 mM N-acetyl-D-glucosamine	10 µl
PME	10 µl
Nikkomycin in PME (when required)	10 µl
Membrane suspension	50 µl

The synthesis of chitin was allowed to proceed at 25°C and was terminated by the addition of 400 µl of 10% trichloroacetic acid. The resulting suspension was pelleted in an Eppendorf microfuge for 1 min, washed by two cycles of sonication and centrifugation with 1% acetic acid and finally resuspended in 500 µl of 1% acetic acid. The acetic acid suspension was then added to 10 ml Instagel scintillant and assayed for ^{14}C using a Searle Mark III 6880 scintillation counter. The radioactivity was related to N-acetyl-D-[^{14}C]-glucosamine incorporated into chitin.

CHAPTER 3: THE ACTION OF NIKKOMYCIN

Introduction

The study of peptide transport systems of both bacteria and fungi has been aided by the use of peptide-drugs which are co-transported with simple peptides. These have been used to create mutants resistant to the action of the drug through a defect in their peptide permease system. An example of this is the dipeptide bacilysin, used against *Saccharomyces cerevisiase* (Nisbet and Payne, 1979a). Resistance to bacilysin removed the ability of the cells to transport and accumulate peptides. A comparison of resistant and sensitive cells revealed the presence of a single peptide permease system.

The peptide-nucleoside antibiotics, the polyoxins, have been shown to be transported by the peptide permeases of certain fungi including *Alternaria kikuchiana* and *Pellicularia sasakii* (Mitani and Inoue, 1968; Hori, *et al.*, 1974,1976 and 1977). Polyoxin A resistance in *A.kikuchiana* has been shown to be mediated via a defect in the ability of the cells to accumulate the drug (Hori, *et al.*, 1977). The possibility of selecting transport-deficient mutants through resistance to polyoxins therefore exists.

Candida albicans is insensitive to most of the available polyoxins. However, the structurally related nikkomycins (Dahn, *et al.*, 1976) are

active against this organism. In the present study the nikkomycins were used to examine the mode of uptake of these drugs and to select a mutant of *C.albicans* resistant to its action through a transport-defect. Nikkomycins have been shown to inhibit the enzyme chitin synthase (Dahn *et al.*, 1976) and so initial studies were possible, on the effects of such drugs on *C.albicans* which have not been previously reported.

Results

Inhibition of growth

Nikkomycin was found to markedly inhibit growth of *C.albicans* at concentrations above 3 μg ml^{-1} when cells were grown in MX at 37°C (Figure 3.1). Total inhibition of growth was observed at 25 μg ml^{-1}. however, the compound was fungistatic at this concentration as the number of viable cells after 48 hours was no greater than the inoculum.

Nikkomycin has been shown to be an inhibitor of chitin synthase isolated from *Mucor rouxii* (Dahn, *et al.*, 1976) and this was also found to be the target enzyme in *C.albicans*. The type of inhibition was determined using the method of Dixon (1953). Nikkomycin showed a competitive inhibition of chitin synthase with the substrate UDP-N-acetyl-D-glucoamine. The K_i for this effect was found to be 0.16 μM (Figure 3.2).

As well as inhibiting a cell-free enzyme preparation a preliminary study showed nikkomycin to have a profound effect upon the morphology of *C.albicans* blastospores.

Effect of nikkomycin on blastospore morphology

When YNB-grown blastospores (Figure 3.4) were compared, using phase contrast microscopy, with those grown in the presence of the drug (50 $\mu g\ ml^{-1}$, Figures 3.7-3.10) a number of morphological abnormalities were observed. In the absence of the drug budding mother cells produced many daughter cells, a small proportion of which appeared as elongate buds or pseudohyphae. In the presence of 50 $\mu g\ ml^{-1}$ nikkomycin mother cells were found to produce extremely large daughter cells after a 6 hour incubation period (Figure 3.7). These contained constrictions and resembled the pseudohyphal form of growth rather than blastospores. In many cases protoplast-like structures were formed (Figure 3.10). Lysis of cells frequently occurred resulting in an extrusion of cytoplasm into the medium (Figure 3.8). Clumping of the cells was observed (Figure 3.9) and although lysis appeared to be occurring any interpretation of the results obtained by phase microscopy was difficult.

Aberrant growth and an altered cell morphology might be expected with an antibiotic whose action is directed against an enzyme producing one of the main structural polymers of the fungal cell wall. This is in agreement with the inhibitory effect of nikkomycin against chitin synthase.

Figure 3.1 The effect of nikkomycin on growth of *C.albicans* 124 (wild type).

Cells were grown to mid-logarithmic phase in YNB and inoculated at 10^3/ml into MX containing serial dilutions of nikkomycin. Viable cell numbers were determined after 48 hours at 37°C by growth on MX plates.

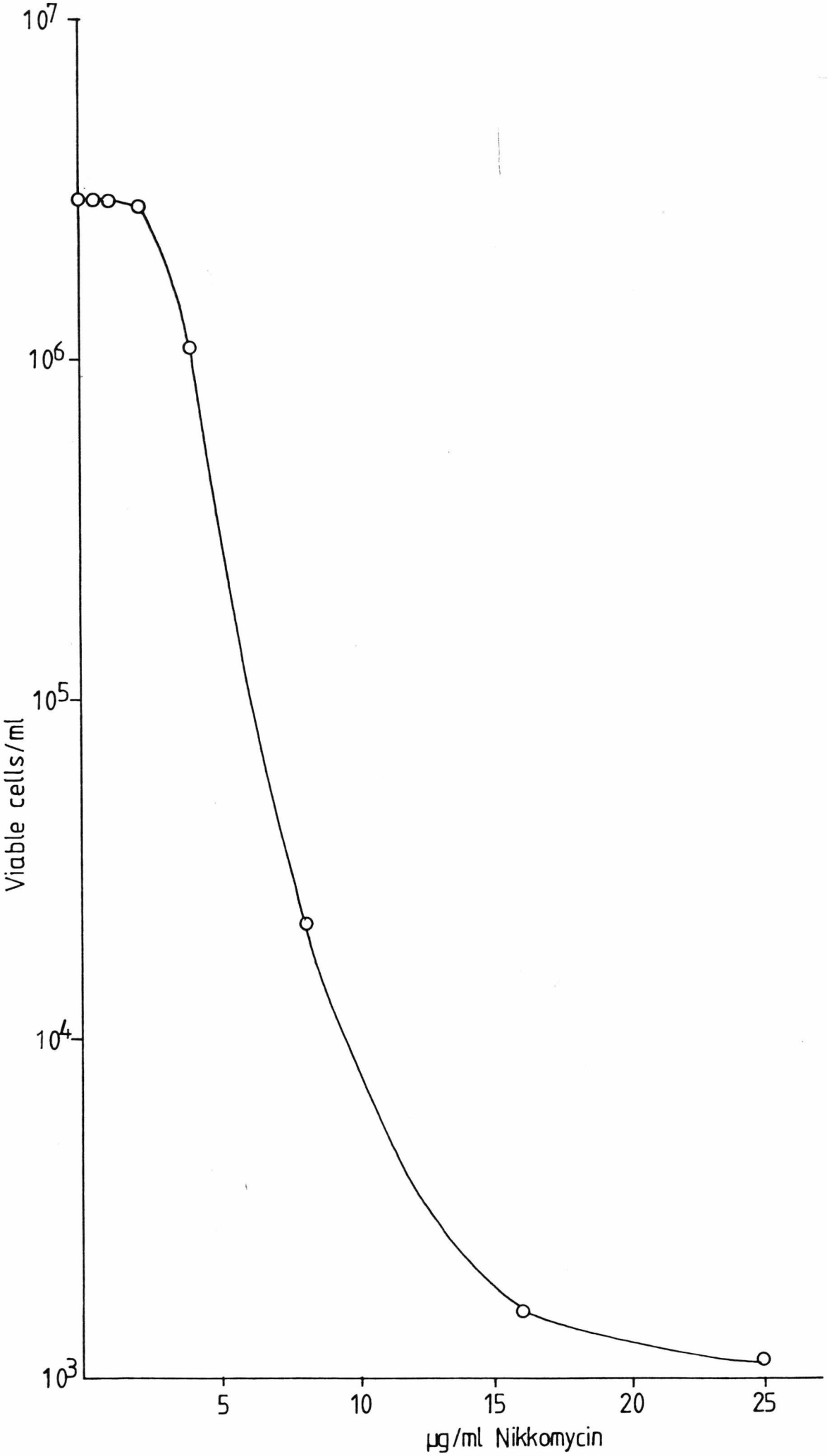

10^7
10^6
10^5
10^4
10^3
Viable cells/ml
5
10
15
20
25
µg/ml Nikkomycin

Figure 3.2 The effect of nikkomycin on *C.albicans* 124 (wild type) chitin synthase

Cells were grown to mid-logarithmic phase, harvested, washed and broken (in PME buffer) using a Braun homogenizer. Whole cells and cell walls were removed by centrifugation (1500 × g). The supernatant was centrifuged at 125,000xg for 30 mins to sediment the membrane fraction. The pellet was incubated with 50 μg ml^{-1} trypsin, proteolysis was terminated by the addition of a 2-fold excess of trypsin inhibitor after 10 mins. The resultant suspension was used as a crude chitin synthase preparation.

Synthesis of chitin was followed by incorporation of UDP-N-acetyl-D-[^{14}C] glucosamine, in the presence of various concentrations of nikkomycin, at two concentrations of unlabelled UDP-N-acetyl-glucosamine (0.3 mM (□) and 0.6 mM (○)). The reaction was terminated by the addition of 10% trichloroacetic acid. The pellet was washed by two cycles of sonication and centrifugation and the radioactivity of the pellet was determined. Initial rates of incorporation of radiolabel into chitin were determined in triplicate and plotted according to the method of Dixon (1953).

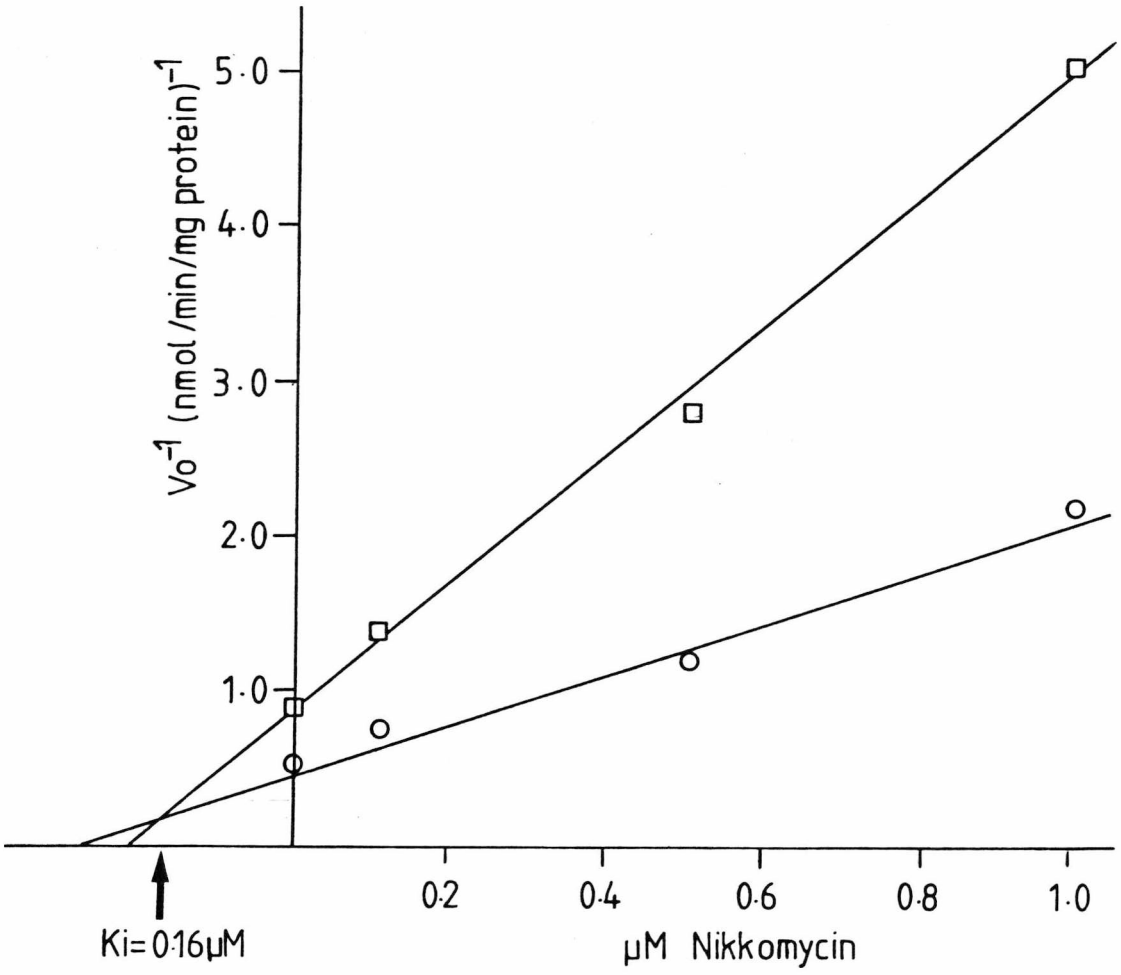


Figure 3.3 The effect of nikkomycin on *C.albicans* 124 (NIK5) chitin synthase.

The method used was identical to that described in Figure 3.2.

Two concentrations of UDP-N-acetyl-D-glucosamine were used: 0.3 mM (□) and 0.6 mM (○).

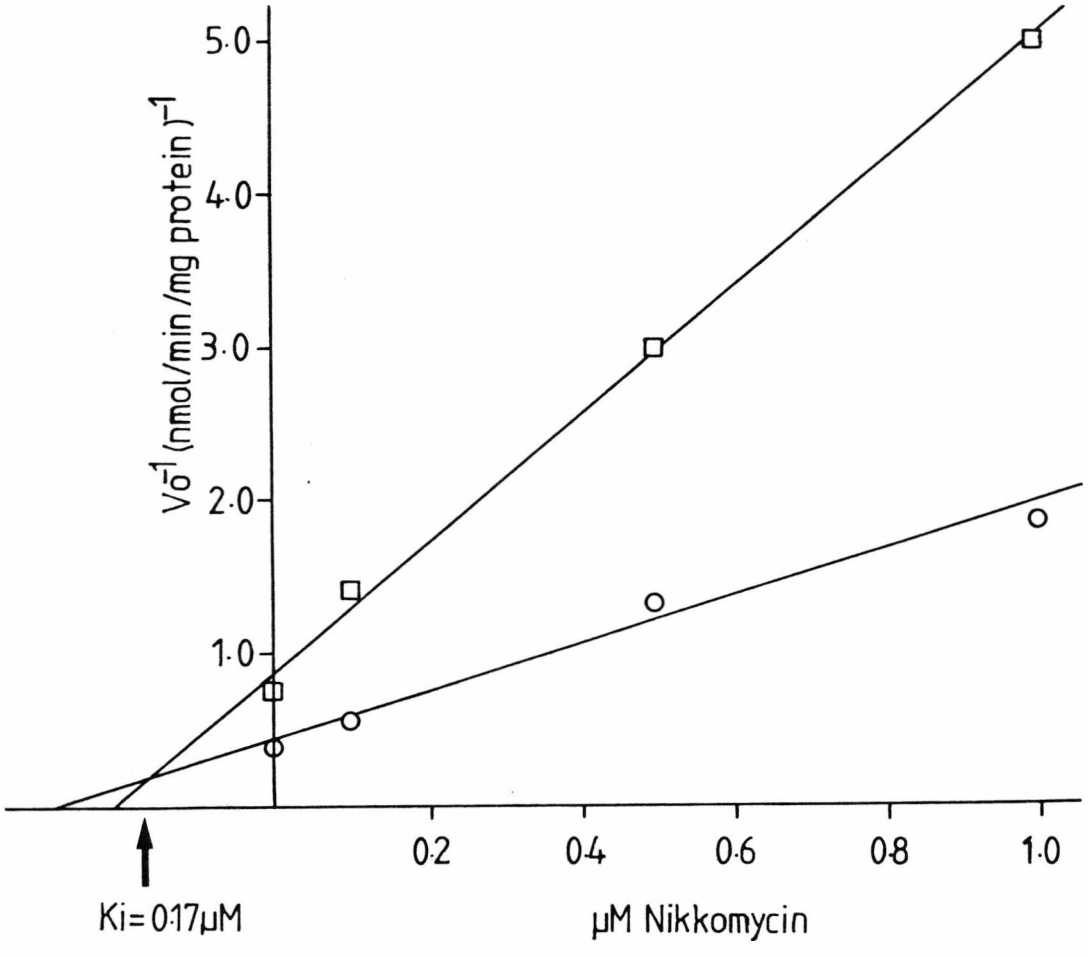

5·0
4·0
3·0
2·0
1·0
$V\delta^{-1}$ (nmol/min/mg protein)$^{-1}$
0·2
0·4
0·6
0·8
1·0
Ki = 0·17μM
μM Nikkomycin

Figure 3.4 *C.albicans* 124 (wild type) blastospores.

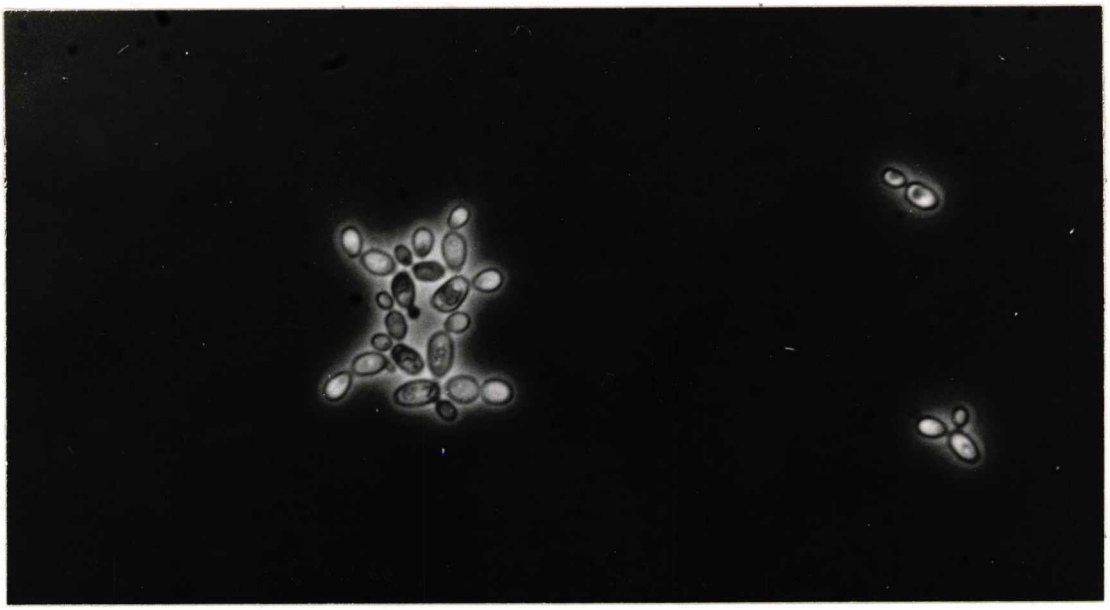
Cells were grown in YNB at 30°C for 10 hours and observed by phase-contrast microscopy. (×750)

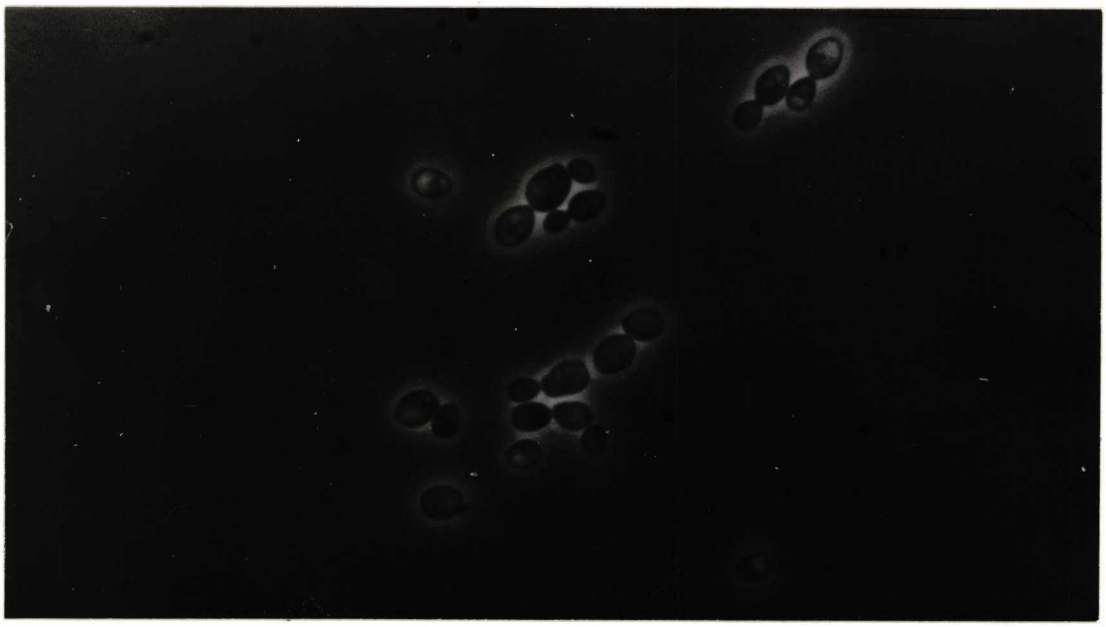
Figure 3.5 *C.albicans* 124 (NIK5) blastospores.

Cells were grown in YNB at 30°C for 10 hours and observed by phase-contrast microscopy. (×1150)

Figure 3.6 *C.albicans* 124 (NIK5) pseudohyphae.

Cells were grown in YNB at 30°C for 24 hours and observed by phase-contrast microscopy. (×1150)





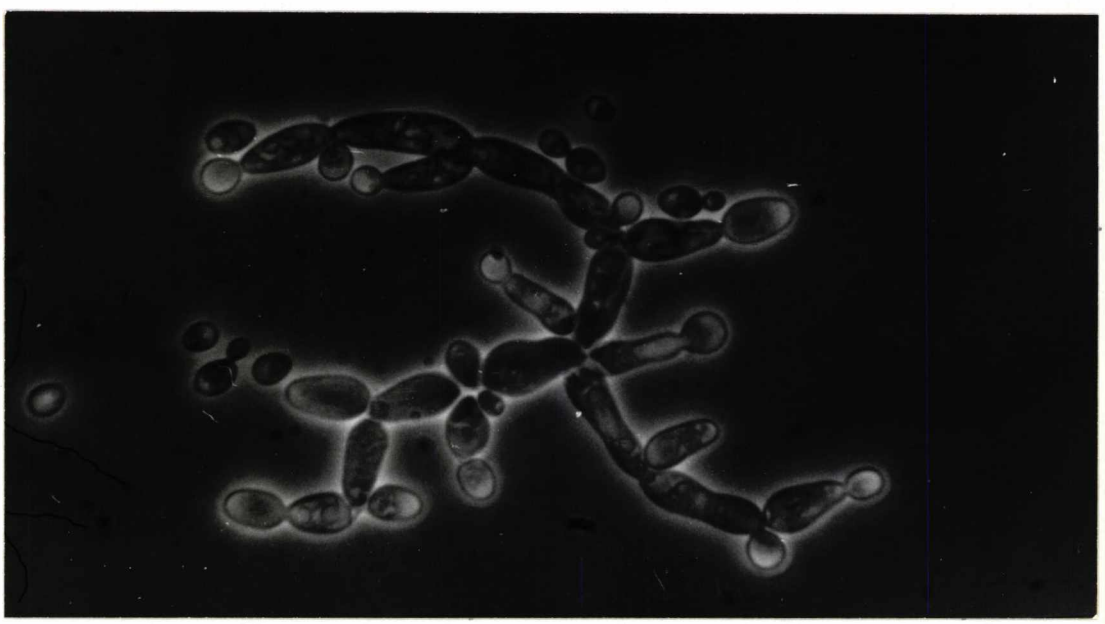


Figure 3.11-3.13 Distribution of primulin-staining regions in *C.albicans*

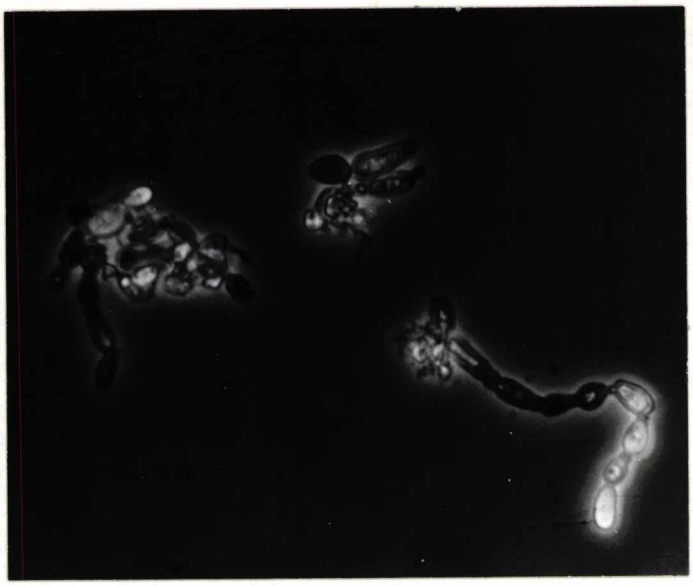
Cells were grown in YNB at 30°C , harvested after a timed period, washed and resuspended in phosphate buffer pH 7.5 containing 0.01 % (w/v) primulin and 0.8% (w/v) mannitol for 1 minute. They were then washed twice and observed by fluorescence microscopy.

3.11 *C.albicans* 124 (wild type) pseudohypha and blastospores following 6 hours incubation. Septa between the mother cells and buds and between pseudohyphal compartments are visible (arrowed). (×1500)

3.12 *C.albicans* 124 (wild type) blastospores following 12 hours incubation. Polar bud scars are clearly visible. (×3000)

3.13 *C.albicans* STM-1 pseudohypha following 6 hours incubation. Polar bud scars are again visible. (×3000)

7



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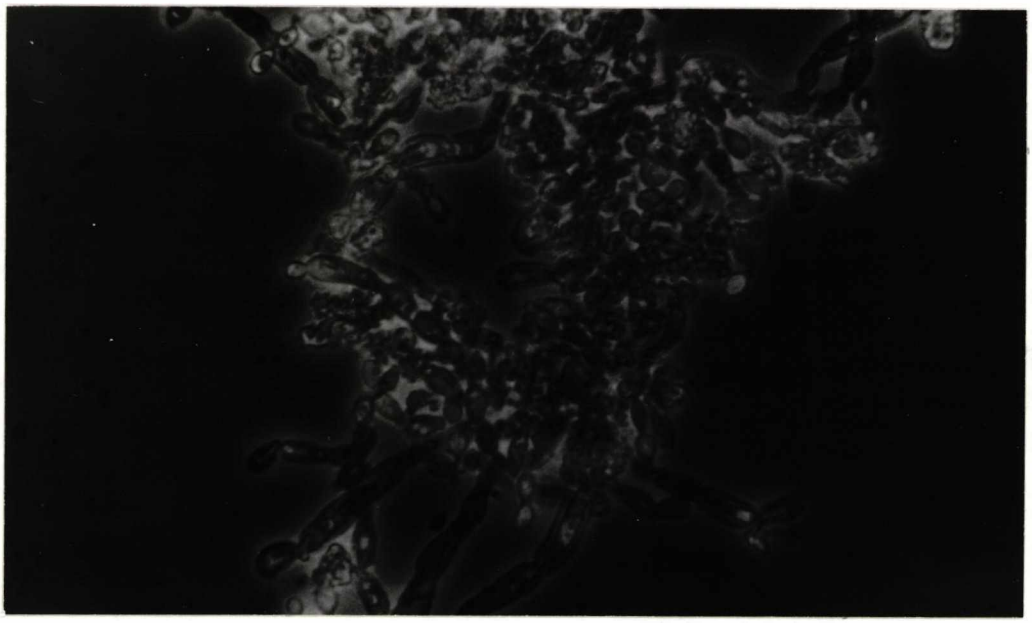

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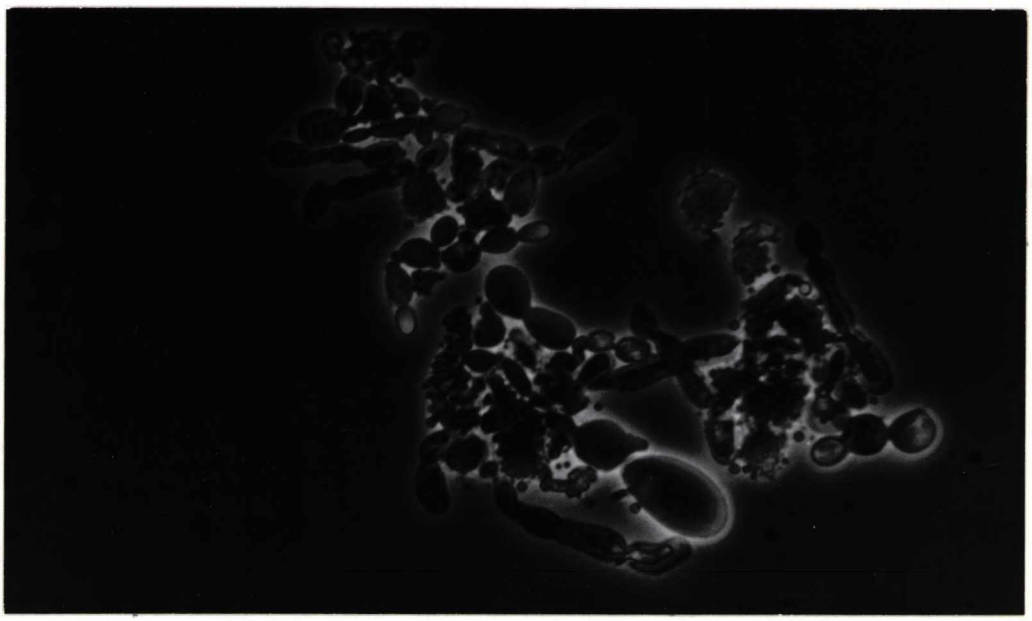


10

Phase-contrast micrographs of *C.albicans* 124 (wild type) growing at $30^{o}C$ in YNB containing 50 $\mu g\ ml^{-1}$ nikkomycin (×950)

Figure 3.7 6 hour incubation

Figure 3.8 6 hour incubation

Figure 3.9 9 hour incubation

Figure 3.10 10 hour incubation

11

12

13

Studies of the morphology of fungal cell walls are aided by the use of optical brighteners. These are dyes which bind to specific walls components causing them to fluoresce brightly when illuminated at certain wavelengths. The components can then be studied throughout the cell cycle or, as in this case, on the addition of a drug to the growth medium. The bud scars of many yeast-like organisms have been observed by the use of primulin (Streiblova and Beran, 1963). In the present study primulin was found to bind to two, highly specific, regions of *C.albicans* cells. In a YNB culture the septa of the hyphae and those between mother and daughter blastopsores showed a brilliant fluorescence while the rest of the cell remained relatively unstained (Figure 3.11). The reason for the brilliant fluorescence of the mother cell is unclear although cell death may have occurred, allowing the stain to enter the cell. The interaction between cytoplasmic constituents and the dye may then have resulted in fluroescence.

Another region of staining was the bud scar. In aged cells (Figure 3.12) many bud scars were found closely packed at the poles of the blastospores . All morphological forms of *C.albicans* appeared to possess polar cells. Pseudohyphae have been found to be formed frequently in certain strains of *C.albicans* such as STM-1 (Figure 3.13). The elongate buds of this growth form followed the developmental pattern of blastospores and budding was again found to occur at the poles of the cells.

AP+ medium was not found to be satisfactory for the production of hyphal cells. However, it did cause production of the pseudohyphal growth form of *C.albicans* 124. These resembled true hyphae only in the

early stages of development. An inoculum of singlet blastospores was found to produce germ tubes in which a septum was laid down at a variable point (Figure 3.14). Further growth then followed with septa being laid down at regular intervals (Figure 3.15). Separation of the cells of the pseudohypha frequently occured. This produced a culture of elongate mother cells which replicated by budding (Figure 3.16).

True hyphae were only induced in the presence of serum (20% (v/v)) at 37°C . These hyphae (Figure 3.17) contained none of the constrictions associated with pseudohyphae (Figure 3.15) and did not separate. The septa were again visualized by primulin staining and were laid down at regular intervals. The fluorescence of the hyphal septum was not as great as that observed in YNB-grown cells due to an interaction between the dye and the serum-proteins leading to a high background fluorescence.

Determination of the primulin staining sites in the various morphological forms of *C.albicans* allowed the effects of nikkomycin to be followed.

Primulin staining of nikkomycin-treated blastospores

Mother cells grown in YNB were found to produce large daughter buds in the presence of 50 $\mu g\ ml^{-1}$ nikkomycin. These buds enlarged to form structures resembling pseudohyphae (Figure 3.19) in which primulin staining regions were restricted to the mother cell wall. Constrictions

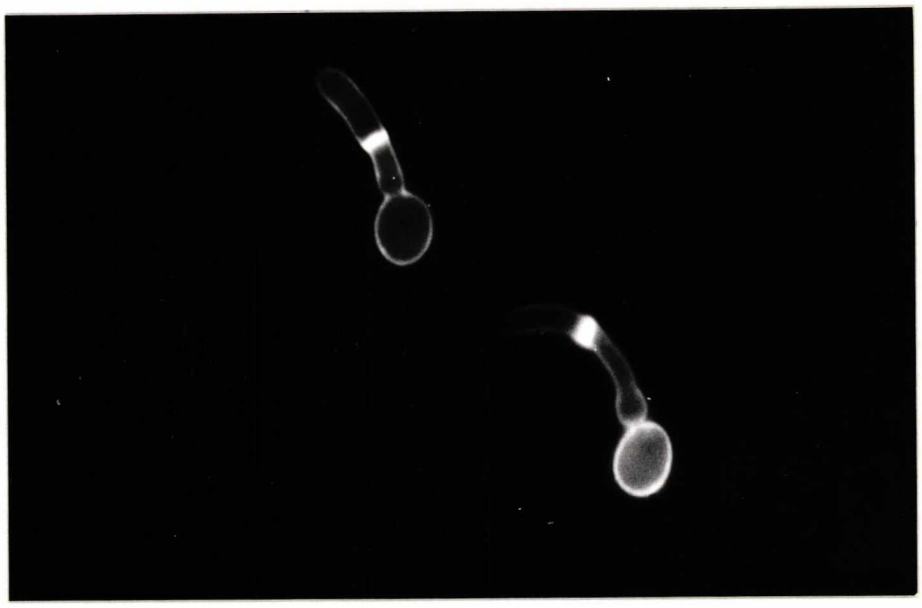
Figrues 3.14-3.16 Distribution of primulin-staining regions in *C.albicans* 124 (wild type)

Cells were germinated in AP+ at 40°C and incubated for a timed period. Harvesting and staining was as described for Figures 3.11-3.13. (×2000)

3.14 3 hours incubation

3.15 6 hours incubation

3.16 8 hours incubation



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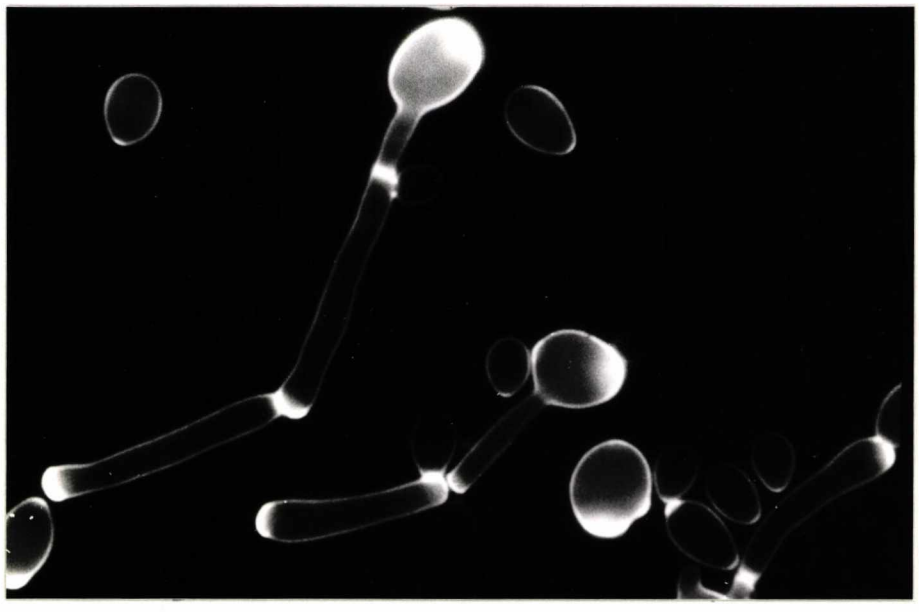

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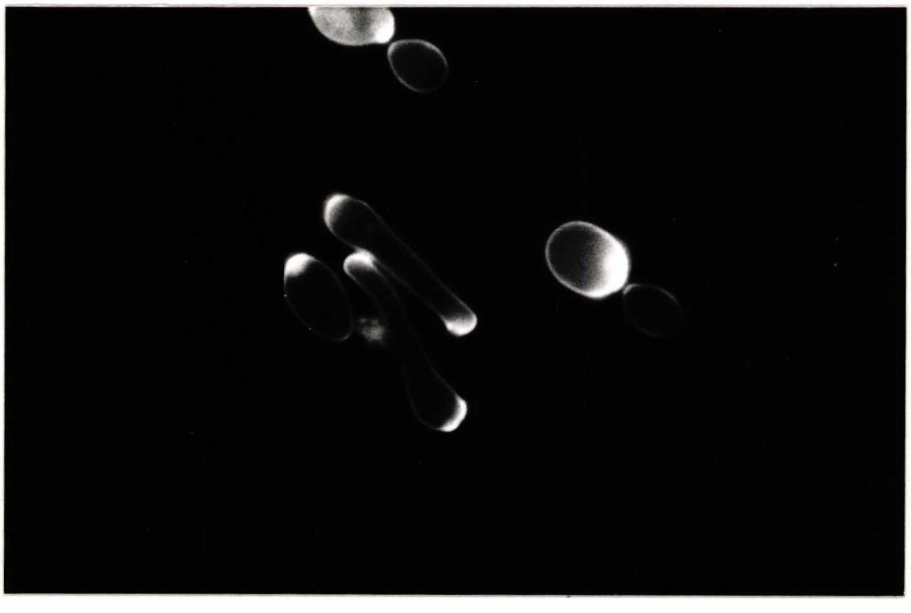
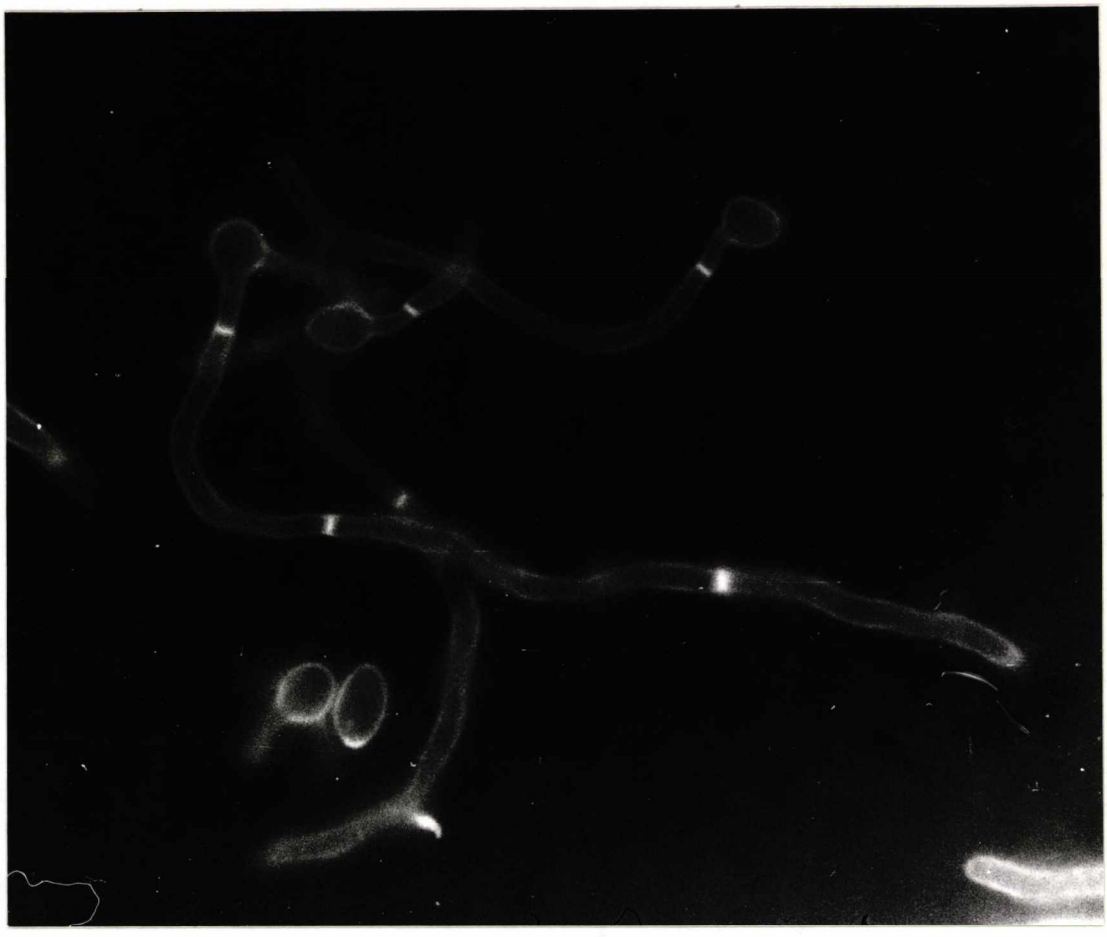


16

Figure 3.17-3.18 True hyphae of *C.albicans* 124 (wild type)

Cells were germinated in 20% newborn calf serum in the presence (Figure 3.18) and absence (Figure 3.17) of 50 μg ml^{-1} nikkomycin. Following 4 hours incubation the cells were harvested and stained as described for Figures 3.11-3.13. (×1750)



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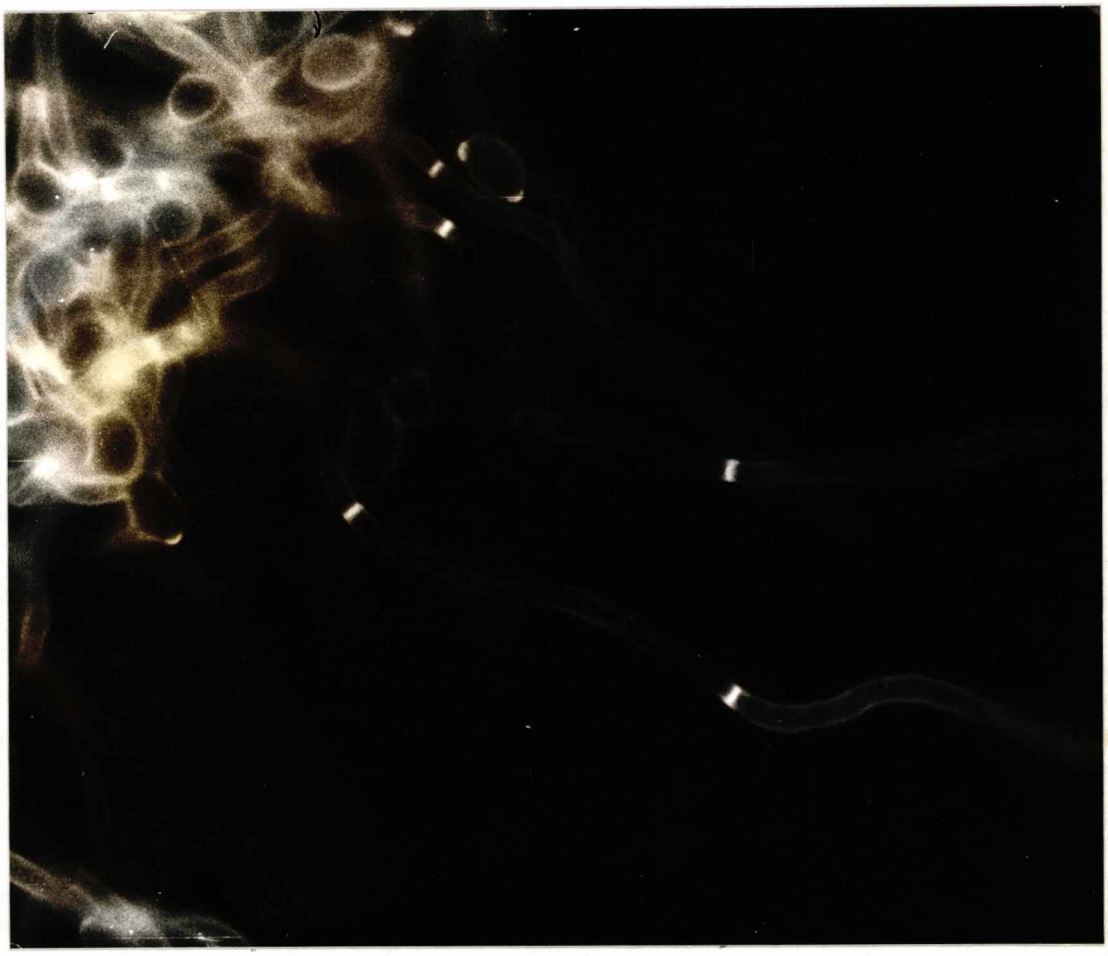


18

Figure 3.19-3.20 The effect of nikkomycin on the yeast form of *C.albicans* 124 (wild type)

Cells were grown in YNB in the presence of 50 $\mu g\ ml^{-1}$ nikkomycin. Following a timed incubation period the cells were harvested and stained as in Figures 3.11-3.13

3.19 Cells harvested after 6 hours incubation. Faint septa are occasionally visible (arrowed). (×2000)

3.20 Cells harvested after 7 hours incubation. Lysis has occurred in many cells with extrusion of cytoplasm into the medium (arrowed). (×1500)

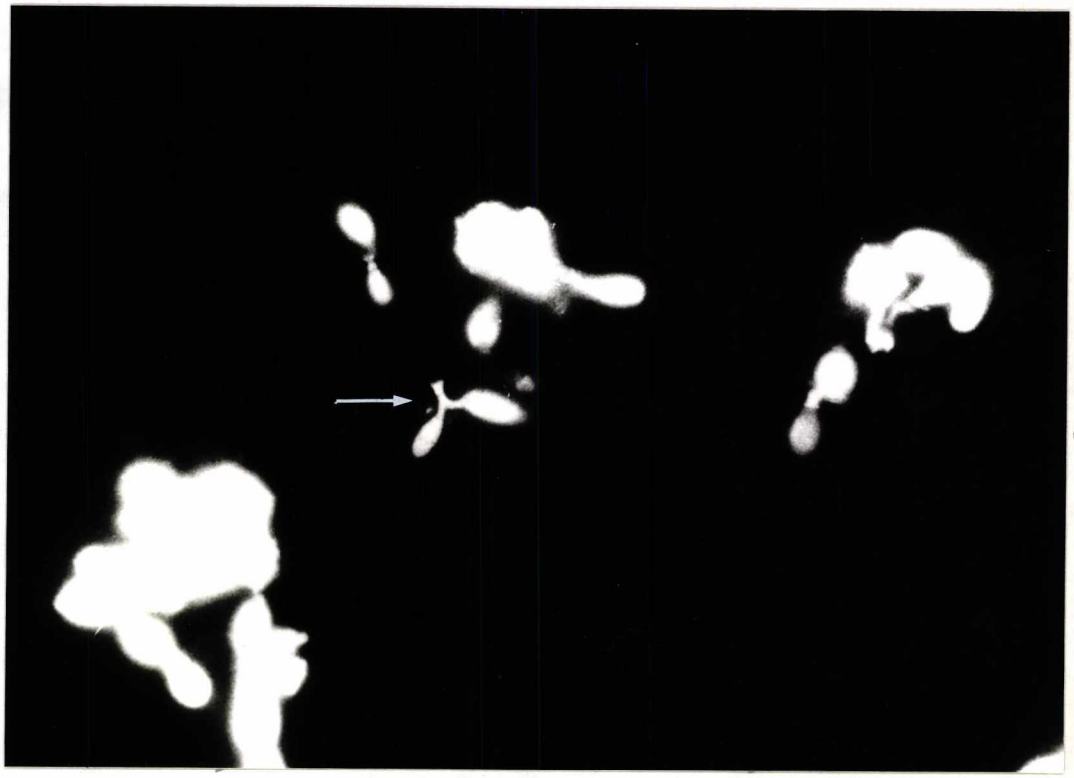


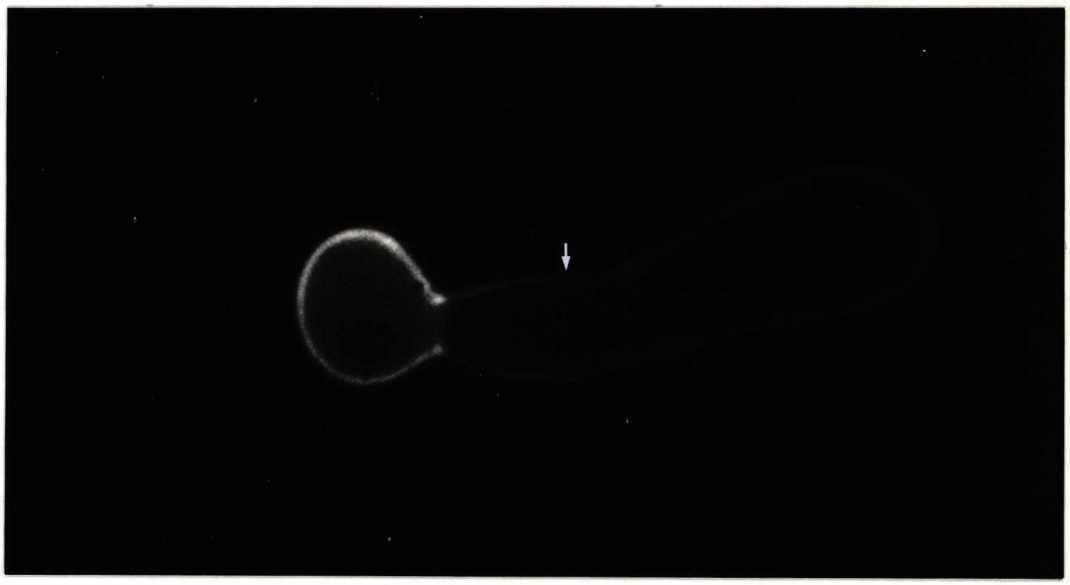
Figure 3.21-3.22 The effect of nikkomycin on the pseudohyphal growth form of *C.albicans* 124 (wild type)

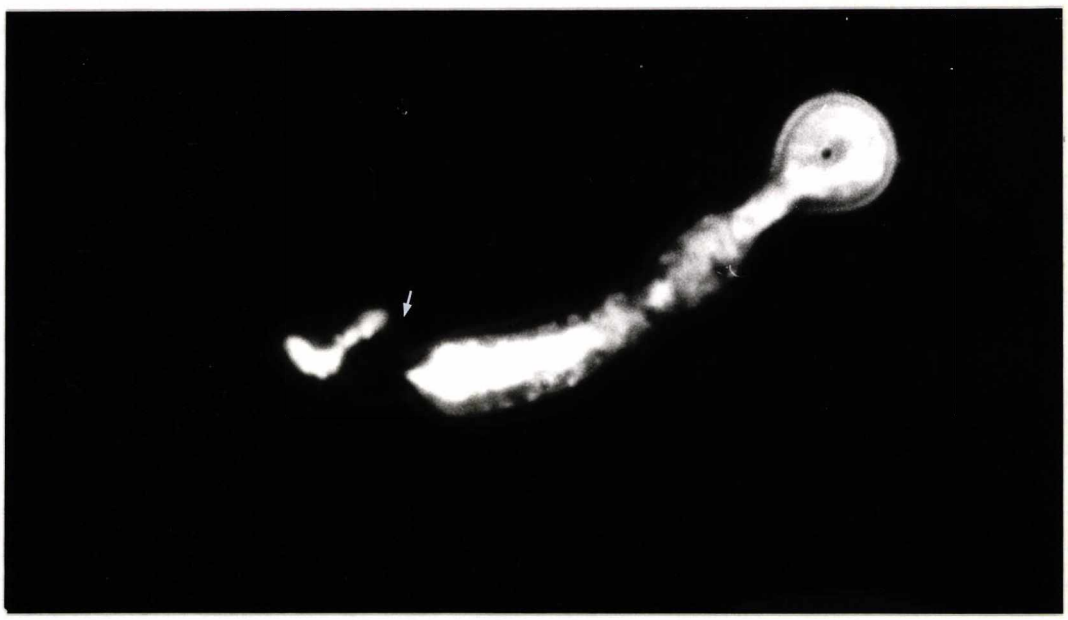
Cells were germinated in AP+ in the presence of 50 $\mu g\ ml^{-1}$ nikkomycin. Following a timed incubation period the cells were harvested and stained as in Figures 3.11-3.13.

(×2500)

3.21 Incubated for 3½ hours. Chitinous regions are restricted to the mother cell while constrictions are visible within the daughter cell (arrowed).

3.22 Incubated for 7 hours. Lysis has occurred through an orifice in the cell wall (arrowed). Constrictions in the daughter cell wall are again visible.





in the bud were apparent with occasional faint septa. Cells rarely separated and after further incubation many were found to lyse, causing an efflux of cytoplasm into the medium (Figure 3.20). Lysis frequently occured at a junction between a pair of cells and resulted in a brilliant intracellular fluorescence.

Primulin staining of nikkomycin-treated pseudohyphae

Inoculation of blastospores into AP+ medium containing 50 $\mu g\ ml^{-1}$ nikkomycin resulted in germination. The protoplasting germ tubes enlarged without the formation of a septum (Figure 3.21). A constriction within the germ tube was visible which correlated with the point at which a septum would have been formed in control cells. All primulin-staining regions were again located on the mother cell wall. Following further incubation the cells enlarged and a second constriction formed (Figure 3.22). Lysis of the cells frequently occured adjacent to this second constriction and resulted in a general fluorescence of the cytoplasm.

The effects of nikkomycin on budding and germination of *C.albicans* 124 were very similar, with the production of distorted pseudo-hyphal forms in which a protoplasting effect was evident. In both cases cell lysis and subsequent extrusion of the cytoplasmic constituents into the medium occurred.

Effect of nikkomycin on serum-induced hyphae

The inclusion of 50 $\mu g\ ml^{-1}$ nikkomycin in 20% serum had no effect upon subsequent germination or growth of the inoculated blastospores

(*C.albicans* 124, Figure 3.18). In both their apparent growth rates and morphology the drug-treated cells were identical to those grown in the absence of the drug.

Peptidase activity in the serum was assayed by the o-phthaldialdehyde method. No activity was found against either ala_2 or nikkomycin. The inactivity of the drug was therefore due to a factor other than the cleavage of its peptide moiety.

Primulin staining suggested that nikkomycin was interacting with septum formation in *C.albicans*. Transmission electron microscopy was used to follow the budding cycle of blastospores in the presence or absence of nikkomycin.

Electron microscopy of *C.albicans* blastospores

(i) Budding cycle

The budding cycle of blastospores (*C.albicans* 124) was demonstrated in a YNB culture grown at $30^{o}C$. Mother cells produced buds which then increased in size (Figure 3.23). Organelle migration from the mother cell to the bud occurred and a scar ring was produced at the point of constriction between the two cells (Figure 3.24). A multilayered septum with a central micropore was produced (Figure 3.25). Membranous material was visible on either side of the septum. In some instances thinning of the cell wall occurred adjacent to the septum (Figure 3.26).

Figures 3.23-3.26 Transmission electron micrographs of budding phase *C.albicans* 124 (wild type)

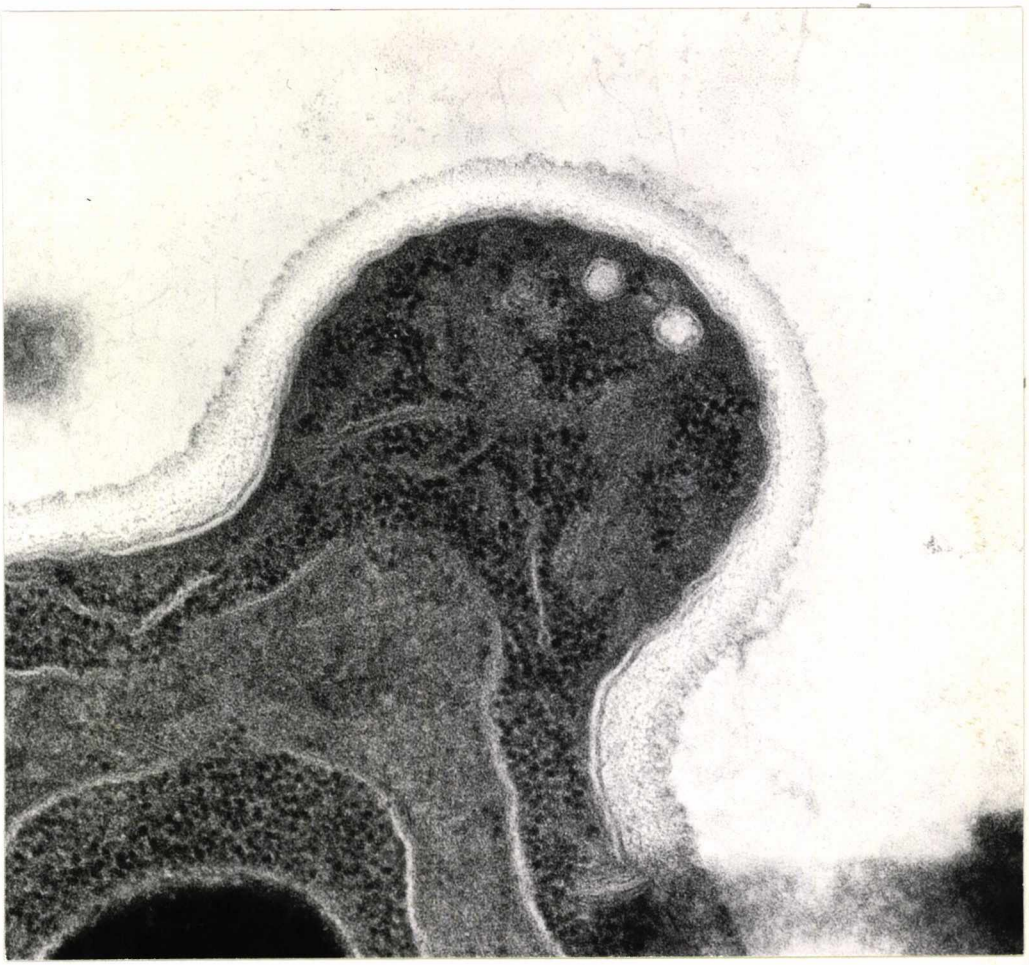
Cells were grown in YNB for 6 hours, fixed in glutaraldehyde and postfixed with osmium tetroxide. Dehydration was thorugh an ethanol series with cells being finally embedded in Spurr's resin.

3.23 A young bud showing organelle migration following the outgrowth of the cell wall. (×80K)

3.24 The start of septum formation. The scar ring can be seen as an outgrowth of the cell wall (arrowed). (×32K)

3.25 The septum. The primary septum (PS) and secondary septa (SS) are visible. The section also reveals the central micropore (MP) and membranous material on either side of the septum. (×80K)

3.26 A pair of cells with a completed spetum. Thinning of the cell wall adjacent to the septum is visible (arrowed). (×50K)



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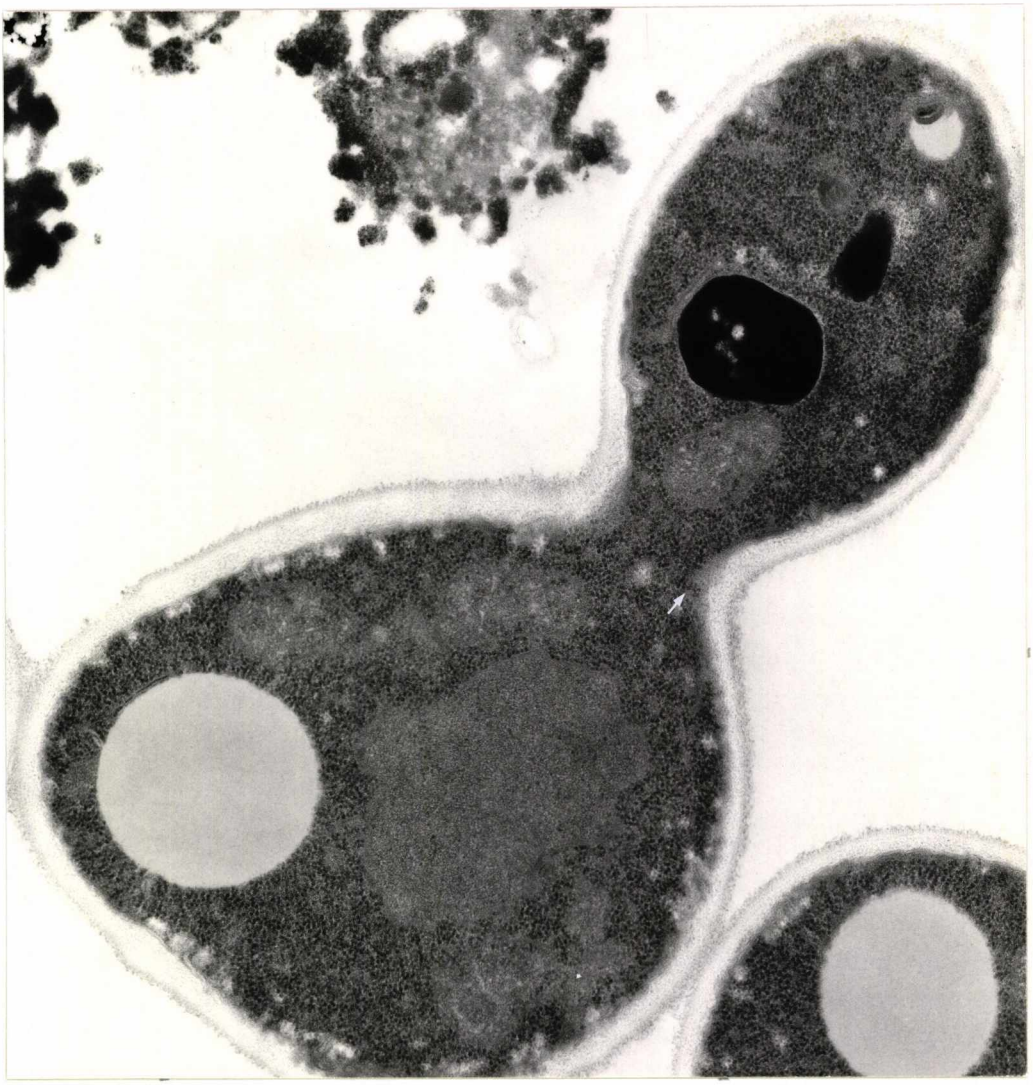

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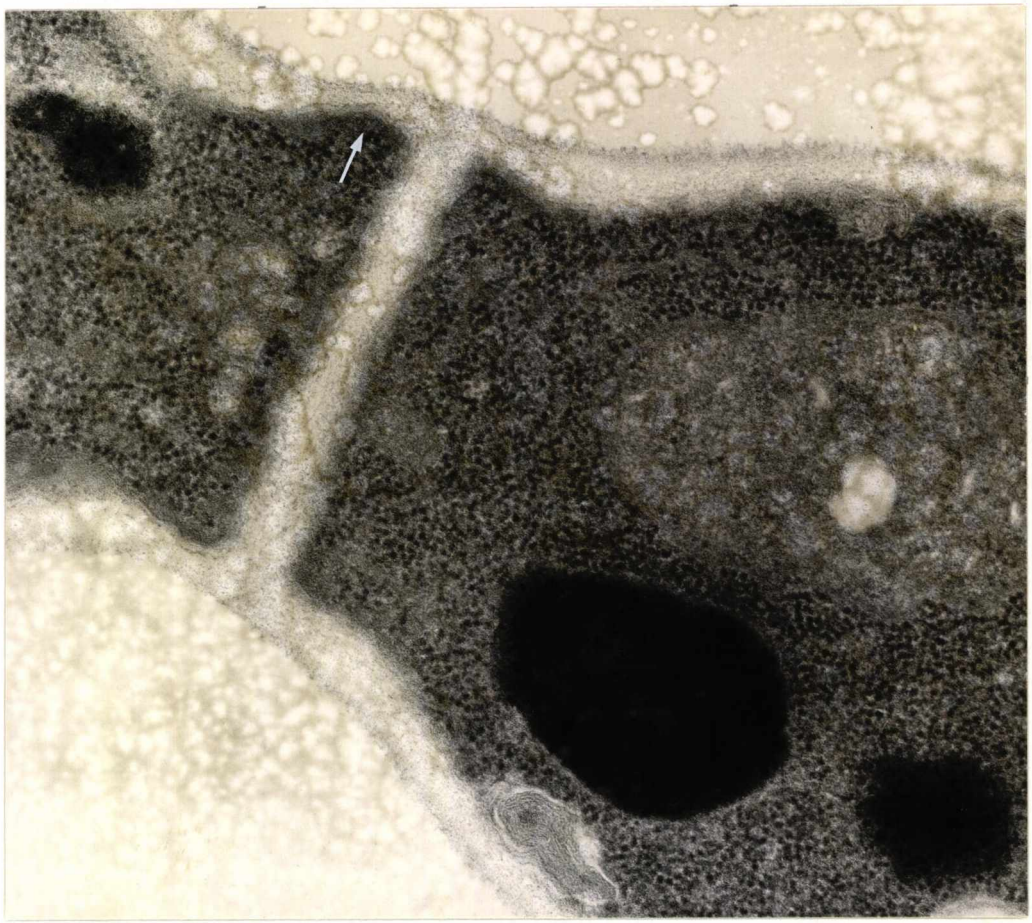

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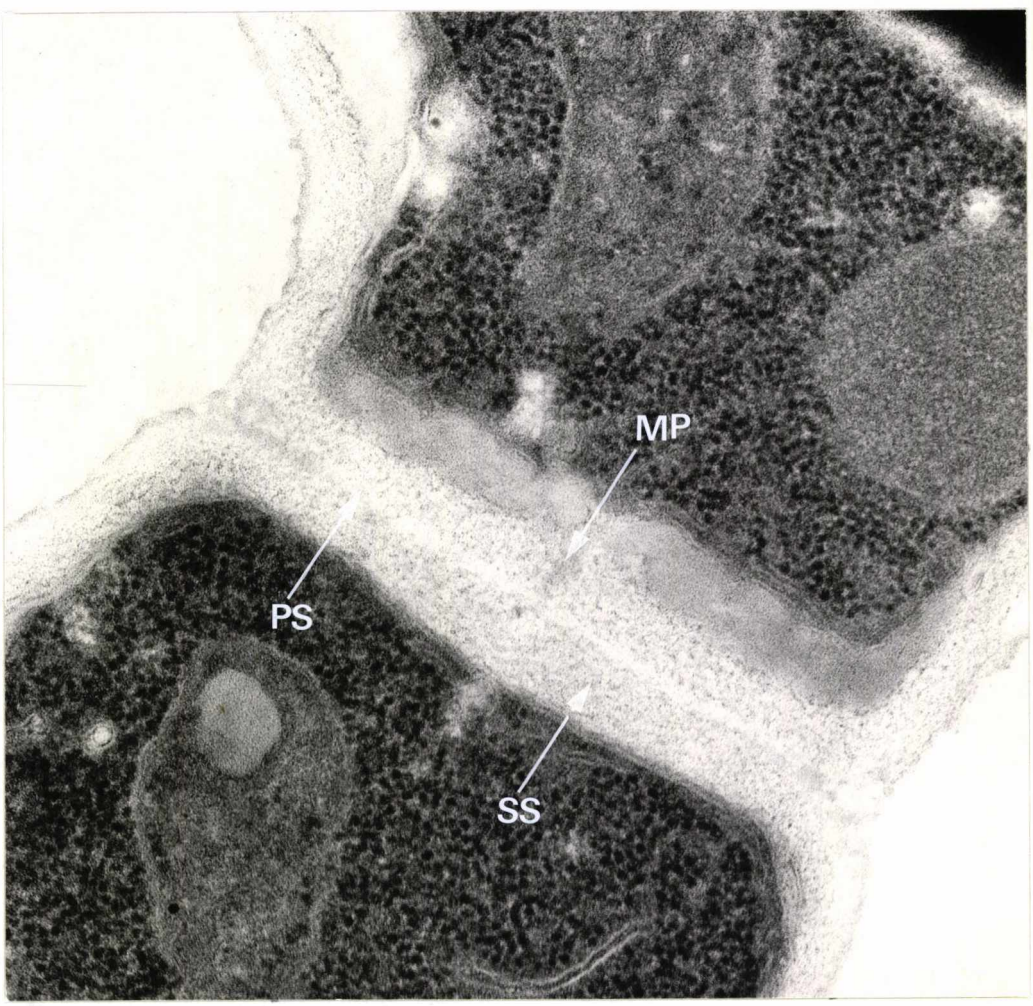


25

Figures 3.27-3.31 The effects of nikkomycin on the budding cycle of *C.albicans* 124 (wild type)

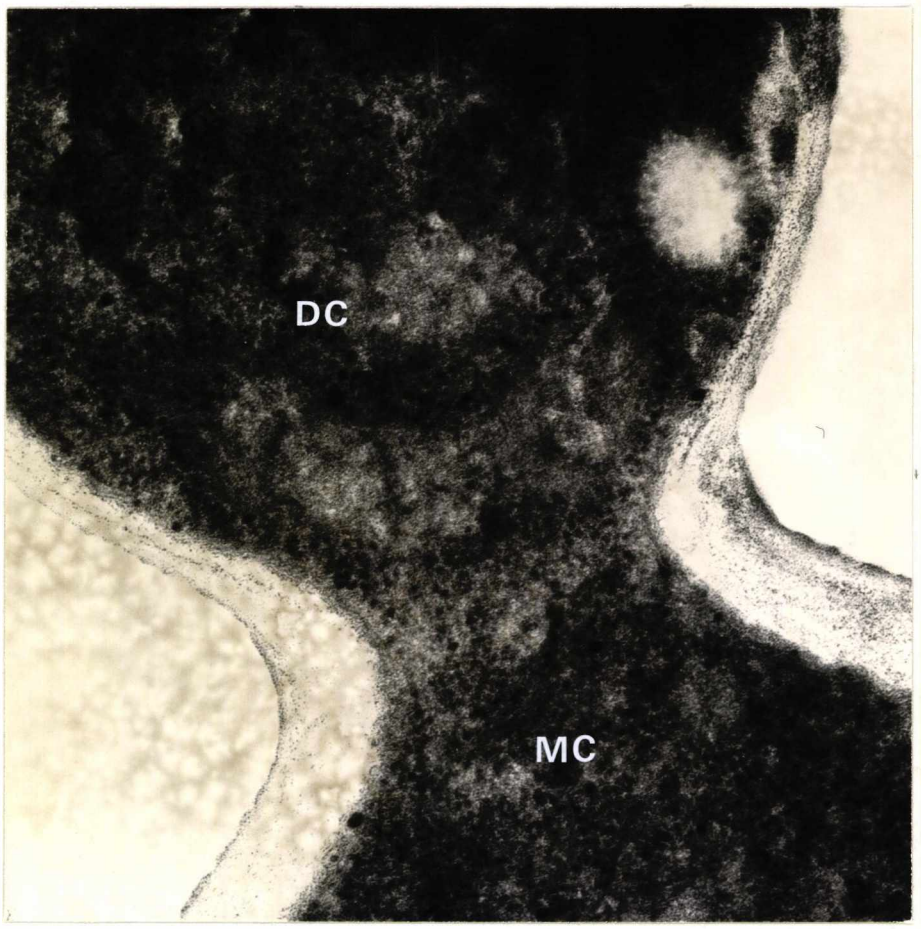
Cells were grown in YNB (containing 50 $\mu g\ ml^{-1}$ nikkomycin) for 6 hours. Fixing and embedding was as described in Figures 3.23-3.26.

3.27 A mother cell (MC) with an enlarged daughter cell (DC). The cell wall of the daughter is far thinner than that of the mother. (×80K)

3.28 A mother cell with an enlarged daughter bud showing the partial formation of the primary septum (PS). (×80K)

3.29 A mother-daughter pair of cells showing the thinning of the cell wall adjacent to the septum (arrowed). (×20K)

3.30-3.31 Mother-daughter pairs of cells showing the lysis of the mother cell wall adjacent to the septum. The intracelluar organelles of the daughter are clearly visible. (3.30 : ×20K; 3.31: ×32K)



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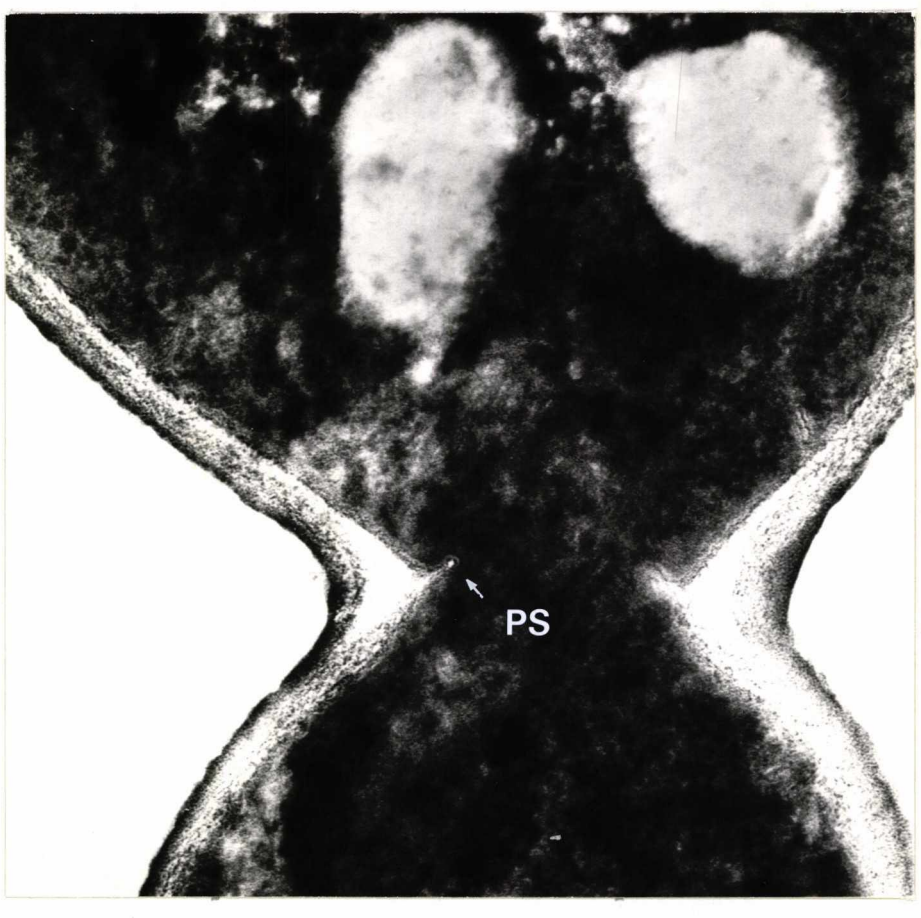

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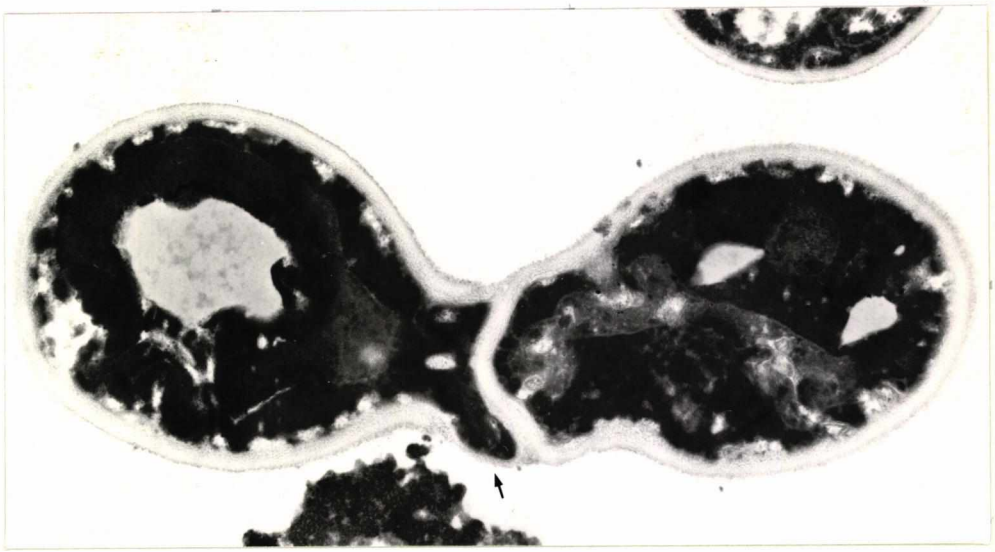
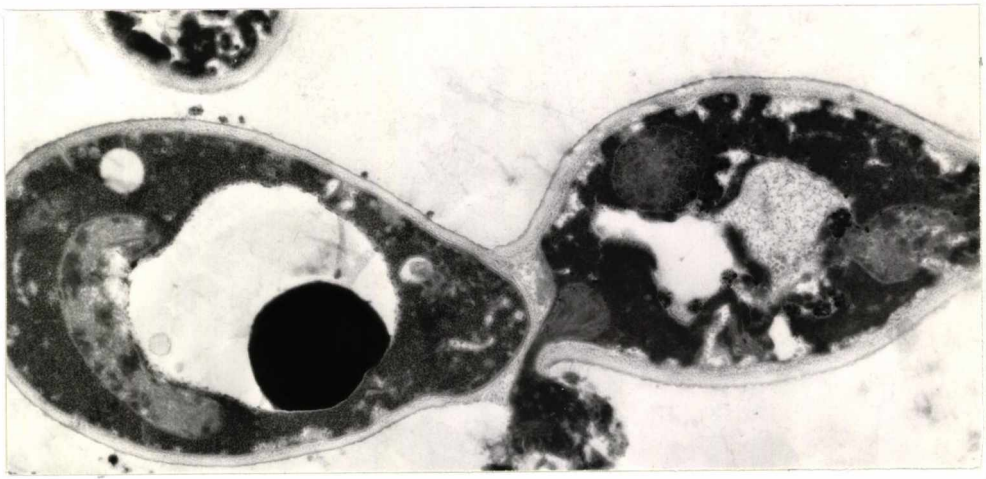
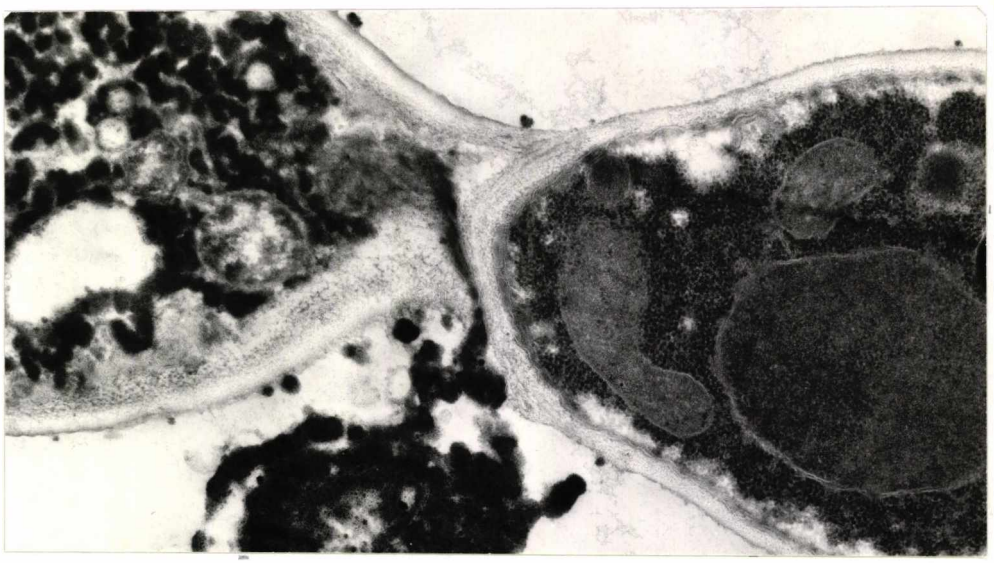


29



30



31

(ii) Effect of nikkomycin

Three major effects of nikkomycin were found in those cells which had been treatedwith the drug (50 μg ml^{-1}) for 6 hours. Firstly the bud cellwall was considerably thinner when compared with the mother cell wall (bud=75 nm; mother=210 nm, Figure 3.27) . Although large buds were visible in these cases a septum had notbeen initiated.

Secondly, a partially formed septum was present (Figure 3.28). This was visible as a small outgrowth from the cell wall at the point of constriction between the mother and daughter cells.

The third effect of nikkomycin was observed in pairs of cells which had formed septa. Thinning of the mother cell wall occurred adjacent to the septum (Figure 3.29) resulting in the formation of a bulge followed by lysis (Figure 3.30). The extrusion of cytoplasm through the orifice in the maternal cell wall resulted in cell death. The daughter cell of the pair remained intact with cytoplasmic organelles clearly distinguishable (Figure 3.31).

Nikkomycin is therefore highly active against *C.albicans* through an interaction with chitin synthase. This brings about morphological distortion and eventual cell lysis. The structural similarity between nikkomycins and polyoxins suggested that they might share a common transport system in addition to the same target enzyme. The polyoxins have ben shown to enter fungal cells via the peptide transport system (Mitani and Inoue, 1968; Hori, *et al.*, 1974, 1976 and 1977). To determine whether nikkomycins utilize this uptake system in *C albicans* the effect of variation of the growth medium upon the subsequent activity of nikkomycin was assayed.

Mode of uptake of nikkomycin

The growth medium was found to have a considerable influence on the action of nikkomycin. On minimal medium (YNB) or a simple undefined medium (MX), the drug was found to inhibit grwoth of a culture at concentrations above 3 $\mu g\ ml^{-1}$. On YNBP , in which peptides serve as the sole nitrogen source, 50 $\mu g\ ml^{-1}$ nikkomycin was found to be inactive (Figure 3.32). The increase in the turbidity of the culture which was observed in minimal medium containing 50 $\mu g\ ml^{-1}$ nikkomycin was due to enlargement and eventual clumping of the cells.

The inclusion of peptone in the medium produced a marked effect on the action of nikkomycin. To show whether this was due to peptides or another factor individual peptides were included in YNB . The effect of the drug was monitored by both turbidity of the culture and by the morphology of the individual cells as observed by phase microscopy.

Effect of individual peptides on nikkomycin activity

50 $\mu g\ ml^{-1}$ nikkomycin was included in YNB and peptides were added to a final concentration of 10 mM . The growth of the culture was followed by turbidity measurements.

It was found that addition of 10 mM ala_2 to YNB inhibited the action of nikkomycin (Figure 3.33). The rate of growth of the culture in the presence of both ala_2 and the drug was the same as that found for aculture in the absence of nikkomycin. Microscopic examination

Figure 3.32: The effect of the growth medium on nikkomycin activity.

C.albicans 124 (wild type) cells were grown overnight in YNB and inoculated at 10^5/ml into YNB (●), YNB containing 50 µg ml^{-1} nikkomycin (■), YNBP (○), or YNBP containing 50 µg ml^{-1} nikkomycin (□). Growth was followed at 30°C by turbidity measurements using an EEL colorimeter.

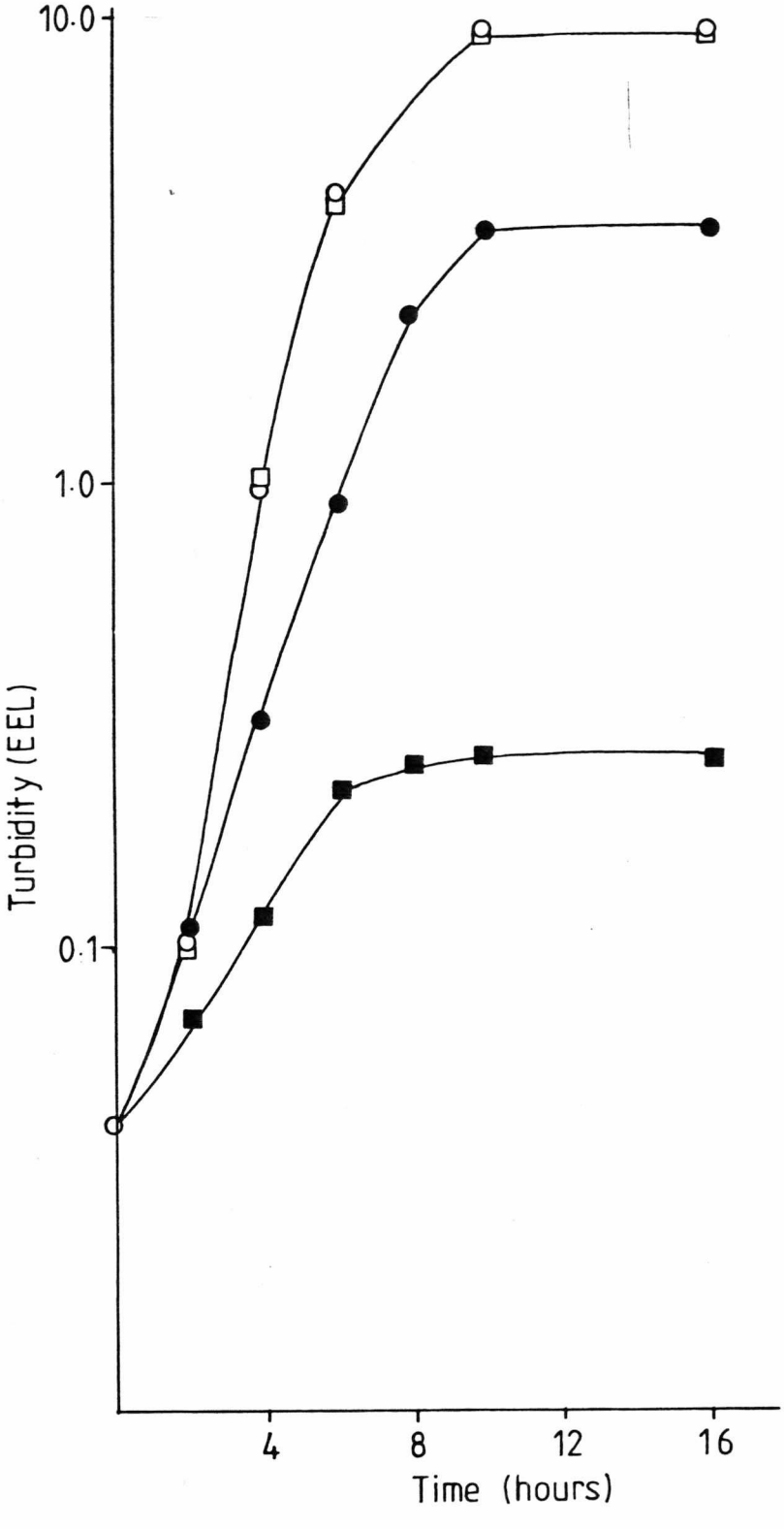
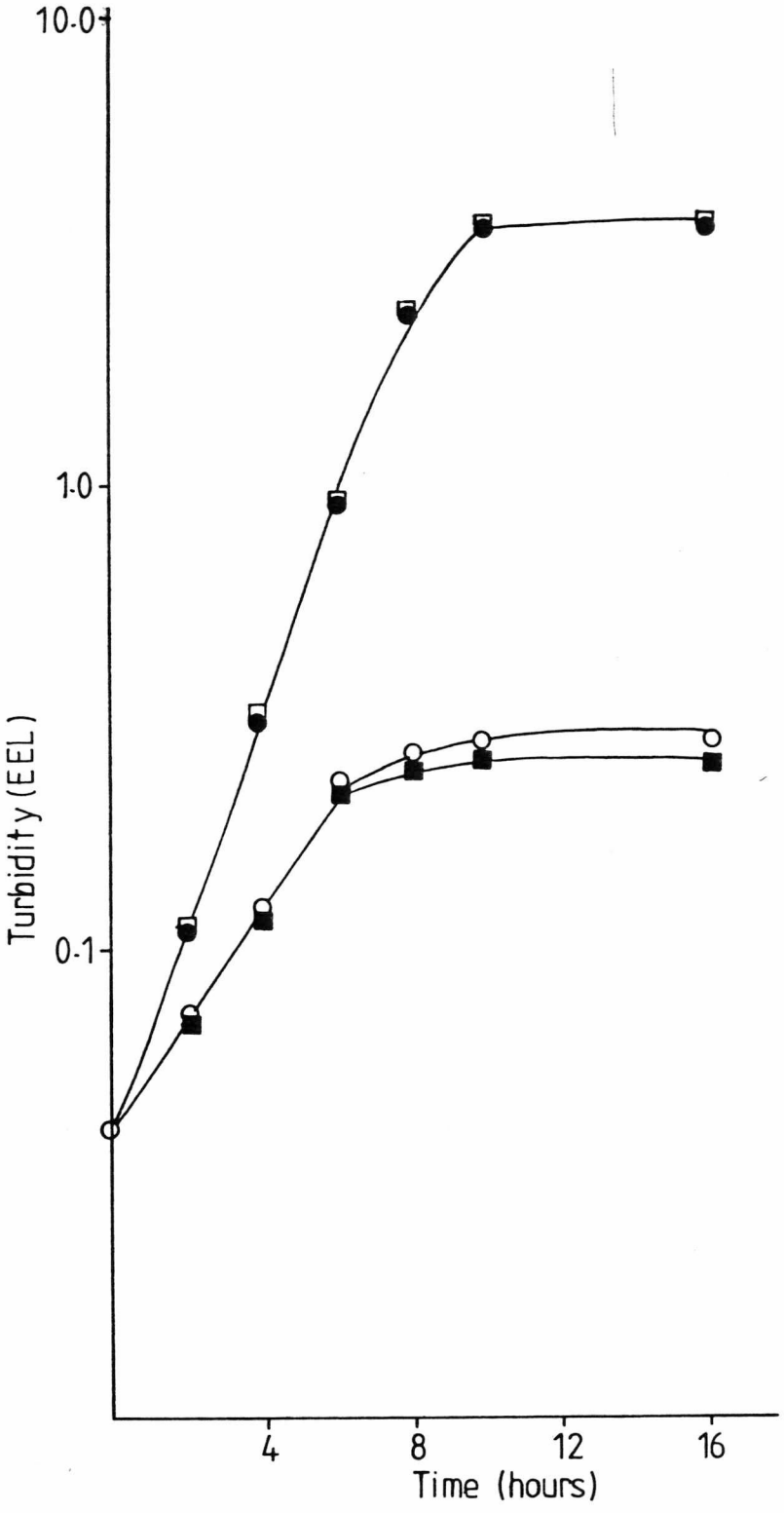

10·0
1·0
0·1
Turbidity (EEL)
4
8
12
16
Time (hours)

Figure 3.33 The effect of simple peptides on the activity of nikkomycin..

C.albicans 124 (wild type) cells were grown overnight in YNB and inoculatedat 10^5/ml into YNB (●), YNB containing 50 µg ml^{-1} nikkomycin (■), YNB containing 50 µg ml^{-1} nikkomycin and 10 mM ala_2 (□), or YNB containing 50 µg ml^{-1} nikkomycin and 10 mM gly tyr (○). Growth was followed by turbidity measurements using an EEL colorimeter.

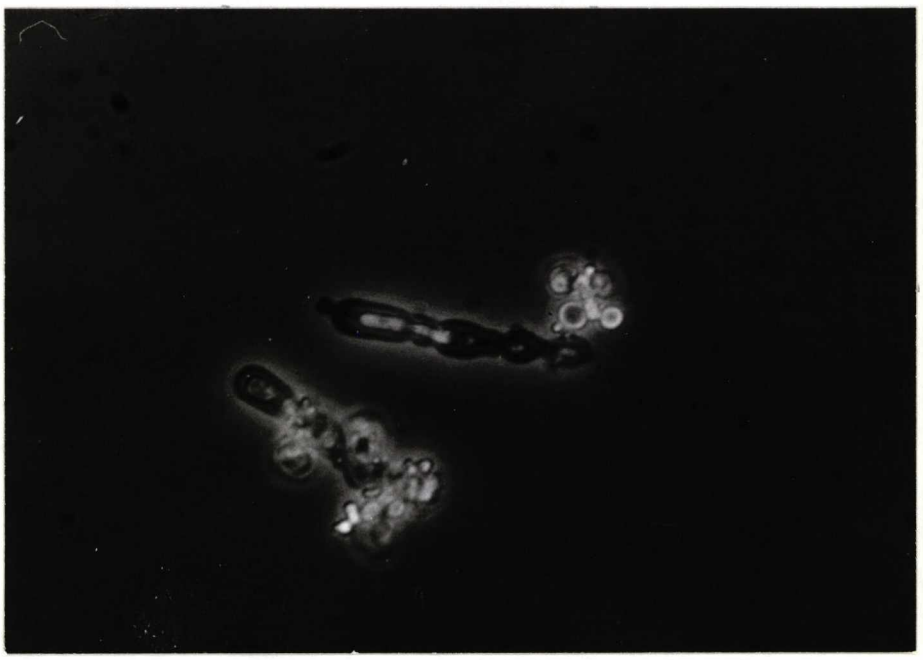

10·0
1·0
0·1
Turbidity (EEL)
4
8
12
16
Time (hours)

Figures 3.34-3.35 The effect of peptides on nikkomycin activity

Cells from the growth curves described in Figure 3.33 were observed by phase-contrast microscopy. (×1300)

3.34 Cells grown for 14 hours in the presence of nikkomycin and gly tyr.

3.35 Cells grown for 14 hours in the presence of nikkomycin and ala_2



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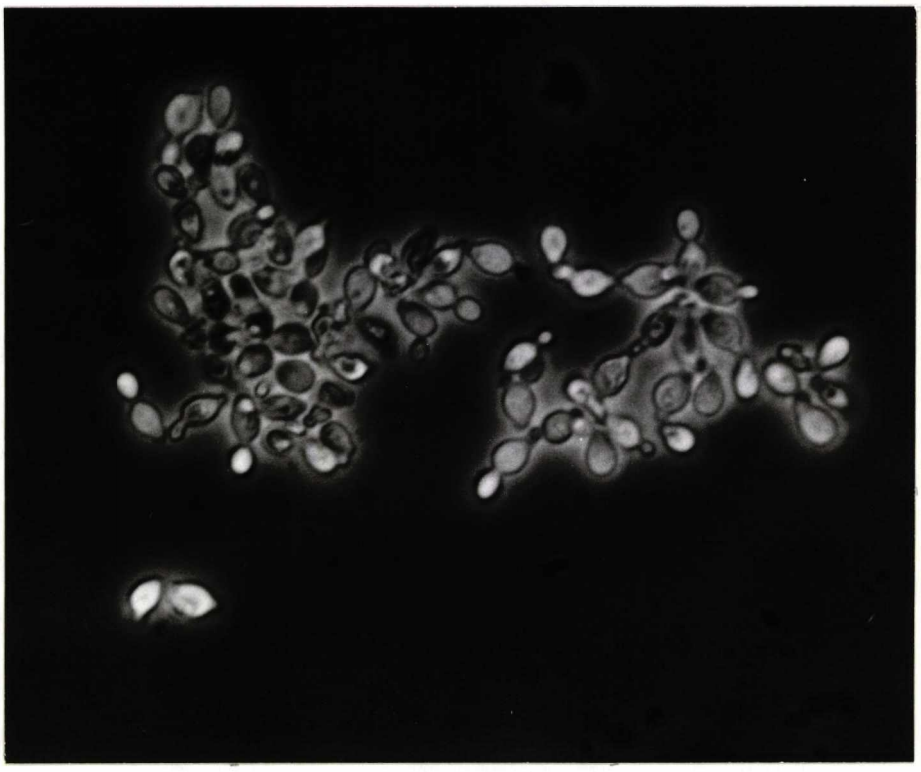


35

Figure 3.36 The effect of leu_2 on the activity of nikkomycin

C.albicans 124 (wild type) cells were grown overnight in YNB and inoculated at 10^5/ml into YNB (●), YNB containing 50 μg ml^{-1} nikkomycin (■) or YNB containing 50 μg ml^{-1} nikkomycin and 10 mM leu_2 (○). Growth was followed by turbidity measurements using an EEL colorimeter.

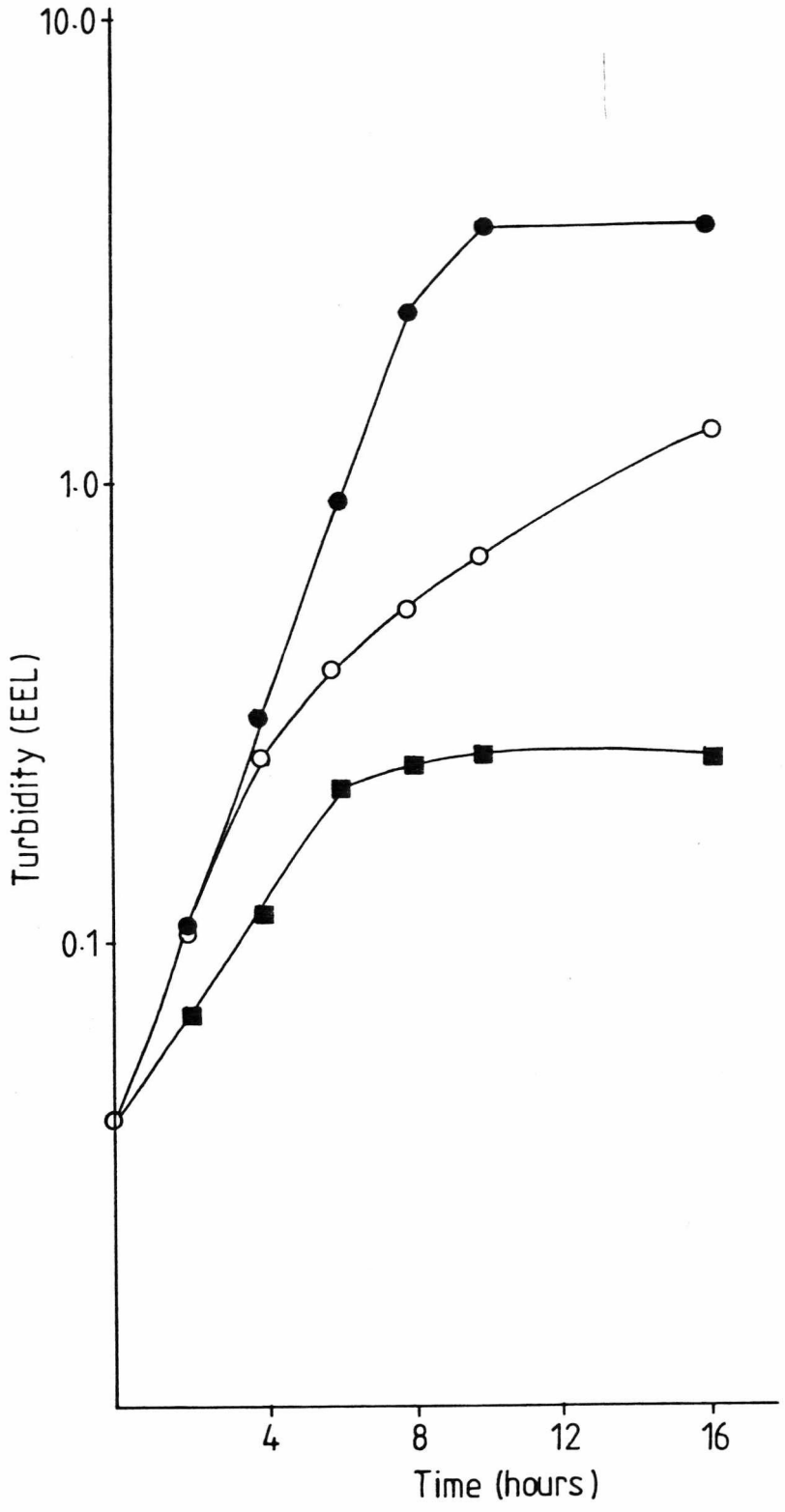

10·0
1·0
0·1
Turbidity (EEL)
4
8
12
16
Time (hours)

Figure 3.37 The effect of oligopeptides onthe activity of nikkomycin

C.albicans 124 (wild type) cells were grown overnight in YNB and inoculated at 10^5/ml into YNB (○) , YNB containing 50 μg ml^{-1} nikkomycin (■) , YNB containing 50 μg ml^{-1} nikkomycin and 10 mM ala_3 (●) , or YNB containing 50 μg ml^{-1} nikkomycin and 10 mM ala_4 (□) . Growth was followed by turbidity measurements using an EEL colorimeter.

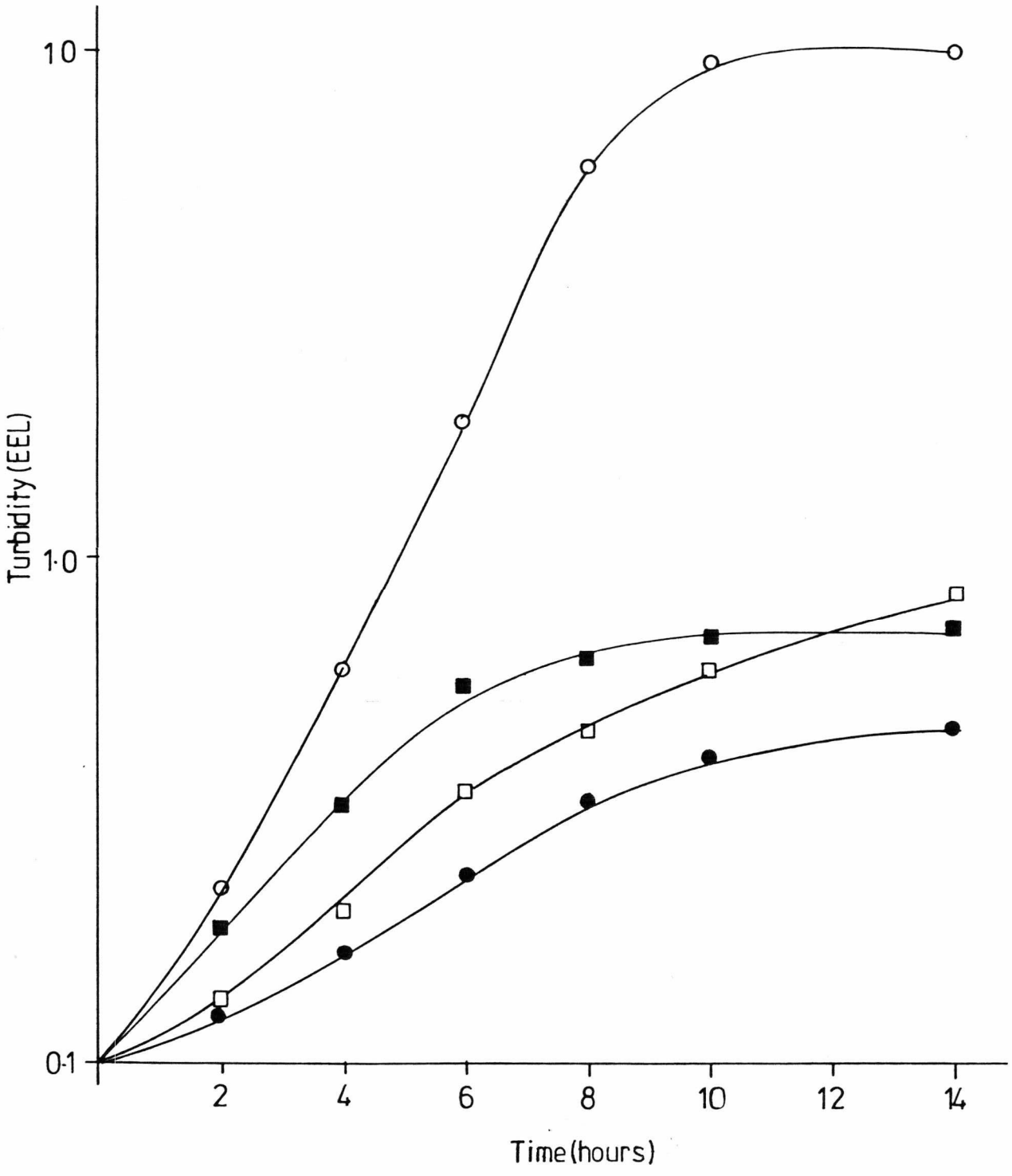

10
1·0
0·1
Turbidity (EEL)
2
4
6
8
10
12
14
Time (hours)

Table 3.1 The effect of peptides on nikkomycin activity

The results form Figures 3.33, 3.36 and 3.37 are tabulated in addition to the effects of certain other dipeptides.

Peptide (10 mM)	Antagonism of Nikkomycin Action
ala_2	+
gly tyr	-
D-leu gly	-
L-leu gly	+
gly_2	-
ala_3	-
ala_4	-
leu_2	+

showed the culture to be composed of blastospores (FIgure 3.35) comparable with those produced in the absence of the drug (Figure 3.4).

The inclusion of 10 mM gly tyr did not show an antagonistic effect. In this case the increase in turbidity was equivalent to that of a culture grown in the presence of nikkomycin alone (Figure 3.33). The aberrant morphology of the cells (Figure 3.34) was similar to the effect of nikkomycin on blastospores which has been previously described (Figures 3.7-3.10).

From the peptides assayed it was apparent that the oligopetpides (ala_3 and ala_4) did not antagonize nikkomycin action at 10 mM (Table 3.1 and Figure 3.37). Of the dipeptides ala_2 and leu gly showed antagonism whereas gly tyr , D-leu gly and gly_2 did not. Leu_2 showed some competitive action although the effect of the drug was not completely reversed (Figure 3.36).

This would suggest that nikkomycin was entering *C.albicans* via the peptide permease, with antagonism being shown by co-transported peptides. To confirm that this was competition at the level of transport it was necessary to show the effect of nikkomycin on $[U^{14}C]$ ala ala transport into blastospores.

Competitive effect of peptide-nucleoside drugs for peptide uptake

Competition for uptake between [U^{14}C] ala ala and the peptide nucleoside antibiotics nikkomycin and polyoxin was studied. It was found that nikkomycin competed with ala_2 for transport into YNBP grown wild type cells. The inhibition constant (K_i) was 4.85 µg ml^{-1} as determined by the method of Dixon (1953) (Figure 3.38).

A crude sample of polyoxin (10% polyoxins A,B,K and L) had only low activity against *C.albicans* with an MIC in excess of 1.6 mg ml^{-1}. The affinity of polyoxin for the peptide transport system was assayed by competition with [U^{14}C] ala ala . This mixture was also a competitive inhibitor but with a K_i of 195 µg ml^{-1} (Figure 3.40).

The effect of various analogues was studied to establish whether this competitive effect was due to the peptide moiety of the drugs.

Competitive effect of analogues of nikkomycin for peptide uptake

Nikkomycins and polyoxins are analogues of UDP-N-acetyl-D-glucosamine. The interaction between this molecule , which is devoid of a peptide group, and the peptide transport system was determined. It was found that at equimolar concentrations no competition occured between UDP-N-acetyl-D-glucosamine and ala_2 . Furthermore, no competitive effect was exerted by uracil or 6-hydroxynicotinic acid.

Figure 3.38 The effect of nikkomycin on $[U^{14}C]$ ala ala incorporation by *C.albicans* 124 (wild type)

Cells were grown in YNBP , harvested, washed and resuspended in PCG . The initial rates of incorporation of $[U^{14}C]$ ala ala were determined in the presence of various concentrations of nikkomycin. Cells were diluted to 1.0 mg dry wt/ml , and $[U^{14}C]$-ala ala added to a concentration of either 0.1 mM or 0.2 mM . The suspension was incubated at $37^{o}C$ with shaking. Samples (1 ml) were removed at timed intervals and the radioactivity incorporated into cells was determined. Initial rates of incorporation were calculated in triplicate. Mean values are plotted according to the method of Dixon (1953).

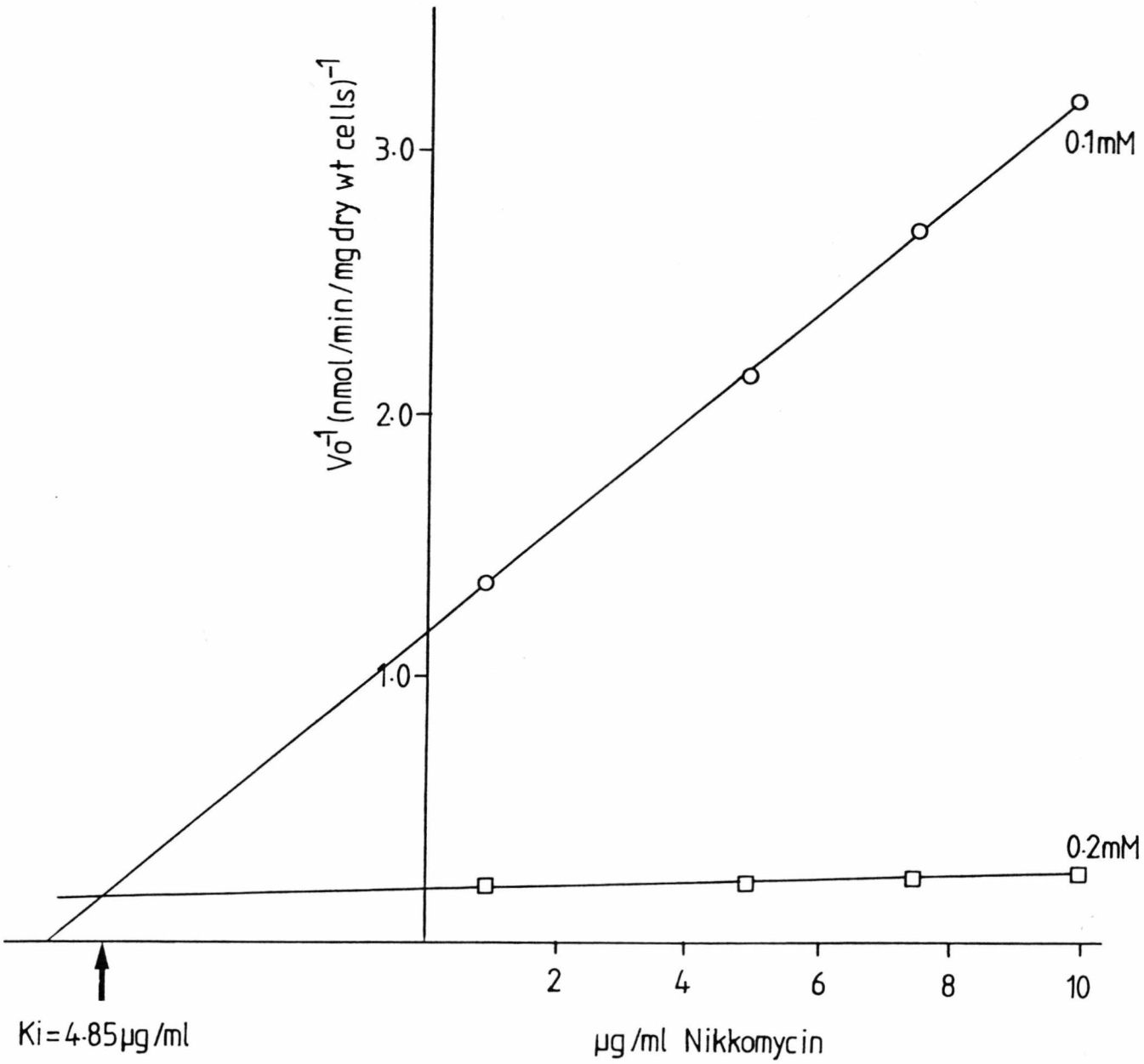

V_o^{-1} (nmol/min/mg dry wt cells)$^{-1}$
3·0
2·0
1·0
0·1mM
0·2mM
2
4
6
8
10
Ki = 4·85 µg/ml
µg/ml Nikkomycin

Figure 3.39 The effect of nikkomycin on [U^{14}C] ala ala incorporation by *C.albicans* 124 (NIK5)

The method employed was identical to that described in Figure 3.38.

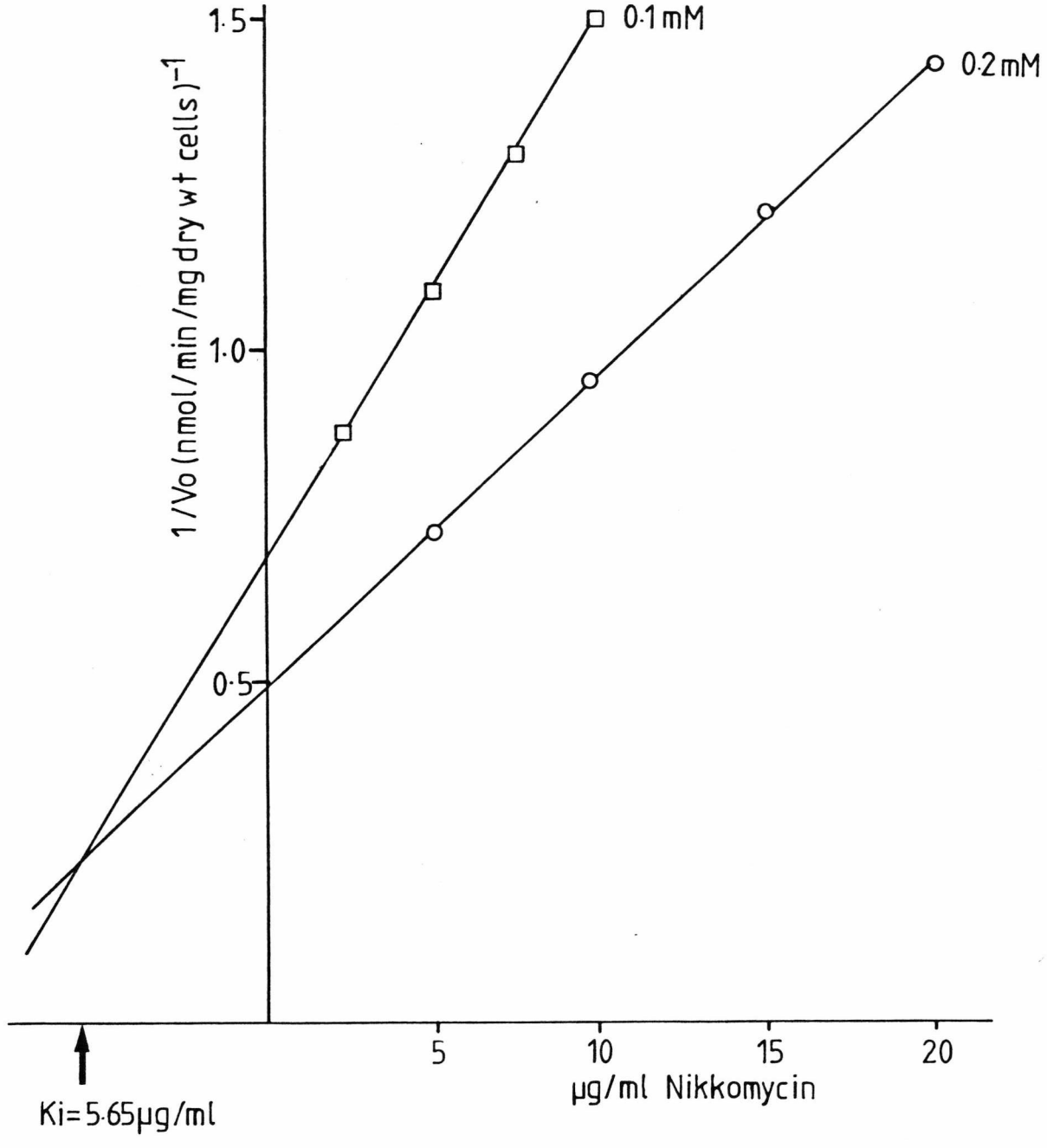
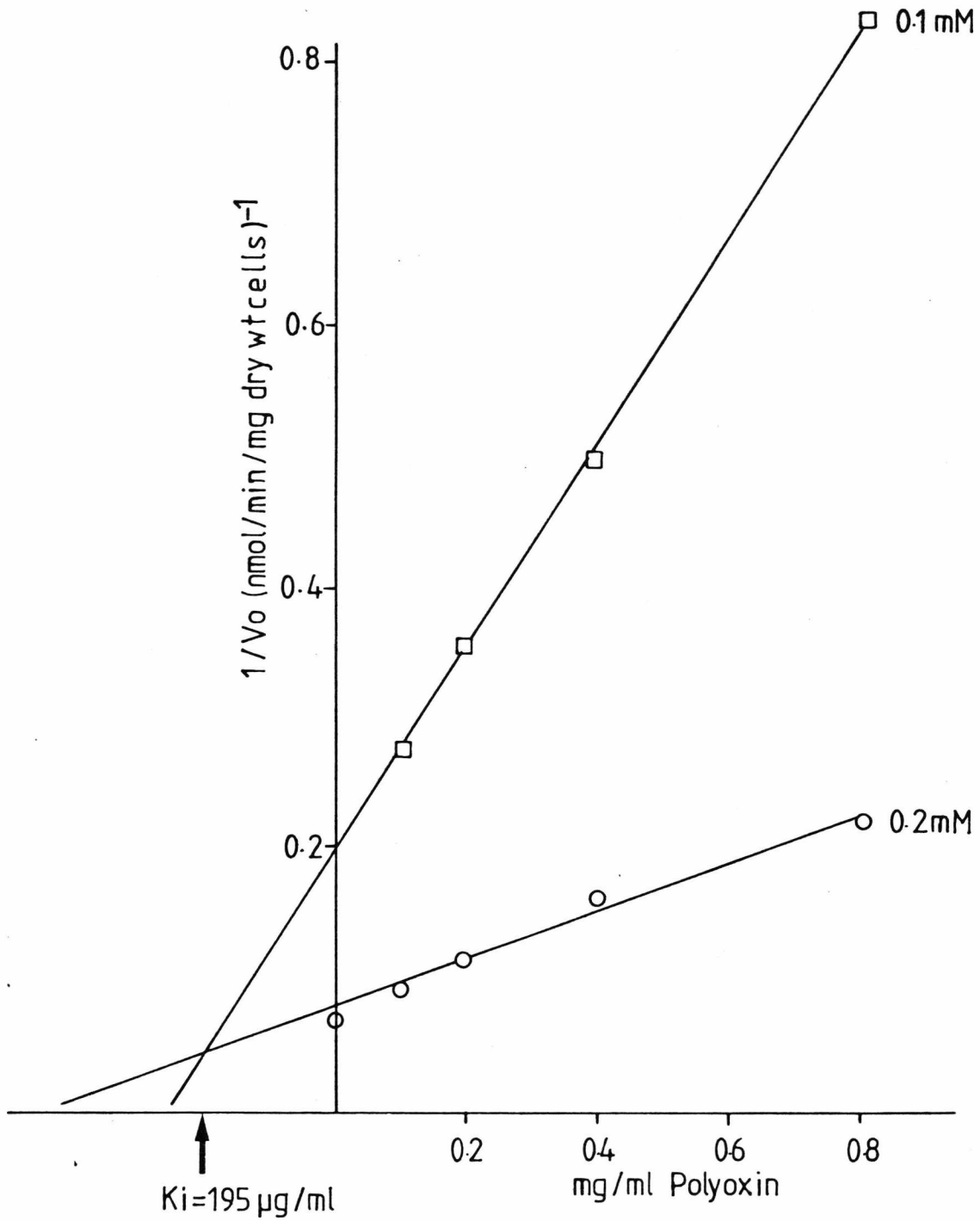

1·5
1·0
0·5
1/Vo (nmol/min/mg dry wt cells)$^{-1}$
0·1 mM
0·2 mM
5
10
15
20
µg/ml Nikkomycin
Ki= 5·65µg/ml

Figure 3.40 The effect of polyoxin (crude) on [U^{14}C] ala ala incorporated by *C.albicans* 124 (wild type)

The method employed was that described in Figure 3.38 except that polyoxin (crude) was substituted for nikkomycin.


0·1 mM
0·8
0·6
0·4
0·2
1/Vo (nmol/min/mg dry wt cells)⁻¹
0·2mM
0·2
0·4
0·6
0·8
mg/ml Polyoxin
Ki=195 µg/ml

The isolation of a peptide-transport deficient mutant

The peptide-nucleoside drug, nikkomycin, entered the cell via a system which also transported peptides. Therefore the possibility existed of creating peptide transport deficient mutants of *C.albicans* by selecting for resistance to nikkomycin.

Ultraviolet irradiation produced six mutants resistant to 100 $\mu g\ ml^{-1}$ nikkomycin. These were designated NIK1 , NIK2 , NIK3 , NIK4 , NIK5 and NIK6 according to their phenotype. Each was assayed for its ability to transport ala_2 . This peptide had been shown to utilize the same transport system as nikkomycin and thus provided a method of screening for transport deficient mutants. Comparison of the ability of mutants to transport ala_2 with that of the wild type (Table 3.2), showed that only one mutant, NIK5 , exhibited such a defect. NIK5 was used in all further experiments. The point of mutation in the other variants was not determined. However, it is possible that some possessed an altered chitinsynthase.

Plate tests showed that NIK5 was totally resistant to nikkomycin in contrast to the wild type, which showed a considerable zone of clearing (Figure 3.41). It is interesting to note the large number of spontaneously resistant colonies which arose from the wild type and appeared within the cleared zone.

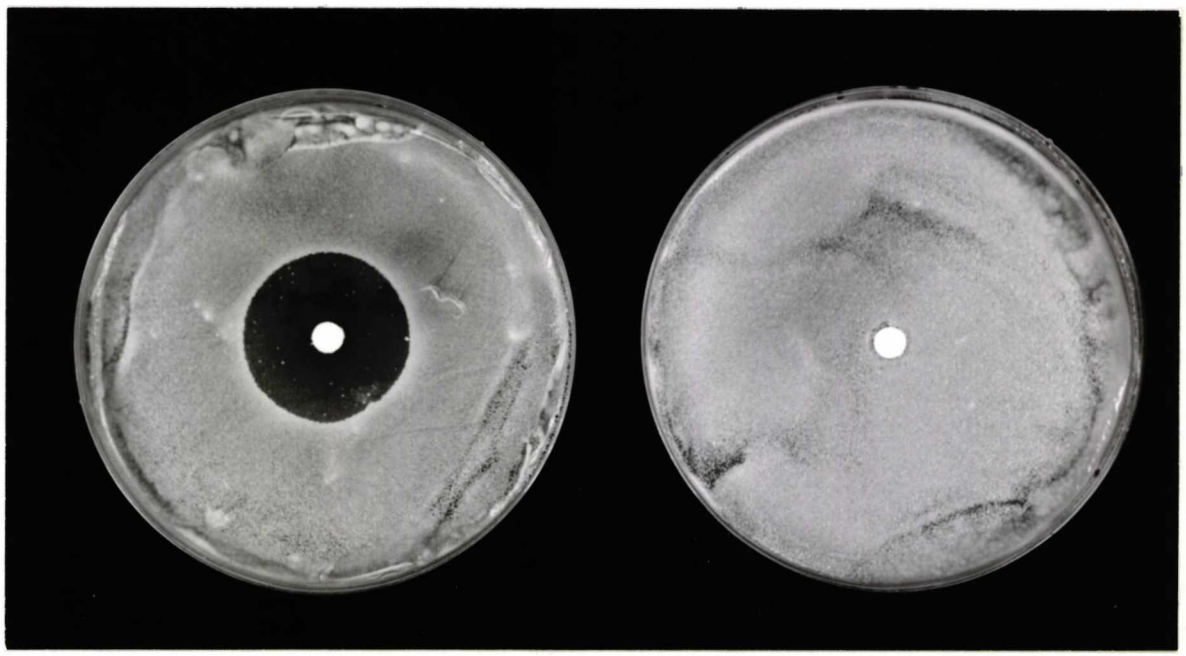
Table 3.2 The ability of the nikkomycin-resistant mutants to transport ala_2

Cells were grown to mid-logarithmic phase in YNBP , harvested, washed and resuspended in PCG . Cells were diluted and ala_2 was added to a concentration of 0.2 mM (1.0 mg dry wt cells/ml). The initial rates of peptide uptake were determined by sampling the suspension at timed intervals during an incubation at $37^{o}C$. The extracellular medium was assayed for ala_2 concentration by the fluorescamine technqiue. Initial rates were determined in triplicate and are expressed as a percentage of the rate obtained with the wild type.

Isolate	ala_2 uptake % of wild type rate
Wild type 124	100
NIK1	100
NIK2	100
NIK3	99
NIK4	96
NIK5	45
NIK6	100

Figure 3.41 The zones of growth inhibition obtained with nikkomycin against the wild type and NIK5

Cells were grown to mid-logarithmic phase in YNB , diluted to 10^6/ml and 500 µl of this suspension was spread over the surface of an MX plate (2% /w/v) agar). A 6 mm diameter disc of chromatography paper containing 50 µg nikkomycin was placed in the centre of each plate. Plates were incubated at $37^{o}C$ for 48 hours and then photographed.



WILD TYPE NIK 5

Figure 3.42 A comparison of the effect of nikkomycin on the growth of the wild type and NIK5

Cells were grown overnight in YNB and inoculated at 10^5 cells/ml into YNB with or without nikkomycin as follows:

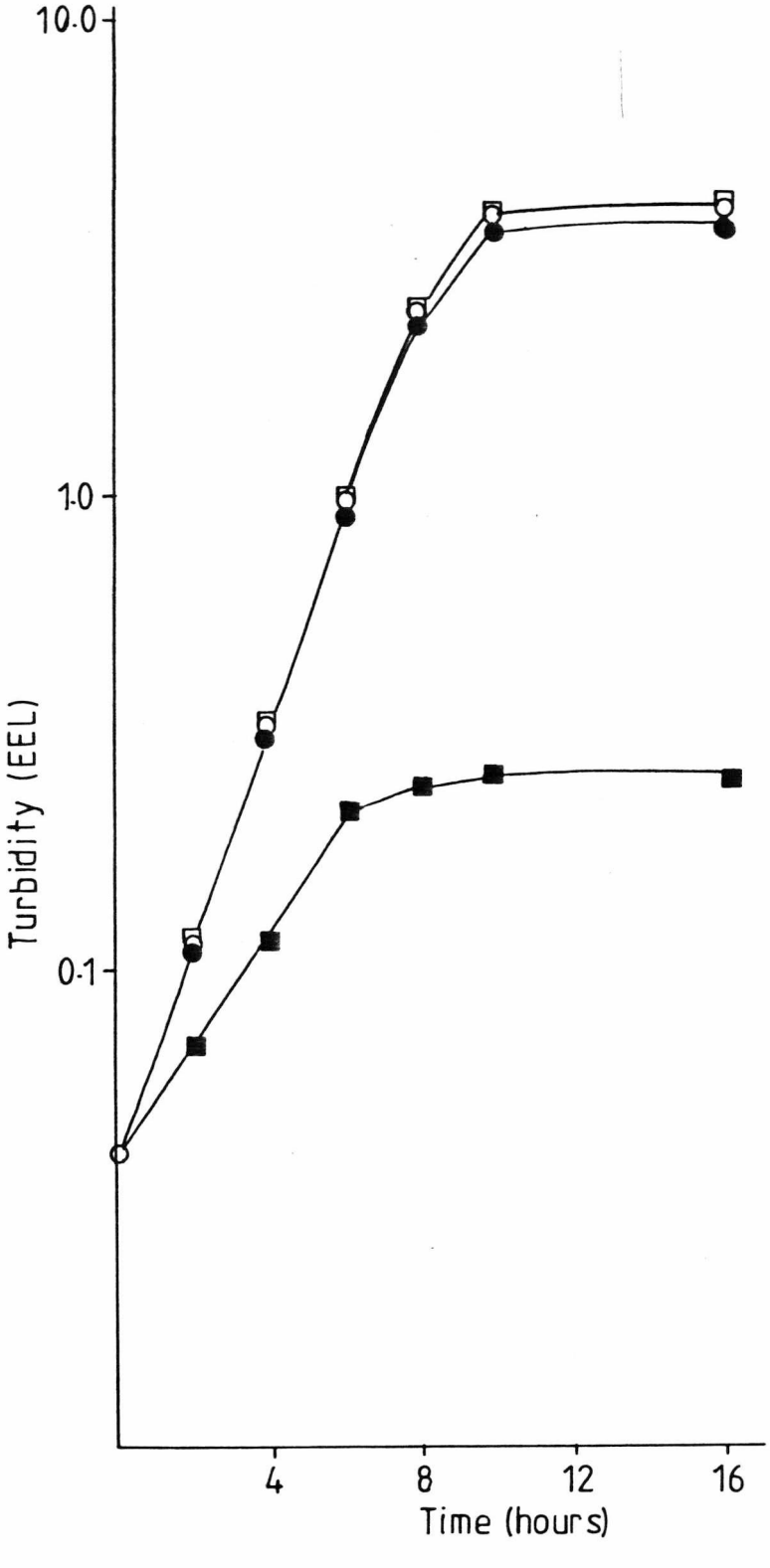
Wild type in YNB (●)

Wild type in YNB containing 50 μg ml^{-1} nikkomycin (■)

NIK5 in YNB (○)

NIK5 in YNB containing 50 μg ml^{-1} nikkomycin (□)

Growth at 30°C was followed turbidometrically using an EEL colorimeter.


10·0
1·0
0·1
Turbidity (EEL)
4
8
12
16
Time (hours)

Sensitivity of NIK5 chitin synthase to nikkomycin

To ensure that NIK5 did not possess an altered chitin synthase (in addition to a defect in transport) the sensitivity of the isolated enzyme to nikkomycin was determined. Nikkomycin was found to competitively inhibit the enzyme with respect to its substrate UDP-N-acetyl-D-glucosame (Figure 3.3, K_i=0.17 μM) . This value was comparable to that obtained for the wild type enzyme (K_i=0.16 μM) and so no alteration in chitin synthase activity was evident.

Effect of nikkomycin on [$U^{14}C$] ala ala incorporation

Ala_2 was transported into YNBP- grown NIK5 cells at a reduced rate when compared with the wild type (Table 4.2). Competition between nikkomycin and ala_2 was again apparent (Figure 3.39). The K_i of 5.65 μg ml^{-1} was similar to that of the wild type (K_i=4.85 μg ml^{-1}). This competitive effect will be discussed in the following chapter on the peptide transport system.

Growth of NIK5 in batch culture

The growth of a batch culture of NIK5 in YNB medium was found to be identical to that of the wild type. In the presence of nikkomycin (50 μg ml^{-1}) the wild type failed to grow whereas NIK5 grew at a rate identical with that found in the absence of the drug (Figure 3.42). Similar results were found for growth on MX .

When grown with peptone as the sole nitrogen source the generation time was found to be identical to that of the wild type (Figure 3.43). However, a lag period of about 4 hours occured when NIK5 was transferred

Figure 3.43 Growth of the wild type and NIK5 on peptone-based medium

Wild type (○) and NIK5 (□) cells were grown overnight in YNB and inoculated at 10^5 cells/ml into YNBP. Growth was followed at $30^{\circ}C$ by turbidity measurements using an EEL colorimeter.

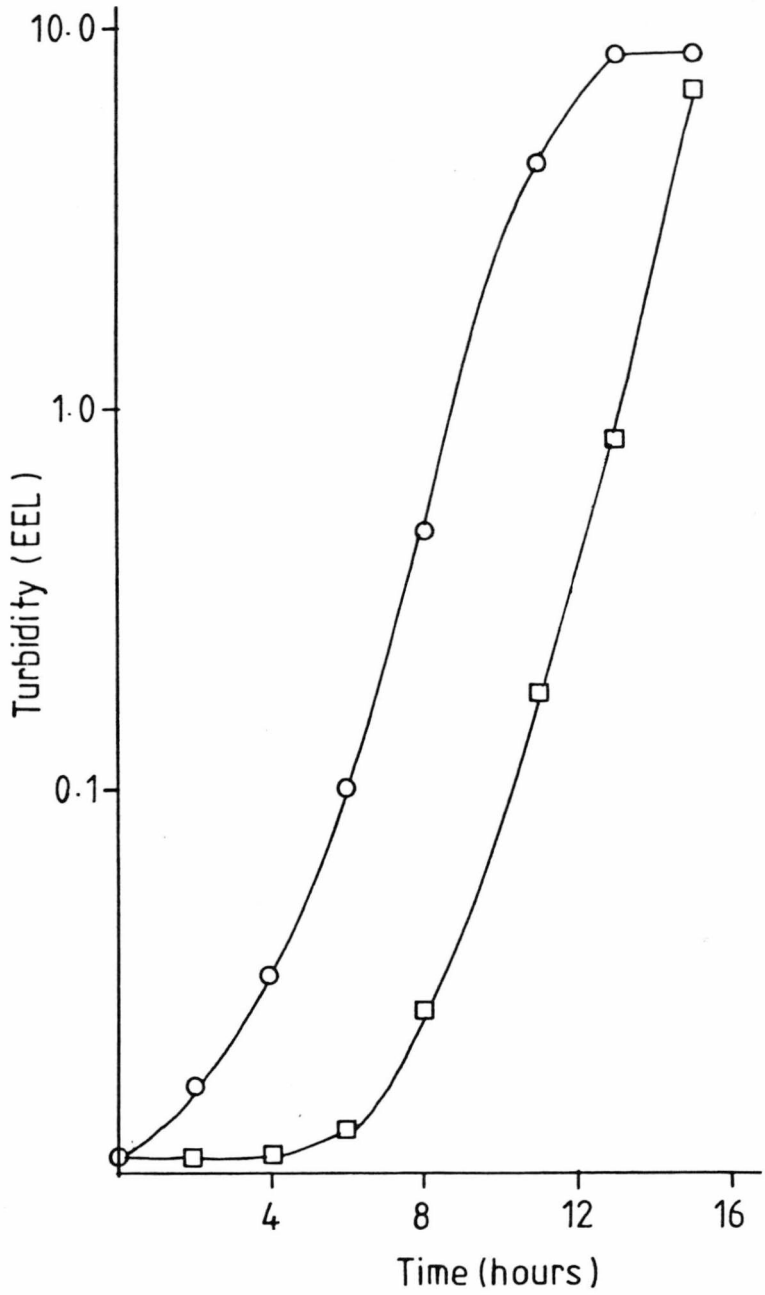

10·0
1·0
0·1
Turbidity (EEL)
4
8
12
16
Time (hours)

Figure 3.44-3.45 The distribution of primulin-staining regions in *C.albicans* 124 (NIK5)

Cells were grown, harvested and stained as described in Figures 3.11-3.14.
(×1400)

3.44 Cells after 7 hours growth in YNB. Brilliant fluorescence of septa is visible at the intersections of the cells.

3.45 Cells after 24 hours growth in YNB. Bud scars (arrowed) are visible at the poles of the cells.

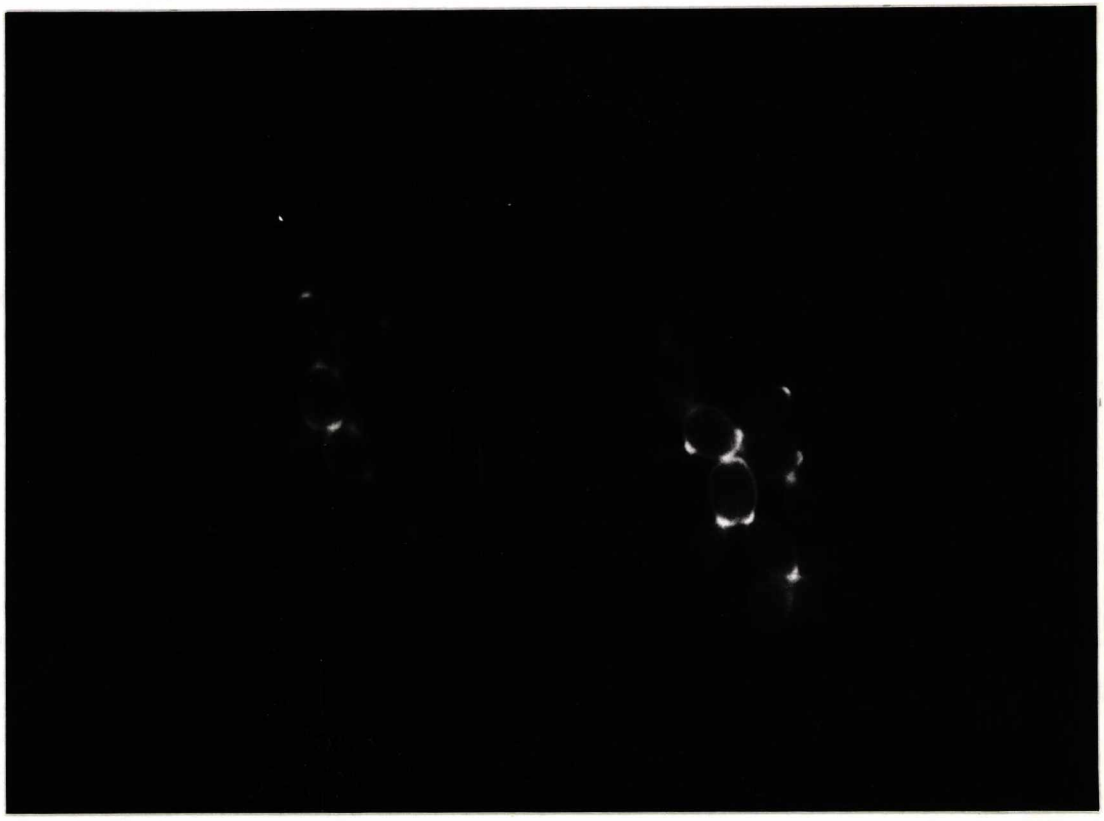


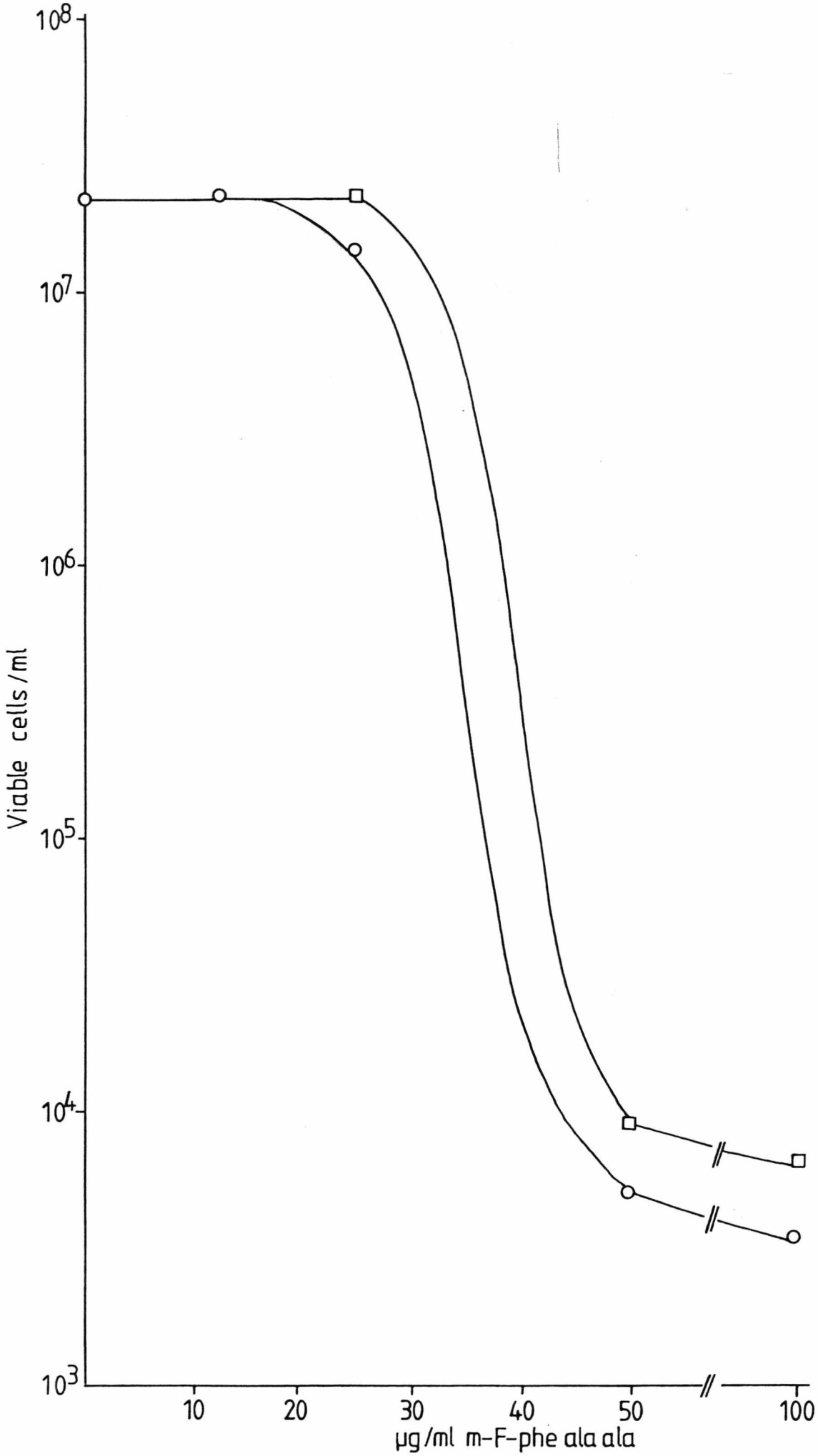
Table 3.3 The effect of various toxic agents on the growth of *C.albicans* 124 wild type and NIK5

The method used was identical to that described in Figure 3.41. Drugs were dried into the paper disc from solution and solvent controls were performed in each case. The quantity of drug used is indicated in parentheses.

Drug	Zone of Inhibition (Diameter, mm) Wild Type	NIK5
Acriflavin (0.5 mg)	15.0	15.0
Ethidium bromide (0.5 mg)	25.0	25.0
5-fluorocytosine (0.5 mg)	37.0	37.0
Actinomycin D (0.5 mg)	10.5	10.5
Cycloheximide (0.5 mg)	0.0	0.0
m-F-phenylalanine (0.5 mg)	0.0	0.0
Nikkomycin (50 µg)	30.0	0.0
Polyoxin (crude) (0.5 mg)	8.5	0.0
Bacilysin (100 µg)	27.0	0.0
5-fluorouracil (50 µg)	10.5	10.5
Miconazole (50 µg)	13.5	13.5
Nystatin (20 µg)	24.0	24.0
m-F-phe ala ala (30 µg)	20.0	11.0

Figure 3.46 The effect of m-F-phe ala ala on the growth of the wild type (○) and NIK5 (□)

Cells were grown overnight in YNB and inoculated at 10^3/ml into MX containing serial dilutions of m-F-phe ala ala. Tubes were incubated at 37°C for 48 hours. Viable cell numbers were determined by growth on MX plates.


10^8
10^7
10^6
10^5
10^4
10^3
Viable cells/ml
10
20
30
40
50
100
µg/ml m-F-phe ala ala

from YNB or MX to YNBP. No equivalent lag period was observed when the wild type was similarly transfered.

The morphology of NIK5

No morphological differences between NIK5 cells grown with or without 50 $\mu g\ ml^{-1}$ nikkomycin were observed under phase microscopy. NIK5 blastospores (Figure 3.5) were also morphologically identical with the wild type. Pseudohyphae similar to those of the wild type were occasionally formed in batch culture (Figure 3.6).

The distribution of the primulin staining regions was identical to that found in the wild type (Figure 3.44 and 3.45).

Cross resistance with other drugs

The wild type and NIK5 showed equivalent sensitivities to the majority of toxic agents tested (Table 3.3). Cycloheximide and m-F-phenylalanine were found to be inactive against this strain of *C.albicans*. Of the drugs found to be active only the dipeptide bacilysin showed total cross resistance with nikkomycin. The crude mixture of polyoxins showed only low activity against the wild type and none against NIK5 .

The toxic tripeptide m-F-phe ala ala was active against both the wild type and NIK5 . However, the latter showed a lower sensitivity in terms of the effect of this drug on growth (Figure 3.46).

No alteration was apparent in the chitin synthase of NIK5 or in the sensitivity of the organism to a range of inhibitors. The only effect noted was in its reduced sensitivity to peptide drugs which enter the cell through the peptide transport system.

Thus NIK5 can be considered to have an altered capacity for peptide transport. This will be considered in the following section of the results.

Discussion

The nikkomycins have recently been isolated from culture filtrate of *Streptomyces tendae* (Dahn, *et al.*, 1976). These peptide-nucleoside antibiotics have been shown to prevent the growth of many fungal species by inhibiting the enzyme chitin synthase, thereby blocking the formation of the cell wall polysaccharidechitin. The effects of nikkomycins on fungal morphology have not been previously reported. However, in both structure and activity the nikkomycins resemble the polyoxins (Zahner, *et al.*, 1982; Isono and Suzuki, 1979). Two fungal species have been used to study the mode of action of polyoxins A,B and D in detail: *Alternaria kikuchiana* and *Pellicularia sasakii*. (Mitani and Inoue, 1968; Hori, *et al.*, 1974,1976 and 1977). The morphological effects of the polyoxins have been reported for several fungal species including *Trichoderma viride* (Benitez *et al.*, 1976) and *Mucor rouxii* (Bartnicki-Garcia and Lippman, 1972). *S.cerevisiae* has also been studied although the sensitivity of this yeast to polyoxin D is low (MIC $\geq$ 1 mg ml^{-1} Bowers *et al.*, 1974).

Until recently the polyoxins were considered inactive against *Candida albicans*. Chiew, *et al.*, (1980) found polyoxin D to be an inhibitor of *C.albicans* cell-free chitin synthase (K_i=1.3 μM). However there was no activity against the intact organism. There has since been a report showing that polyoxin B is highly active against *C.albicans* on certain media (MIC varies between 0.1 μg ml^{-1} and >1000 μg ml^{-1}. Mehta *et al.*, 1982). Details of this work are not currently available and therefore its significance has yet to be established.

The nikkomycin used in the present study was a mixture of mainly nikkomycins X and Z (X/Z mixture. Zahner, personal communication). and was found to be active against *C.albicans* (MIC on MX=25 μg ml^{-1}). Dahn *et al.* (1976) found an equivalent X/Z mixture to be active against a broad range of fungi including *Basidiobulus microsporus* (MIC=10 μg ml^{-1}) , *Mucor hiemalis* (+) (MIC=1 μg ml^{-1}) , and *Ustilago maydis* (MIC=50 μg ml^{-1}). *S.cerevisiae* was less sensitive to nikkomycin (MIC=100 μg ml^{-1}) which reflects the low sensitivity of this organism to polyoxin D (Bowers, *et al.*, 1974).

A report of the activities of nikkomycins X and Z has been published (Muller, *et al.*, 1981) showing that these drugs are potent inhibitors of a cell-free chitin synthase preparation isolated from *M.hiemalis* (nikkomycin X, K_i=0.5 μM; nikkomycin Z, K_i=3.5 μM) and are of comparable activity to certain polyoxins (polyoxin A, K_i=0.6 μM). The remaining nikkomycins have not been studied. *Pyricularia oryzae* chitin synthase gave K_i values of 11 μM for polyoxin A , 5.6 μM for polyoxin B , 11 μM for polyoxin K , and 2.7 mM for polyoxin C (Hori, *et al.*, 1971). Polyoxin C , which shows only low activity, is the only polyoxin lacking a peptide

side-chain, suggesting that this moiety is needed for the inhibitory action against chitin synthase. Ohta *et al.* (1970), reported that polyoxin C had no antifungal activity: This could be due not only to the low activity against the target enzyme but also poor penetration of the plasma membrane, as the polyoxins are known to enter the cell via the peptide permease (Hori, *et al.*, 1977; Mitani and Inoue, 1968). There are no reports concerning the activities of nikkomycin C or C_X which also lack the peptide moiety (Zahner, *et al.*, 1982).

In the present study the X/Z mixture was found to competitively inhibit incorporation of UDP-N-acetyl-D-glucosamine into chitin by a cell-free chitin synthase preparation isolated from both the wild type (K_i=0.16 µM) and the nikkomycin resistant mutant (NIK5 , K_i=0.17 µM). Thus resistance to the drug did not arise through an obvious alteration of chitin synthase, as had been observed in a polyoxin D resistant mutant of *Schizophyllum commune* (wild type K_i=16 µM; resistant mutant K_i= 100 µM . MacGruder, 1979). The inhibition constant for nikkomycin and *C.albicans* chitin synthase was slightly lower than that previously described for pure nikkomycin X (*M.heimalis* K_i=0.5 µM . Muller *et al.*, 1981). However, there may be a difference in the sensitivities of chitin synthase isolated from different sources. Such a difference is analogous to the variation in the activity of polyoxin D against chitin synthase from *Neurospora crassa* (K_i=1.4 µM . Endo, *et al.*, 1970) and *Pyricularia oryzae* (K_i=12.0 µM. Hori, *et al.*, 1971).

The effects of a chitin synthase inhibitor on *C.albicans* have not been reported and therefore a study of the cellular morphology was undertaken. However, before the effects of the drug can be discussed the normal developmental patterns of *C.albicans* in relation to other fungi must be considered.

Chitin has been shown to be restricted primarily to the septa and bud scars in *C.albicans*, although further depositions are found within the inner layers of the wall in association with β(1,6) glucans, where they may contribute to wall rigidity (Tronchin, *et al.*, 1981). A similar distribution pattern has been proposed for *S.cerevisiae* (Cabib and Bowers, 1971). Streiblova and Beran (1963) have shown that primulin binds to the bud scars of a variety of yeast-like fungi including *S.cerevisiae*. Restriction of the brilliant fluorescence to the septum between mother and daughter cells, mother and germ tube and to the mother cell bud scars in *C.albicans* suggests that primulin is also binding to the exposed chitinous regions in this fungus.

The developmental pattern in budding yeasts (*S.cerevisiae*) and filamentous fungi (such as *N.crassa*) have been studied in detail. These will be compared with *C.albicans* on the blastospore, pseudohyphal and hyphal forms induced by variation of media and temperature: Blastospores were produced by *C.albicans* on YNB at 30°C; AP+ at 40°C induced largely pseudohyphal cells, induction of true hyphae occured only on 20% serum at 37°C .

An early study of budding in *S.cerevisiae* demonstrated that very little of the maternal cell wall was found in the newly synthesized daughter cell (Chung, *et al.*, 1965). Thus bud formation requires the synthesis of the cell wall polysaccharides glucan and mannan. However the periodic synthesis of these polymers during the cell cycle is unclear. The chitin of the cell wall has been found to be concentrated in the bud scars (Cabib and Bowers, 1971) with the total cell content increasing once per division cycle (Cabib and Farkas, 1971) beginning at a point shortly after bud emergence (Cabib and Bowers, 1975). Initiation of chitin deposition occurs from an annular structure, the scar ring, composed of glucan (Holan, *et al.*, 1981) which cannot be found in cells at points in the cell division cycle preceding bud emergence (Vrasanka, *et al.*, 1979). Formation of a primary septum consisting of chitin (Holan, *et al.*, 1981) commences from the scar ring by centripetal growth. Secondary septa are then formed on opposing faces of the primary septum. Electron microscopy has shown the appearance of the secondary septa to be equivalent to that of the cell wall, implying that they are composed mainly of glucan and mannan (Cabib, *et al.*, 1974). Asymmetric separation of the two cells then follows with the primary septum remaining embedded in the mother cell wall. This accounts for the brilliant fluorescence of the bud scar in the presence of optical brighteners (Streiblova and Beran, 1963).

An identical pattern of budding has been demonstrated for *C.albicans* (Shannan and Rothman, 1971; the present study). Bud outgrowth is followed by centripetal primary septum formation and ultimately secondary septum formation. The major difference between *S.cerevisiae* and *C.albicans*

is the presence of a central 25 nm pore in the septum of the latter. The function of the micropore has not been defined and yet it appears to be present in both the blastospore and hyphal forms of growth (Gow, *et al.*, 1980; Odds, personal communication). Budding was highly polar in *C.albicans*. In aged cells the bud scars were closely packed, which might be a limiting factor in the number of division cycles through which a mother cell can pass.

C.albicans is able to grow by both budding and filamentous cycles. However, there are a variety of intermediate cell-types which are termed pseudohyphae and which are differentiated from true hyphae by the presence of constrictions at the point of septum formation (Odds, 1979). In the present study it was found that the modification of Eagle's medium (AP+), which had been described as a defined medium for the induction of hyphae (Schwartz and Larsh, 1980), induced pseudohyphae of *C.albicans* strains 124 and STM-1. Mother cells produced small outgrowths (germ tubes) which were differentiated from buds by the site of initial septum formation. This was laid down at a variable point within the neck of the germ tube but seldom at the intersection of the two cells (as in budding). Mitchell and Soll (1979) also found a variation in the site of initial septum formation. Although the outgrowth of bud and germ tube occured synchronously, initial septum formation in the budding form took place 30 mins earlier than in the mycelial form. The mechanism of site determination in septum formation remains to be elucidated. However, it could give information concerning phenotypic regulation. The germ tubes produced on AP+ elongated and septa were laid down at regular intervals. Buds

were formed adjacent to septa. Separation of the pseudohyphal compartments frequently occured at the septum resulting in elongate mother cells which replicated by budding.

True hyphal growth was only observed when blastospores of *C.albicans* 124 were incoulated into serum at 37°C . Hyphae were initiated as germ tubes which elongated and produced septa at regular intervals. No constrictions were observed between hyphal compartments and production of buds or secondary hyphae rarely occured. Frequent secondary hyphae have been reported (Gow and Gooday, 1982 a and b), however, these were induced on solidified serum in contrast to the present study. It would therefore appear that, as yet, no defined medium has been described which can induce the stable hyphal form of many *C.albicans* strains.

The structure of the cell wall of such filamentous fungi as *N.crassa* has been studied in depth and appears to be similar to that of *C.albicans:* An inner chitinous region surrounded by outer regions containing glucan and protein (Hunsley and Burnett, 1970). The hyphal apex (extension zone) is composed mainly of chitin covered by a layer of protein (Hunsley and Kay 1976) . The apex wall is only α 50 nm thick. However, wall thickness increases to 125 nm at 250 µm from the tip with thicker walls in older hyphae (Trinci and Collinge, 1975). This increase in thickness arises from the addition of further wall components after extension of a region of the hypha has ceased. In certain fungi such as *Botrytis cinerea* the extension zone stains with the optical brightener calcofluor (Gull and Trinci, 1974). However, such an effect was not observed in *C.albicans* hyphae in the present study, indicating that the chitinous

layer is not exposed in this region. Nuclear division within hyphae is followed by septation (*C.albicans:* Gow and Gooday, 1982a). The septa of the filamentous fungi resemble those of *S.cerevisiae* in that they consist of a central chitinous plaque with R-glucan / chitin secondary septa (*N.crassa*: Hunsley and Gooday, 1974; Mahadevan and Tatum, 1967). Pores are generally present. *N.crassa* has a simple pore of 350-500 nm diameter, however the complexity varies between species. The *N.crassa* septal pore is far larger than that of *C.albicans* (25 nm) and will therefore allow cytoplasmic streaming with the passage of mitochondria and nuclei (Hunsley and Gooday, 1974).

C.albicans is able to grow as either a budding yeast or as a true filamentous fungus. It was therefore possible to study the effects of nikkomycin on the developmental cycles of the organism and to compare these with the morphology of the yeast *S.cerevisiae* and the filamentous fungi *Mucor rouxii* and *Trichoderma viride* in the presence of polyoxin D .

In the presence of nikkomycin both blastospores and pseudohyphae produced buds which enlarged to form structures resembling protoplasts. Loss of wall rigidity was also noted in *M.rouxii* (Bartnicki-Garcia and Lippman, 1972); *T.viride* (Benitez *et al.*, 1976); *Agaricus bisporus* (Wood and Hammond, 1977) and *Coprinus cinereus* (Gooday *et al.*, 1976) when treated with polyoxin D. The extension zones of both *M.rouxii* and *T.viride* mycelia were observed to swell producing apical bulges which lysed unless the medium was osmotically stabilized. Bursting tips were not observed in *C.albicans* pseudohyphae suggesting that pseudohyphal growth resembles budding, with growth over the entire cell surface, rather than true hyphal elongation. Electron microscopy revealed that the wall of the enlarged daughter cell was thinner than that normally observed.

This is in contrast to *T.viride* in which the wall of the bulge was of a similar thickness to that of the normal wall (Benitez, *et al.*, 1976). Chitin has been shown to be deposited in the inner wall of *C.albicans* (Tronchin, *et al.*, 1981). Thus inhibition of chitin synthase results in a weakened cell wall. Cells treated with nikkomycin contained some wall constituents whereas none were present in true protoplasts of this organism (Torres-Bauza and Riggsby, 1980). The thinning of the cell wall suggests that chitin may be involved in the maintenance of wall structure in addition to rigidity. Rigidity may be the major function of chitin in *T.viride*. Hence thinning of the wall would not be observed on treatment with polyoxin D, since wall structure is maintained by other polysaccarides.

Enlarged cells of *C.albicans* did not immediately lyse and, in many cases, further growth results in highly distorted forms. Chitinous regions, as observed by primulin staining, were restricted to the mother cells, however occasional faint septa were seen at the points of constriction in the daughter cells. Such feint septa have also been described during the course of normal septum development (Mitchell and Soil, 1979). The initial formation of "light" septa was followed by their modification to full septa within 30 mins. Nikkomycin apparently blocks septum formation at an early stage of development, with the constrictions of enlarged cells corresponding with potential sites of septum deposition. Partial formation of the septum, due to a chitin synthase inhibitor, has not been reported previously. However, this was evident in *C.albicans* observed by electron microscopy. The primary septum is composed of

chitin in *S.cerevisiae* (Holan, *et al.*, 1981) and appears to be similar in *C.albicans* (Tronchin, *et al.*, 1981). Hence, inhibition of chitin synthase during primary septum formation would result in the presence a partially formed chitinous plaque which would correspond with the faint septum observed by light microscopy. It is also probable that the outgrowth contains the scar ring composed of glucan (Vrasanka, *et al.*, 1979; Holan, *et al.*, 1981). Therefore nikkomycin-grown cells would be able to produce scar rings, a process independent of chitin synthesis. This may explain why the constrictions observed in enlarged cells occurred without subsequent septum formation. However, further work is necessary to obtain useful information concerning site determination in septum formation.

Prolonged incubation in the presence of the drug resulted in some lysis, either at a point of intersection of two cells or at a point of constriction within the enlarged cell. The inclusion of the osmotic stabilizer mannitol (0.8 M) in the staining medium had no effect upon the occurrence of lysis, which was therefore due to a weakening of the cell wall followed by lysis within the growth medium brought about by the turgor pressure of the cell.

The lytic effect of nikkomycin on *C.albicans* closely resembles the effect of polyoxin D on *S.cerevisiae* (Bowers, *et al.*, 1974). Two aberrant forms of cells were found in *S.cerevisiae:* Firstly the sudden lysis of a mother-daughter pair followed by loss of cytoplasm into the medium; secondly the production of a highly refractile pair when observed by phase microscopy, these separated to produce two cells with pointed ends. Enlarged cells were not observed in *S.cerevisiae* indicating

that chitin is of lesser importance in the maintenance of cell rigidity in this organism. No equivalent of the refractile pair was observed in *C.albicans*; however lysis occured at a point adjacent to the septum and this closely resembles the event observed in *S.cerevisiae* (Bowers, *et al.*, 1974). The major difference between the two organisms was that in *C.albicans* the primary septum was present whereas that of *S.cerevisiae* was aberrant. Further studies of septum deposition in the two organisms may account for this difference. The control of cell separation in yeasts has yet to be elucidated. However, it is probable that partial hydrolysis of the mural polysaccharides is required. Under abnormal conditions, such as the presence of a chitin synthase inhibitor, uncontrolled lytic action could damage the wall and hence result in lysis.

The ultimate morphological form induced by nikkomycin appears to be determined by the point in the cell cycle at which the inhibitory intracellular concentration of the drug is achieved. If budding has commenced, the inhibition results in a weakened cell wall. Scar rings can be produced thus forming constrictions within enlarged cells. Lysis may be due to two factors: either the cleavage of the weakened walls following prolonged incubation or, alternatively, due to autolysis occuring at a site of potential cell separation. If the primary septum has been produced before the inhibitory concentration of nikkomycin is reached, septum formation continues. Lysis of cells then occurs due to degradation of polysaccharides adjacent to the septum. Initial weakening of the cell wall thereby leads to ultimate cell death. The daughter cell of such a pair appears to be unaffected indicating that

separation is controlled by the mother cell and that the daughter is not dependent upon chitin synthesis at this stage of the cell cycle.

The hyphae produced on 20% serum were resistant to nikkomycin. The apparent rates of hyphal elongation in the presence and absence of the drug were equivalent. There are three possible explanations for the absence of drug action under these conditions.

1. Hydrolysis of nikkomycin at the peptide moiety by enzymes within the serum could produce an inactive nikkomycin C or C_X) resembling the inactive polyoxin C (Hori, *et al.*, 1971). However, no peptidase action towards either ala_2 or nikkomycin was found in the serum. Degradation of the drug at the nucleoside residue cannot be eliminated.
2. Polyoxins have been shown to enter the fungal cell via the peptide permease (Mitani and Inoue, 1968; Hori, *et al.*, 1977) and this is also the case for the nikkomycins. It is possible that there is a variation in the peptide transport systems in the yeast and hyphal forms thereby giving natural resistance in the hyphal form.
3. Serum proteins and peptides may be interacting with the peptide permease and thus competing for the uptake of nikkomycin. It has been shown that large, non-transported peptide conjugates such as met_3-poly(ethylene-glycol) compete for uptake of met_3 into *S. cerevisiae* (Naider, *et al.*, 1980). The possibility that serum proteins may interact with nikkomycin uptake will be of considerable importance in the development of peptide

drugs as antifungal agents.

Competition for nikkomycin action

Although nikkomycin was highly active against *C.albicans* on simple media (YNB or MX , MIC=25 μg ml^{-1}) , the presence of bacteriological peptone as the sole nitrogen source eliminated the activity of the drug at 50 μg ml^{-1}. The effect is similar to that described for the growth of *Pellicularia sasakii:* Growth inhibition by low concentrations of polyoxins was antagonized by the addition of peptone, yeast extract or casein to the medium (Mitani and Inoue, 1968).

Inclusion of certain simple dipeptides in YNB also inhibited the action of nikkomycin against *C.albicans*. Ala_2 or leu gly were antagonistic while gly_2 , ala_3 , ala_4 , gly tyr and D-leu gly were ineffective. The antagonism was apparent in both the rate of growth of a batch culture and in the morphology of the cells. Peptides have also beeen shown to antagonize the action of a polyoxin mixture against *P.sasakii* (Mitani and Inoue, 1968) and polyoxin A inhibition of *Alternaria kikuchiana* (Hori, *et al.*,1977). In the latter case 10 mM gly_2 , gly ala , or gly leu inhibited the action of 20 μM polyoxin A while amino acids and bases showed little effect. Hori *et al.*, (1977) also demonstrated that peptides had no competitive effect for polyoxin A at the level of cell-free chitin synthase whereas they did inhibit the uptake of [^{3}H] polyoxin A by intact cells. Competition between the peptides and polyoxin A was therefore at the level of co-transport into cells.

The uptake of [$U^{14}C$] ala ala was inhibited by nikkomycin (K_i = 4.85 μg ml^{-1}), confirming that competition for drug action in *C.albicans* also occurs at the level of transport. Previous studies have clearly demonstrated the presence of a peptide-specific transport system in *C.albicans* (Lichliter, *et al.*, 1976; Logan, *et al.*, 1979; Davies, 1980). The specificity of this system with regard to those peptides able to antagonize nikkomycin action will be considered in the following section. The antagonistic action of peptides against nikkomycin is the first evidence that these peptides enter the cell via the peptide permease.

Nikkomycins are analogues of UDP-N-acetyl glucosamine. However, only the nikkomycins were able to compete with uptake of [$U^{14}C$] ala ala. Therefore the peptide moiety is necessary for an interaction with the transport system and hence entry to the cell. Equally the uracil moiety of UDP-N-acetylglucosamine was unable to inhibit peptide uptake showing that peptide and base permeases are independent. 6-hydroxynicotinic acid, an analogue of the N-terminal amino acid side-chain residue of the nikkomycins showed no competition for peptide uptake. Hence the inhibitory action of nikkomycin is dependent upon the intact peptide moiety and not simply upon a single side-chain residue.

The transport deficient mutant NIK5

As nikkomycin is transported by the peptide permease of *C.albicans* selection for resistance to the drug allowed the production of mutants defective in transport. Only one of the six mutants produced showed a stable deficiency in peptide transport. The remaining five variants may show an alteration in chitin synthase such as that described for polyoxin D

resistance in *Schizophyllum commune*(MacGruder, 1979). The purpose of selecting for resistance was to create a mutant defective in peptide uptake and therefore the point of mutation in the remaining variants was not determined. NIK5 is analogous to a mutant of *Alternaria kikuchiana* which was found to be unable to accumulate either [^{3}H]-polyoxin A or [^{14}C] gly gly (Hori, *et al.*, 1977). However, further details of the peptide transport capacity of this mutant are not available.

Ala_2 uptake was used as a screen for a transport deficient mutant since this peptide had been shown to be co-transported with nikkomycin. Transport of nikkomycin could not be measured even though the presence of a primary amine group allows assay with fluorescamine. It was found that the addition of the drug to a suspension of blastospores of either the wild type of NIK5 in PCG resulted in a rapid efflux of E_{260} absorbing material containing free amine groups. The material was not identified and the efflux masked any transport which might have occured during the incubation period. This efflux may have occured due to an interaction between the drug and the plasma membrane and would therefore be analogous to the effect of miconazole (Swamy, *et al.*, 1974) which resulted in the release of many intracellular constituents. No radiolabelled nikkomycin was available for this study and therefore the precise rates of uptake of the drug into the wild type and mutant could not be determined. The recent development of an HPLC technique for the separation of nikkomycins (Fiedler, 1981) may allow quantitation of the drugs even in the presence of effluxed material and hence show whether transport is occuring.

The defect in transport was the only mutation evident in NIK5. There was no alteration in the growth rate on simple media or on a peptone based medium when compared with the wild type. However, the mutant showed a lag-period on transfer from simple to peptone-based medium which was not observed in the wild type. The significance of this will be discussed in the following section. Chitin deposition, revealed by primulin staining, was the same in both isolates as was the sensitivity to most of the antifungal agents tested.

The peptide drugs showed cross-resistance indicating that both bacilysin and polyoxins are transported exclusively by the system through which nikkomycin enters the cell. The tripeptide m-F-phe ala ala did not show clear cross-resistance. The peptide inhibited growth of NIK5 and this is the first indication that all peptides do not share a common permease in *C.albicans*.

CHAPTER 4: PEPTIDE TRANSPORT

Introduction

The recent development of the fluorescamine technique has allowed direct monitoring of the peptide transport systems of bacteria (Payne and Bell, 1977 and 1979) and *Saccharomyces cerevisiae* (Nisbet and Payne, 1979). The advantage of this technique over methods employing radiolabels is that any peptide possessing a free primary amino group may be assayed. There is therefore no dependence upon implied rates of uptake from competition studies. The rates of transport obtained by the fluorescamine method have been found to be higher than that found for uptake of radiotracers. This has been ascribed either to efflux of the constituent amino acids or to further metabolism resulting in the loss of radiolabel from the cell (Payne and Nisbet, 1980).

Peptide transport in *Candida albicans* has been studied using auxotrophic growth responses (Lichliter, *et al.*, 1976) and competition for transport of radiolabelled peptides (Logan, *et al.*, 1979; Davies, 1980). The results were sometimes contradictory for example the transport of N-acetyl peptides which Lichliter *et al.* (1976) and Logan *et al.* (1979) found to occur while Davies (1980) did not. As the molecular determinants of transport must be clearly defined before any rational approach to peptide-drug development is possible, the fluorescamine assay was used in the present study to describe the peptide transport system of *C.albicans* in more detail.

Nikkomycin, a compound which is taken into the cell via the peptide permease, aided this study by allowing the creation of a mutant of *C.albicans* deficient in the transport of certain peptides. The rationale behind the selection of the mutant NIK5 has been discussed in the preceeding chapter. This is the first study to describe the actual transport of peptides into *C.albicans* and to use mutants of this organism to identify multiple components of the system.

Results

Section 1 Peptide transport by wild type *C.albicans*

Strain dependence

The rate of transport of 0.2 mM ala_2 was found to vary greatly between various strains grown on YNBP (Table 4.1). Strain 124 showed the greatest rate of transport (38.0 nmol/min/mg) whereas four others (113,495,539 and STM-1) showed rates of approximately one-third of that found for strain 124. In consequence strain 124 was used in subsequent experiments.

Both Logan *et al.* (1979) and Davies (1980) stated that the medium on which cells are grown has a considerable influence upon the subsequent capacity of the cells to transport peptides. They also found the system to be pH dependent. It was therefore necessary to determine the effect of such environmental factors on strain 124.

Growth medium

Uptake of ala_2 and ala_4 was assayed with cells grown on YNB , MX and YNBP (Table 4.2). There was a considerable difference in the rates observed. Those grown on an ammonium ion based medium (YNB) or a simple undefined medium (MX) exhibited comparable rates while cells grown with peptone as the sole nitrogen source (YNBP) had a considerably incrased capacity. YNBP-grown cells were therefore used in the routine assay of peptide transport except where otherwise stated.

pH dependence

The uptake of 0.2 mM ala_2 by wild type cells showed a marked pH dependence. Rates of uptake were determined in a series of PCG buffers of differing pH . Optimum uptake occured at pH 5.0 and half-optimal rates were observed at pH 4.7 and pH 5.7 (Figure 4.1).

The peptide uptake rates of YNBP-grown strain 124 at pH 5.0 were studied with respect to the effects of stereochemistry, side-chain residues, chain length and alteration of the amino and carboxyl terminals.

Stereochemistry

Uptake of ala_2 was found to be highly stereospecific. D-ala-L-ala and D-ala-D-ala were not transported at a detectable rate (Table 4.3) while L-ala-D-ala was transported at a very low rate (1.6 nmol/min/mg). This was in contrast to L-ala-L-ala which was transported at 38.0 nmol/min/mg. In addition L-leu gly was transported at 32.0 nmol/min/mg whereas the D-stereoisomer was not taken into cells.

Table 4.1 Strain dependence of ala_2 uptake

Six strains of *C.albicans* were grown to mid-logarithmic phase in YNBP at 30°C. Cells were harvested, washed and resuspended in PCG . Uptake at 0.2 mM ala_2 was assayed at pH 5.0 and 37°C (cell density=1.0 mg dry wt./ml) by sampling the suspension and determining the peptide concentration of the extracellular medium by the fluorescamine technique. Standard deviations from mean initial rates of uptake were determined from triplicate assays of two separate experiments.

C. albicans strain	Rate of ala_2 transport (nmol/min/mg dry wt. cells) $[ala_2]_o$ = 0.2 mM
113	8.0 ± 0.4
124	38.0 ± 1.3
125	20.0 ± 1.6
495	12.0 ± 0.8
539	10.0 ± 1.1
STM-1	12.0 ± 0.7

Table 4.2 Effect of growth medium on peptide transport

C.albicans 124 and the mutant NIK5 were grown in YNB, MX or YNBP to mid-logarithmic phase at 30°C. Cells were harvested, washed and resuspended in PCG. Peptide uptake was assayed as described in Table 4.1 at at initial concentration of 0.2 mM.

Peptide (0.2 mM)	Growth Medium	Wild type	NIK5
ala_2	YNB	11.0	0
ala_2	MX	12.0	0
ala_2	YNBP	38.0	7.4
m-F-phe ala ala	MX	18.0	7.0
m-F-phe ala ala	YNBP	22.0	38.0
ala_4	YNB	18.0	8.0
ala_4	MX	21.0	12.0
ala_4	YNBP	52.0	90.0

Figure 4.1 pH dependence of peptide transport

Cells were grown to mid-logarithmic phase in YNBP, harvested, washed and resuspended in PCG. Initial velocities for the uptake of ala_2 by the wild type (□) and of ala_3 by NIK5 (○) were determined as described in Table 4.1 using a series of 10 mM phosphate citric acid buffers (supplemented with 0.8% (w/v) glucose) of differing pH.

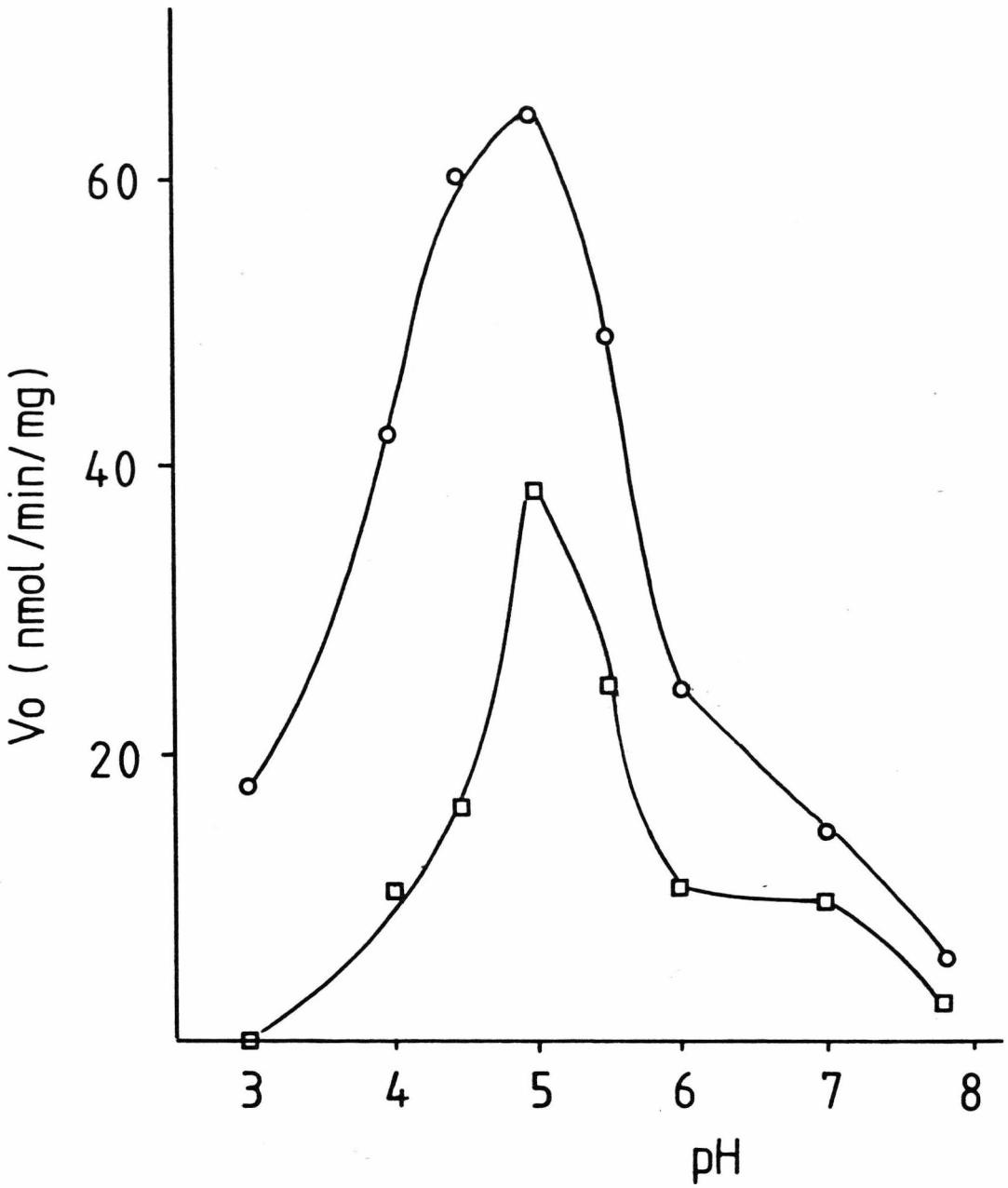

60
40
20
Vo (nmol /min/ mg)
3
4
5
6
7
8
pH

Table 4.3 Rates of peptide uptake by *C.albicans* 124 wild type

Cells were grown in YNBP to mid-logarithmic phase at 30°C harvested, washed and resuspended in PCG . Uptake of peptides was assayed as in Table 4.1 from an initial concentration of 0.2 mM.

Peptide (0.2 mM)	Rate of Transport (nmol/min/mg dry wt. cells)	Mean Rate of Transport % rate of ala_2
ala_4	52.6 ± 1.2	138.5
ala met	48.0 ± 1.3	126.3
m-F-phe ala ala	38.0 ± 1.5	100.0
ala_2	38.0 ± 1.3	100.0
leu gly	32.0 ± 1.4	84.2
ala_3	30.3 ± 1.0	79.7
tyr ala	26.0 ± 1.2	68.4
gly leu tyr	23.0 ± 1.3	60.5
ala leu	22.0 ± 1.6	57.8
ser ala	22.0 ± 0.8	57.8
bacilysin	20.0 ± 0.5	52.6
tyr gly	14.0 ± 0.6	36.8
met ala ser	13.0 ± 0.5	34.2
gly leu	13.0 ± 0.7	34.2
gly phe	12.0 ± 0.3	31.5
met glu	11.5 ± 0.3	30.2
ala trp	11.0 ± 0.4	28.9
ala glu	11.0 ± 0.7	28.9
leu pro	10.0 ± 0.6	26.3
glu ala	6.5 ± 0.3	17.1
leu ala	5.0 ± 0.2	13.1
met ala	3.0 ± 0.2	7.8
L-ala-D-ala	1.6 ± 0.2	4.2

Non-transported peptides: gly asp , gly pro , gly tyr , D-leu gly , ala ala ONH_2 , D-ala-L-ala , D-ala-D-ala , leu_2 , leu_3 , gly_2 , gly_3

Table 4.4 Effect of alteration of N- and C-terminal amino acids on the rates of dipeptide transport

Initial rates of transport taken from Table 4.3.

Alteration of the N-terminal amino acid residue

Peptide (0.2 mM)	Mean Rate of Uptake (nmol/min/mg dry wt. cells)	Nature of the Residue
ala_2	38.0	Neutral
tyr ala	26.0	Polar
ser ala	22.0	
glu ala	6.5	Hydrophilic
leu ala	5.0	Hydrophobic
met ala	3.0	

Alteration of the C-terminal amino acid residue

Peptide (0.2 mM)	Mean Rate of Uptake (nmol/min/mg dry wt. cells)	Nature of the Residue
ala met	48.0	Neutral/ Hydrophobic
ala ala	38.0	
ala leu	22.0	
bacilysin	20.0	Polar
ala trp	11.0	
ala glu	11.0	Hydrophilic

Thus, the presence of D-steroisomers of amino acids reduced the affinity of the peptide for the uptake system. However, if the D-isomer is present at the carboxyl terminal a low rate of transport is still possible.

Effect of side-chain residues

Uptake of a large number of dipeptides and oligopeptides was assayed and the resultant rates of transport showed a considerable dependence upon the nature of the side-chain residues (Table 4.3). In addition to the amino acid residues normally found in proteins unusual ones such as the anticapsin residue of baciylsin and the m-F-phenylalanyl residue of the tripeptide m-F-phe ala ala were also tolerated.

Consideration of the ala-x group of peptides showed the influence of the carboxyl terminal residue of a dipeptide upon transport (Table 4.4). Ala met and ala leu possess hydrophobic residues at this point. The rates of transport of these peptides were higher than those found for either the polar side chain residues of bacilysin (ala anticapsin) and ala trp or the hydrophilic residue of ala glu. Ala_2 possesses a methyl side chain in the carboxyl residue and was transported at a rate comparable with those of the hydrophobic groups.

An equivalent analysis of the x-ala series showed the influence of the amino terminal residue (Table 4.4). In this case polar side chain residues enhanced the rate of transport: tyr ala and ser ala were transported at far higher rates than leu ala or met ala with hydrophobic side chains. Ala ala was transported at a higher rate than the polar

residue-containing peptides which again indicates that the presence of small side chain residues enhanced the affinity of the peptide for the transport system.

Many glycyl peptides (gly asp , gly pro , gly tyr , gly_2 and gly_3) were not transported at a detectable rate. Thus, the presence of a glycyl amino terminus in conjunction with a polar or hydrophilic carboxyl terminal amino acid eliminated transport. Gly leu and gly phe , which possess hydrophobic C-terminal side chain residues, were transported into cells emphasizing the influence of the carboxyl terminal residue upon transport. This has also been demonstrated for the ala-x series.

It was found that the highly hydrophobic peptides leu_2 and leu_3 were not transported at a detectable rate. Davies (1980) had found leu_2 to interact with the transport of radiolabelled peptides and had interpreted this as the transport of leu_2 . In the present study leu_2 has been shown to interfere with the transport of nikkomycin and to antagonize the action of this drug (Figure 3.36). However, this cannot be interpreted in terms of competition for transport since the direct assay using fluorescamine indicated that leu_2 was not entering the cell. To ascertain whether leu_2 could interfere with the uptake of simple dipeptides in this strain of *C.albicans* the uptake of [$U^{14}C$] ala ala was assayed in the presence and absence of leu_2 (Figure 4.2). It was found that even at equimolar concentrations the rate of ala_2 incorporation was considerably reduced by the presence of leu_2 (rate of ala_2 incorporation=16 nmol/min/mg; rate of ala_2 incorporation+leu_2=1.0 nmol/min/mg). Although leu_2 interacted with the peptide transport system subsequent transport did not

Figure 4.2 Effect of leu_2 on [$U^{14}C$] ala ala incorporation

C.albicans (wild type 124) was grown to mid-logarithmic phase in YNBP, harvested, washed and resuspended in PCG. Cells were incubated in PCG pH 5.0 (1.0 mg dry wt./ml) at 37°C containing 0.2 mM [$U^{14}C$] ala ala (O) or 0.2 mM [$U^{14}C$] ala ala and 0.2 mM leu_2 (□) . 1 ml samples were removed at intervals and the radioactivity determined.

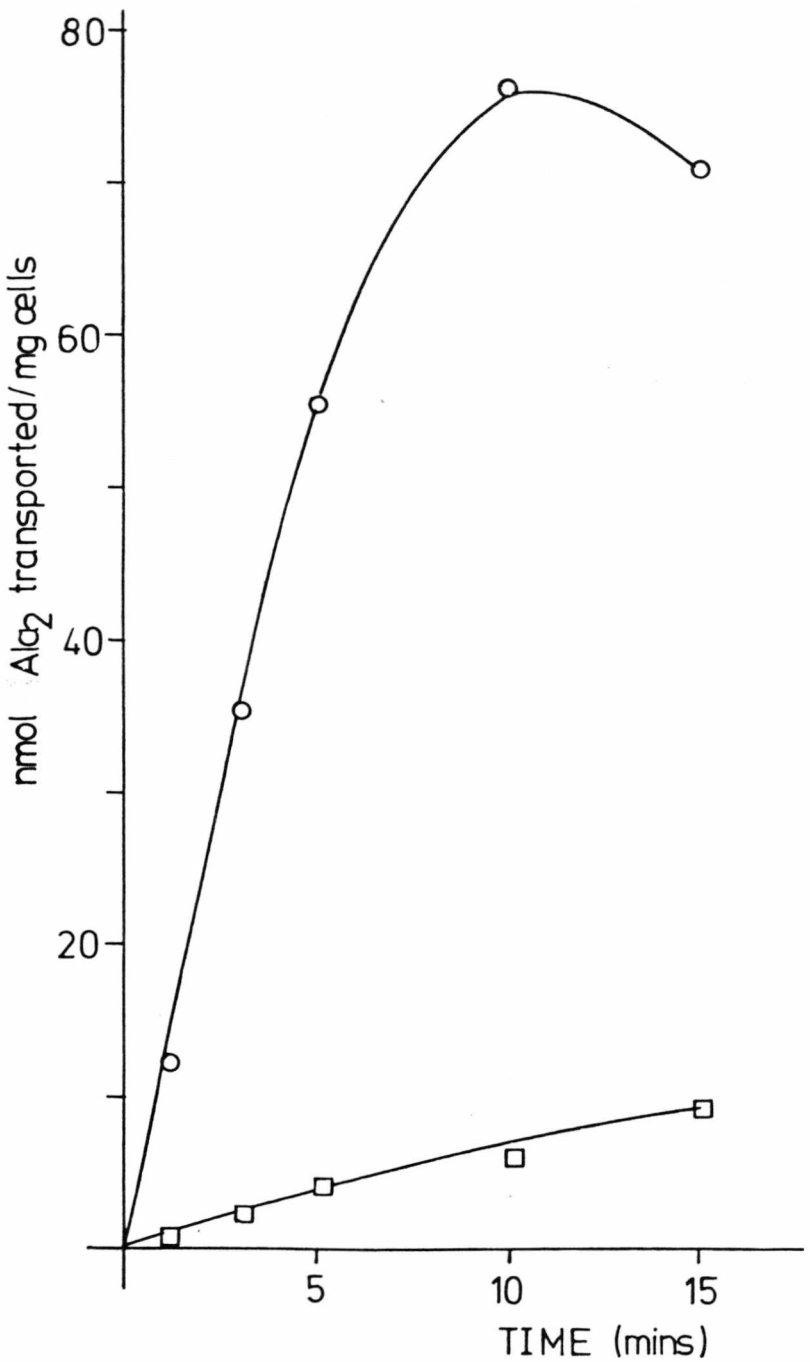
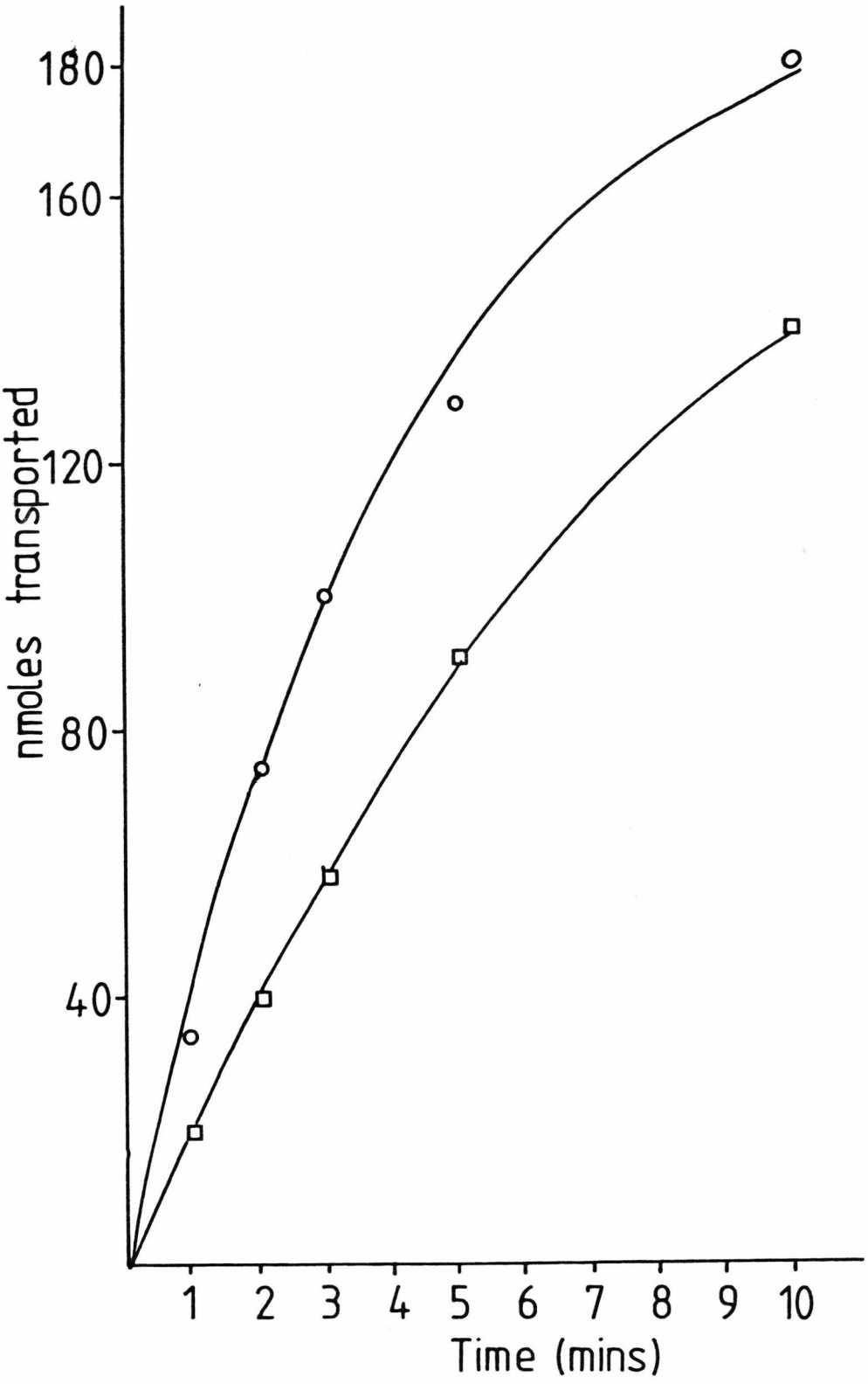

80
60
40
20
nmol Ala2 transported/mg cells
5
10
15
TIME (mins)

Figure 4.3 Effect of N-acetyl ala_2 on ala_2 transport

C.albicans 124 was grown to mid-logarithmic phase in YNBP, harvested, washed and resuspended in PCG . Uptake of ala_2 (0.2 mM) was assayed as described in Table 4.1 in the presence (□) and absence (o) of 0.2 mM N-acetyl ala_2 .


180
160
120
80
40
nmoles transported
1
2
3
4
5
6
7
8
9
10
Time (mins)

occur. This is the first direct evidence of such an effect and emphasizes that competition studies cannot necessarily be used to assess peptide transport. It also shows that, even though peptides may have a high affinity for a binding site, they may have low affinity for the ensuing translocation step.

Effect of chain length

It was found that oligopeptides were transported by *C. albicans* at rates comparable to those of dipeptides. The series ala_2, ala_3, ala_4 showed rates of 38.0, 30.3 and 52.6 nmol/min/mg respectively. It was not possible to assay the transport of ala_5 due to its insolubility. Thus, the upper size limit for the peptide transport system could not be determined. While the series did not show a clear progression it would appear that dipeptides and tripeptides may have similar transport rates whereas, although only one tetrapeptide (ala_4) was studied, these may enter the cell at a higher rate.

Amino- and carboxyl-terminals

The amide of ala_2 was not transported into the wild type cells indicating that alteration of the carboxyl terminus eliminates transport. As the N-acetyl derivative of ala_2 cannot be assayed directly by the fluorescamine technique its interaction was determined by competition for transport with ala_2. Even at an equivalent concentration it was found that a reduction in the rate of ala_2 transport occured (Figure 4.3). N-acetyl ala_2 can therefore interact with the transport system through which ala_2 enters the cell. This is of importance when considering the

potential attachment sites for toxic residues in the development of peptide-drugs. It is evident that the C-terminus cannot be used as such an attachment site whereas the N-terminus can be blocked without eliminating its ability to interact with the peptide transport system. However, this interaction may not be followed by transport and may reflect the situation previously described for leu_2.

The data presented so far suggests that *C.albicans* 124 transports a large number of peptides. The system is highly stereospecific with the nature of the side chain residues affecting the rate of uptake. The nikkomycins have large, unusual side chain residues and yet are still able to enter the cell. No data are available concerning the actual rate of uptake of nikkomycins and so no direct comparison is possible between the peptide-nucleosides and simple peptides.

From this point it was necessary to determine the means of energization of peptide transport. Consideration of the kinetics of transport will be presented in conjunction with those of NIK5.

Metabolic inhibitors

The effect of various inhibitors upon the rate of incorporation of $[U^{14}C]$ ala ala was determined (Table 4.5). Replacement of the D-glucose energy source in the medium (PCG) with 2-deoxy-D-glucose prevented ala_2 uptake, while disruption of the membrane's integrity with Triton-X-100 reduced the rate of transport by 84%. Inhibitors of ATPases (potentially membrane bound enzymes) were also effective. Thus sodium arsenate and *ortho*-vanadate reduced the uptake rates by 90% and 30% respectively when compared with control cells. It is of interest to

Table 4.5 Effect of metabolic inhibitors on [U^{14}C] ala ala incorporation

Cells were grown to mid-logarithmic phase in YNBP at 30°C, harvested, washed and resuspended in PCG†. Rates of incorporation of [U^{14}C] ala ala were determined, after preincubation in the presence of inhibitors, by taking 1 ml samples and determining radioactivity incorporated into cells. Initial rates of incorporation are expressed as % rate of incorporation of ala_2 in the absence of inhibitors.

†To determine the effect of arsenate the buffer used was 10 mM dimethylglutaric acid-NaOH pH 5.0 supplemented with 0.8% (w/v) glucose.

In the case of 2-deoxy-D-glucose this compound replaced the D-glucose in PCG.

Inhibitor	Concentration	Rate of [U^{14}C] ala ala Incorporation (% of control)
Triton X-100	0.1% (v/v)	16.6
Sodium azide	0.1 mM	91.2
	1.0 mM	1.5
Sodium *o*-vanadate	0.01 mM	83.3
	0.1 mM	70.8
2,4-DNP	0.02 mM	51.5
	0.2 mM	0.7
2-deoxy-D-glucose	0.8% (w/v)	0.4
Dithioerythritol	2.0 mM	100.0
DTNB	0.5 mM	100.0
Sodium arsenate	0.2 mM	20.0
	2.0 mM	10.0
2-mercaptoethanol	0.1 mM	100.0
	1.0 mM	100.0

note that none of the thiol reagents tested, some of which might be expected to modify the cell wall and possibly the plasma membrane, had any effect upon the rate of ala_2 transport. In contrast uncouplers of oxidative phosphorylation (NaN_3 and 2,4-DNP) completely abolished transport.

Section 2. Peptide transport by the *C.albicans* 124 mutant NIK5

The isolation and characterization of the mutant have been discussed in the preceding chapter. NIK5 was found to be deficient in peptide transport, an observation which will now be examined in more detail.

Peptide transport by NIK5

The ability of the mutant NIK5 (grown on YNBP) to transport dipeptides and oligopeptides at an initial concentration of 0.2 mM was assayed at pH 5.0 (Table 4.6). The rates of uptake of dipeptides were greatly reduced. Thus, ala_2 was transported at 19.5% of the rate found in the wild type while ala met was taken up at only 5.2% of the wild type velocity. None of the other dipeptides assayed were transported at a detectable rate. However, rates of oligopeptide transport were greatly increased to amounts between 137% for ala_3 and 253% for met ala ser.

The major components of the nikkomycin used were nikkomycins X and Z both of which are dipeptides. The loss of dipeptide transport capacity

therefore apparently correlates with resistance to nikkomycin.

The effect of environmental factors upon the transport of peptides by NIK5 were determined by the methods used for studies of the wild type.

pH dependence

Transport of ala_3 was found to be pH dependent (Figure 4.1). As for the wild type, peptide transport showed a peak of activity at pH 5.0 with half maximal velocities at pH 4.0 and pH 5.7.

Effect of the growth medium

NIK5 cells grown on MX , YNB or YNBP were each assayed for their ability to transport ala_2 and ala_4 (Table 4.2). The effect of growth medium reflected that found in the wild type. The rates of uptake by cells grown on YNB or MX were again comparable whereas the rates for cells grown on YNBP were considerably increased. Therefore the presence of peptides in the growth medium appears to either activate or induce additional capacity for peptide transport.

The toxic tripeptide m-F-phe ala ala was used to obtain more data concerning the nature of the mutation. This peptide facilitates the transport of m-F-phenylalanine into the cell which, following intracellular cleavage, is released and hence incorporated into protein. m-F-phe ala ala

Table 4.6 Rates of peptide transport by NIK5

NIK5 cells were grown to mid-logarithmic phase in YNBP. Rates of peptide uptake (initial concentration 0.2 mM) were determined as in Table 4.1.

Results are expressed as initial rates of transport and as % initial rate of uptake observed with the wild type taken from Table 4.3.

Peptide (0.2 mM)	Rate of Uptake (nmol/min/mg dry wt cells)	Mean (% of wild type rate)
ala_2	7.4 ± 0.6	19.5
met ala ser	33.0 ± 1.2	253.8
tyr ala	0	0
tyr gly	0	0
ala met	2.0	5.2
bacilysin	0	0
gly leu tyr	48.0 ± 1.8	208.7
ala_3	41.6 ± 1.3	137.5
ala_4	90.9 ± 2.0	173.5

is therefore an excellent example of illicit transport since m-F-phenylalanine is inactive against *C.albicans* unless incorporated into a peptide (Table 3.3).

Activity of m-F-phe ala ala against the wild type and NIK5

m-F-phe ala ala was found to inhibit growth of the wild type grown on MX at concentrations above 46 µM (Figure 3.46). However, NIK5 was unaffected at concentrations below 77 µM . In neither case was total inhibition of growth achieved at 300 µM m-F-phe ala ala.

The rates of transport of oligopeptides into NIK5 cells grown on YNBP were found to be higher than the corresponding rates shown by the wild type. However, the mutant showed a lower sensitivity to m-F-phe ala ala than did the wild type; a result not predictable from the transport rates of YNBP-grown cells. As the activity of m-F-phe ala ala was determined in MX the effect of the medium on transport of this peptide was determined.

Transport of m-F-phe ala ala

Transport of m-F-phe ala ala showed the same medium dependence found for other peptides (Table 4.2). MX-grown wild type cells transported m-F-phe ala ala at a greater rate than MX-grown NIK5 . This would explain the reduced sensitivity of NIK5 to the drug. YNBP-grown cells displayed the converse situation : NIK5 cells transported the peptide at a greater rate than the wild type cells.

Induction of peptide transport capacity

It has been shown that the growth medium greatly influences the subsequent rate of peptide transport by both the wild type and NIK5 . MX-grown NIK5 exhibited a reduced rate of transport when compared to an equivalent batch of cells grown on YNBP (Table 4.2). Furthermore it was noted that on transfer of NIK5 cells from a simple medium (YNB or MX) to YNBP there was a 4 hour lag period before logarithmic growth resumed (Figure 3.43). No equivalent lag period was observed wtih wild type cells.

To determine whether activation or induction of peptide transport capacity was occuring NIK5 cells were transfered from MX to YNBP at the mid-logarithmic phase of growth. Following a further period of incubation the cells were harvested and their capacity to transport ala_3 was determined. Control cells were transfered from MX to fresh MX and from MX to YNBP containing the protein synthesis inhibitor blasticidin S.

Blasticidin S (Figure 4.4) is produced by *Streptococcus griseochromogenes*, it has the structure of a novel nucleoside (cytosinine), linked via a peptide-bond to the amino acid blastic acid. The drug possesses some antifungal activity and has been reported to inhibit the incorporation of amino acids into protein in *Pyricularia oryzae* (Huang, *et al.*, 1964). It has also been shown to bind to the 60S ribosomal subunit of *S.cerevisiae* thereby inhibiting peptidyl transferase and preventing chain elongation (Battaner and Vazquez, 1971). Blasticidin S has been found to inhibit

Figure 4.4 Molecular structure of blasticidin S

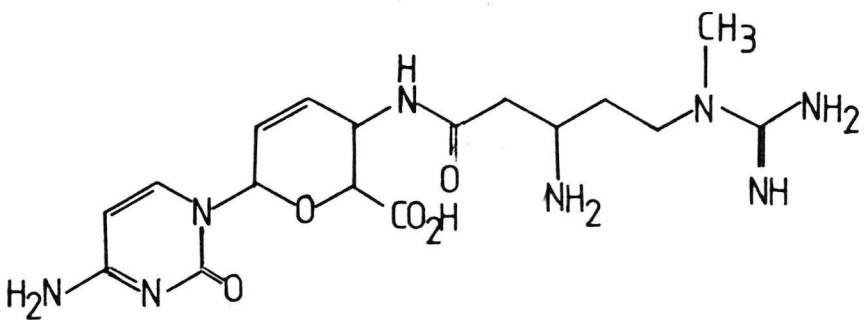

CH3
H
N
N
NH2
O
NH2
NH
N
O
CO2H
O
H2N
N

Figure 4.5 Effect of blasticidin S on the growth of the wild type (○) and NIK5 (□)

Cells were grown to mid-logarithmic phase in YNB and inoculated at 10^3 cells/ml into MX containing serial dilutions of blasticidin S . Viable cell numbers were determined after 48 hours at $37^{\circ}C$ by growth on MX plates.

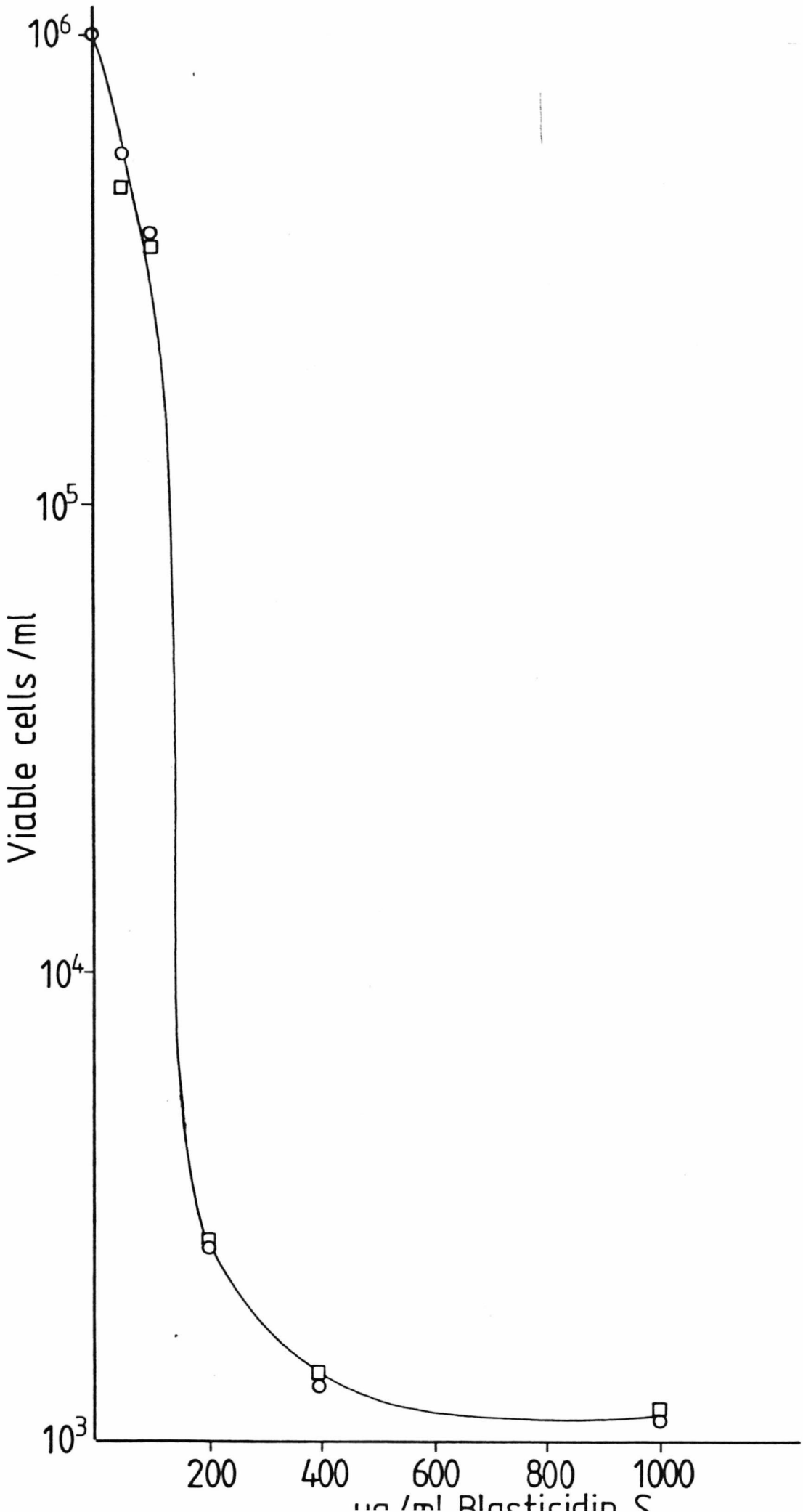
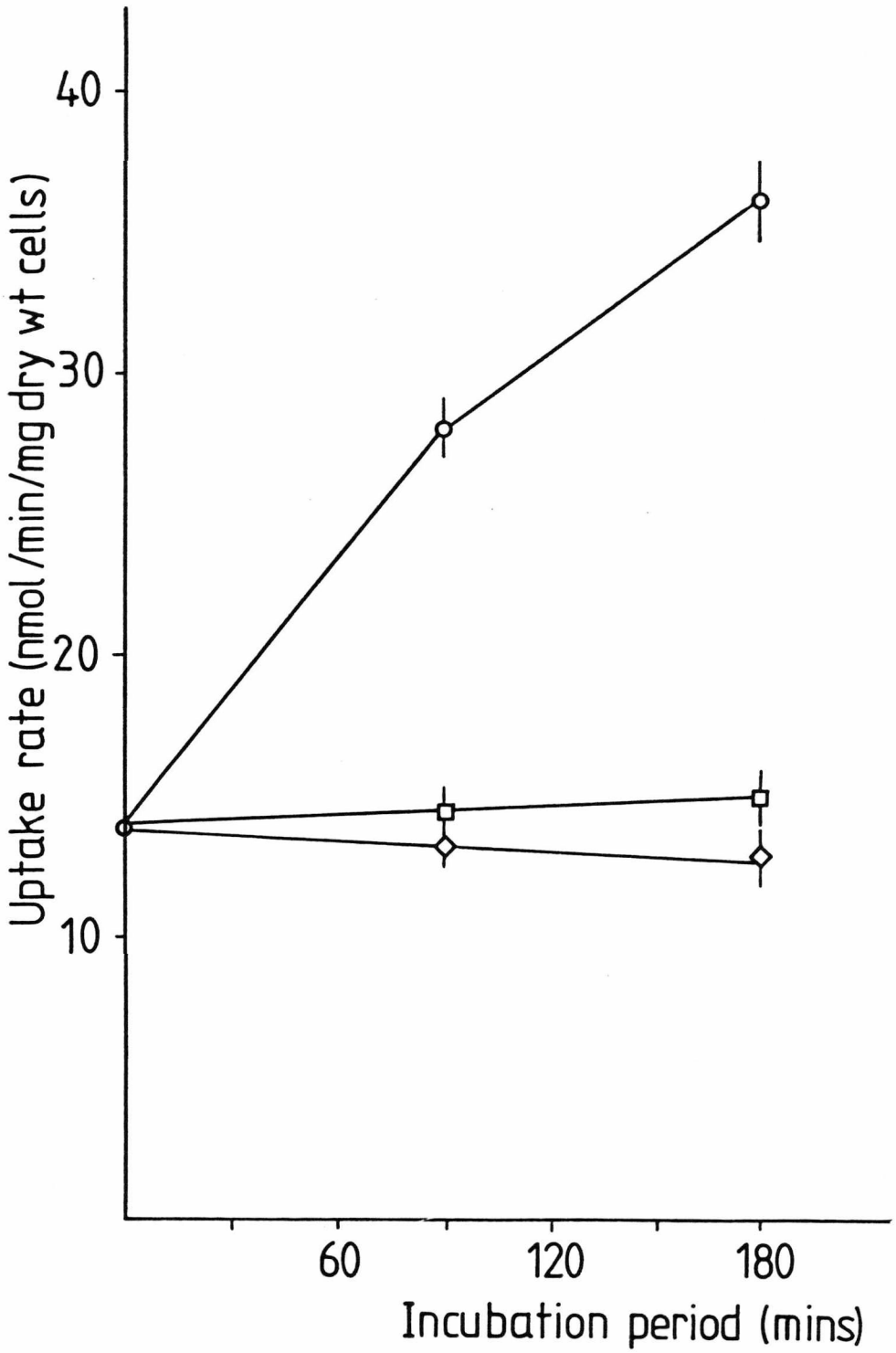

10^6
10^5
10^4
10^3
Viable cells /ml
200
400
600
800
1000

Figure 4.6 Induction of NIK5 peptide transport capacity

NIK5 was grown to mid-logarithmic phase in MX , harvested and resuspended in MX (□) , YNBP (○) or YNBP supplemented with 500 μg ml^{-1} blasticidin S (◇) for $1\frac{1}{2}$ hours or 3 hours.

Cells were harvested, washed and resuspended in PCG . Initial uptake rates of ala_3 (initial concentration 0.2 mM) were determined as described in Table 4.1.


40
30
20
10
Uptake rate (nmol/min/mg dry wt cells)
60
120
180
Incubation period (mins)

protein synthesis in *C.albicans* (Yamaguchi and Iwata, 1970), and to prevent cell division at 500 $\mu g\ ml^{-1}$ (Figure 4.5). This concentration was therefore used to inhibit any protein synthesis which might occur on transfer of cells from MX to YNBP.

NIK5 cells transfered from MX to YNBP showed a marked increase in their ability to transport ala_3 after 1½ hours incubation (Figure 4.6), this was increased further after 3 hours incubation (rate of ala_3 transport by the inoculum=14 nmol/min/mg ; rate after 1½ hours incubation=28 nmol/min/mg; rate after 3 hours incubation=36 nmol/min/mg). NIK5 cells transferred from MX to fresh MX showed no such increase in their peptide transport capacity, demonstrating that the increase observed on transfer to peptone was not a result of the supply of fresh growth medium. In the presence of 500 $\mu g\ ml^{-1}$ blasticidin S and peptone there was no increase in the rate of ala_3 uptake, indicating that protein synthesis was required for the increase peptide transport capacity. Failure to detect an increased transport rate was not due to cell death since an equivalent number of viable cells were present after 3 hours incubation in YNBP with or without blasticidin S.

The effect of the growth medium on the subsequent capacity of both wild type and NIK5 to transport peptides can therefore be attributed to the induction of either the complete transport system or of a component of that system.

The differences between the peptide transport capacities of the wild type and NIK5 , when grown on YNBP, still required examination. This was aided by a study of the kinetics of ala_2 , ala_3 and ala_4 transport into cells.

Kinetics of peptide transport

The kinetics of transport of ala_2, ala_3 and ala_4 were determined using the fluorescamine technique (Figures 4.7-4.9). The rate of uptake of each peptide was found to be dependent upon its initial concentration and exhibited Michaelis-Menton kinetics (Table 4.7). The transport of $[U^{14}C]$ ala ala was also studied as a comparison (Figure 4.10).

K_M values for both isolates revealed that the increase in chain length corresponded with an increased affinity for the peptide transport system. The V_{max} values reflected those results obtained using a single peptide concentration.

In both cell types the results obtained by the incorporation of radiolabelled ala_2 gave results considerably lower than those generated with the fluorescamine technique.

This appears to be due to the metabolism of the peptide and the significance of this finding will be discussed subsequently.

Discussion

There have been two major studies of peptide transport in *C.albicans* (Logan, *et al.*, 1979; Davies, 1980) both of which have followed the uptake of a limited number of radiolabelled peptides. These data were supplemented by competition studies using non-*labelled* substrates. The work presented here uses the relatively new technique of assaying the peptide concentration in the extracellular medium with fluorescamine, and

Figure 4.7 Lineweaver-Burk plots for the uptake of ala_2 by wild type cells (□) and NIK5 cells (○)

Cells were grown in YNBP to mid-logarithmic phase of growth, harvested, washed and resuspended in PCG. Cells were incubated at 1.0 mg dry wt./ml in PCG at $37^{o}C$ containing ala_2 at various concentrations. Samples were removed at intervals and the extracellular peptide concentrations determined by the fluorescamine method to establish the initial rate of uptake. A double reciprocal plot is illustrated.

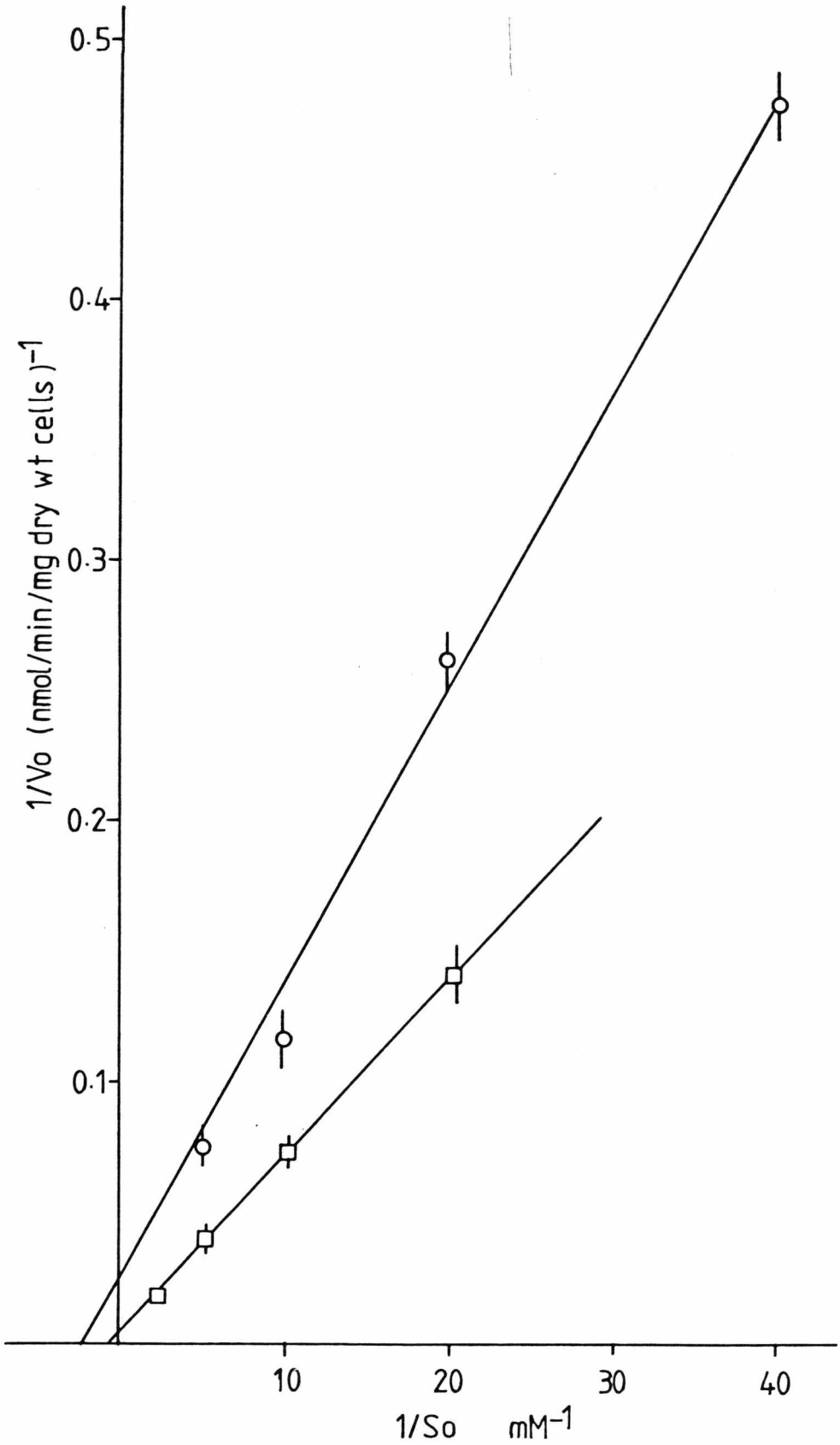

0·5
0·4
0·3
0·2
0·1
1/Vo (nmol/min/mg dry wt cells)$^{-1}$
10
20
30
40
1/So mM^{-1}

Figure 4.8 Lineweaver-Burk plots for the uptake of ala_3 by wild type (□) and NIK5 (○) cells

The method used was that described in Figure 4.7.

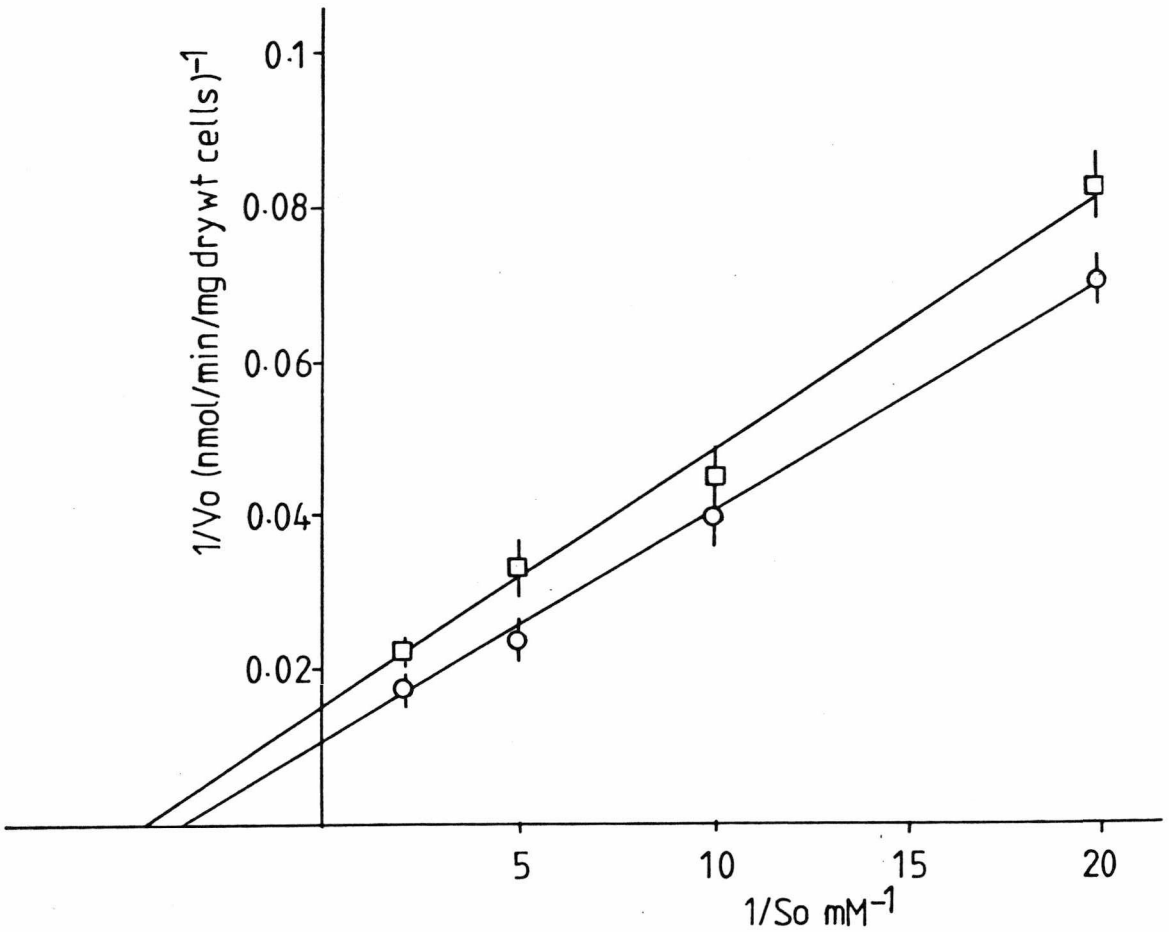

0·1
0·08
0·06
0·04
0·02
1/Vo (nmol/min/mg dry wt cells)$^{-1}$
5
10
15
20
1/So mM^{-1}

Figure 4.9 Lineweaver-Burk plots for the uptake of ala_4 by wild type (□) and NIK5 (○) cells

The method used was that described in Figure 4.7.

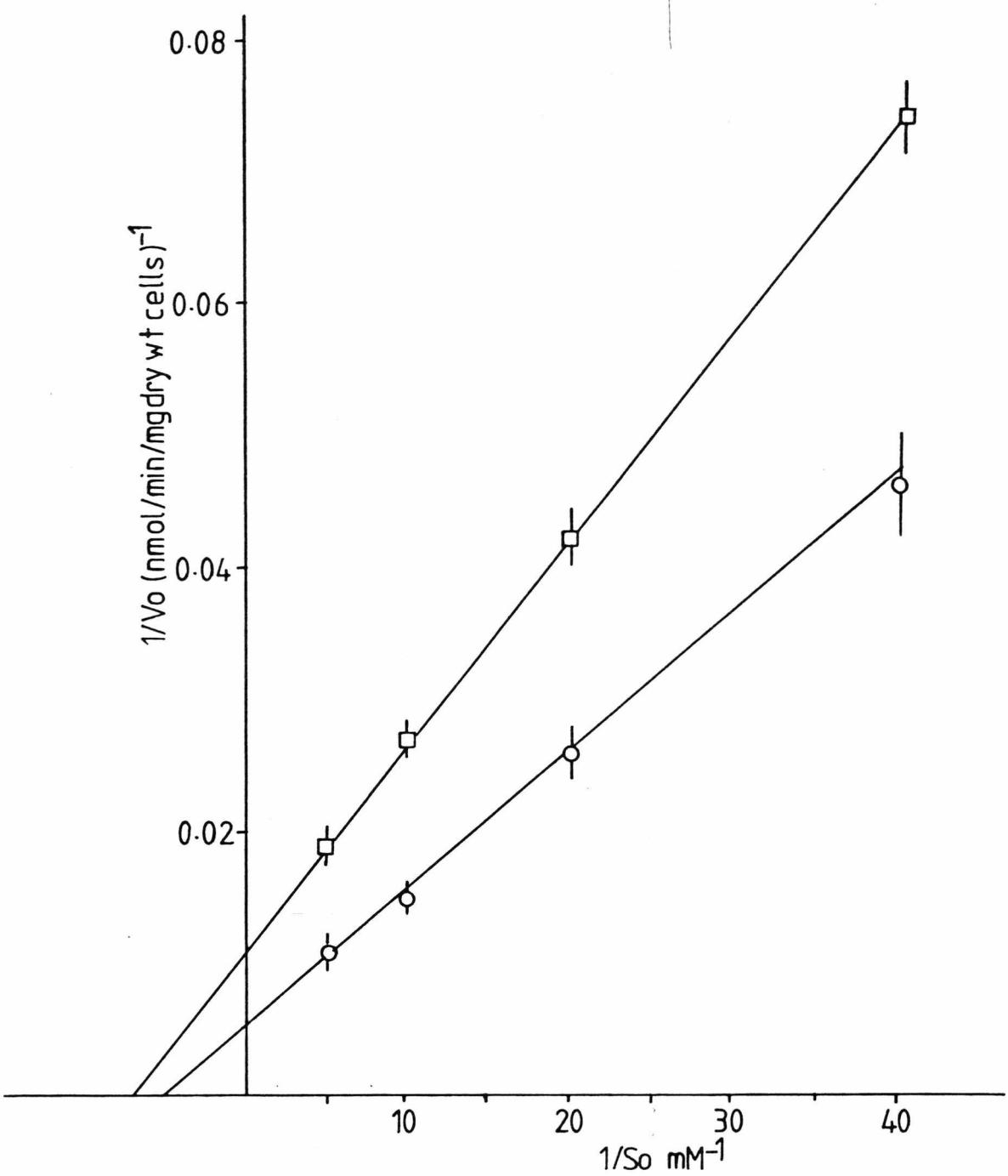

0·08
0·06
0·04
0·02
1/Vo (nmol/min/mg dry wt cells)$^{-1}$
10
20
30
40
1/So mM^{-1}

Figure 4.10 Lineweaver-Burk plots for the incorporation of [U^{14}C] ala ala by the wild type (O) and NIK5 (□) cells

Cells were grown in YNBP to mid-logarithmic phase of growth, harvested, washed and resuspended in PCG . Cells were incubated at 1.0 mg dry wt./ml in PCG at 37^{o}C containing [U^{14}C] ala ala at various concentrations. Samples were removed at intervals and the radioactivity incorporated into the cells was determined to establish the initial rate of incorporation. A double-reciprocal plot is illustrated.

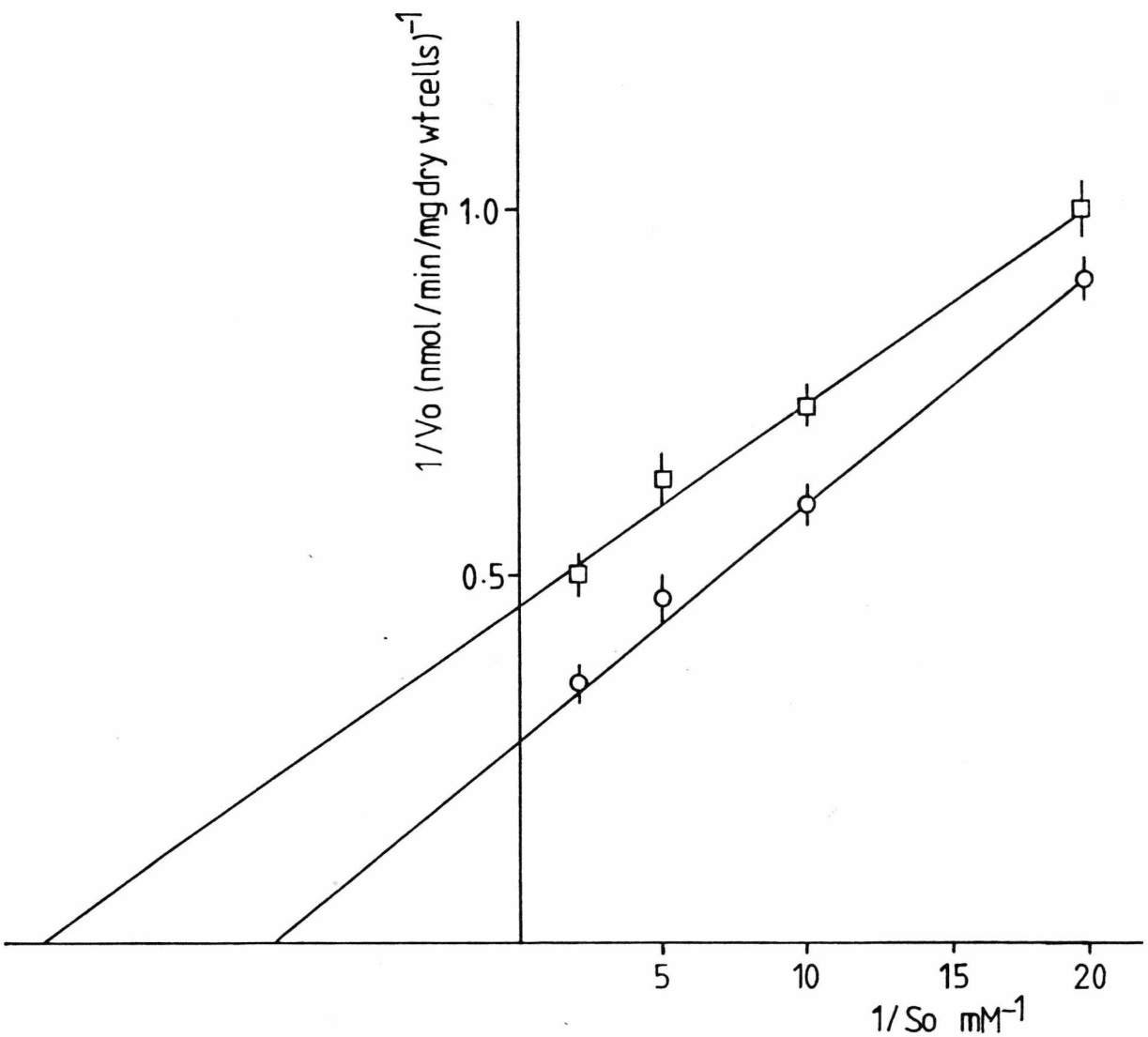

1/Vo (nmol/min/mg dry wt cells)$^{-1}$
1·0
0·5
5
10
15
20
1/So mM^{-1}

Table 4.7 Kinetic values for peptide transport of the wild type and NIK5

The K_M (μM) and V_{max} (nmol/min/mg dry wt. cells) from Figures 4.7-4.10 are summarized.

Peptide	Wild Type		NIK5	
	K_M	V_{max}	K_M	V_{max}
[U ^{14}C] ala ala	110	3.54	62	2.20
ala_2	1225	179	440	39.0
ala_3	219	67	268	91.0
ala_4	141	90	181	189

relating it to transport (Nisbet and Payne, 1979a). This has the considerable advantage of not being limited by the availability of labelled substrate, since fluorescamine will react with any substrates possessing a free primary amine group. The selective assay of peptides, even in the presence of amino acids, is possible since the fluorescence of the product is pH dependent. As very few competition studies were necessary this method gives the first direct study of peptide transport in *C.albicans*.

To assist in this investigation a mutant of *C.albicans* 124 resistant to the effects of nikkomycin (NIK5), was produced by ultraviolet-irradiation. The properties of the NIK5 peptide permease will be discussed together with those of the wild type.

The transport of peptides by both the wild type and NIK5 was found to be pH dependent. This shows that either ala_3 was transported into NIK5 by a system equivalent to that transporting ala_2 into the wild type, or that the separate systems shared a common component possibly involved in energy coupling. The pH dependence is in partial agreement with previous work with *C.albicans* which have described pH optima at pH 3.5 (Logan, *et al.*, 1979) and pH 4.5 (Davies, 1980). Peptide uptake has also been shown to be pH dependent in *S.cerevisiae* (pH opt 5.5, Becker and Naider, 1977) and bacteria such as *Salmonella typhimurium* (pH opt 6.0, Yang, *et al.*, 1977). The pH may play two roles in transport, firstly the ionization of the substrate will be affected by the pH of the environment and hence the affinity of the peptide for the permease will be altered. Secondly, alteration of the extracellular pH may affect the

pH gradient across the cell membrane and thus alter the proton motive force (pmf). The dependence of transport upon a pmf will be discussed at a later point.

The use of different *C.albicans* strains had a profound effect upon the rate of transport of a single peptide (ala_2). Six strains gave initial rates of uptake ($[S]_o$=0.2 mM) varying between 8.0 nmol/min/mg (strain 113) and 38.0 nmol/min/mg (strain 124). Either variation in the absolute capacity for peptide transport or differing pH optima may account for the range of rates obtained. In the latter case the rate of uptake may not have been assayed at the pH optimum for the strain. Reference to Figure 4.1 shows that alteration of the pH by 1.0 unit from the pH optimum reduces the rate of ala_2 transport (wild type) to 10.0 mnol/min/mg at pH 4.0 or pH 6.0, thus a small variation in the pH optimum would produce a marked effect upon the apparent rate of transport. This is reflected by the differing pH optima reported for strains WD18-4 (pH 3.5 Logan, *et al.*, 1979) and 6406 (pH 4.5. Davies, 1980). There is therefore the need for a standard strain of *C.albicans* to be used in comparative studies of this nature.

Ala_2 transport was found to be sensitive to the presence of sodium arsenate, sodium azide and 2,4-DNP. Arsenate reduces the cellular ATP levels and implies the involvement of an ATPase in energization, while 2,4-DNP and azide are both uncouplers of electron transport. The effects of these inhibitors is in agreement with the findings of Davies (1980) for gly phe uptake and of Logan *et al.*(1979) for met_3 transport. Both

reports also found CCCP and DCCI to be inhibitors of the *C.albicans* peptide permease. The uptake system of *S.cerevisiae* has also been found to be sensitive to these inhibitors in addition to the ATPase inhibitor diethylstilbestrol (Payne and Nisbet, 1981).

ortho-Vanadate has been found to be a potent inhibitor of ATPases from various sources including the H^+-ATPase of *N.crassa* plasma membane (Addison and Scrbrough, 1981), the gastric H^+/K^+-ATPase (Faller *et al.*, 1981) and the renal Na^+/K^+-ATPase (Grantham and Glynn, 1979). The inhibitory effect of vanadate on peptide transport in *C.albicans* could be through inhibition of either a H^+-ATPase or the Na^+/K^+-ATPase which is inhibited by DCCI.

Peptide permeases are generally assumed to be located within the plasma membrane together with the amino acid, monosaccharide and other substrate permeases. The presence of amino acid and monosaccharide permeases in the plasma membrane has been confirmed by the finding that plasma membrane vesicles (*S.cerevisiae*) are able to transport glycine and proline (Merkel, *et al.*, 1980) in addition to the monosaccharides glucose and galactose (Fuhrman, *et al.*, 1976). There is also evidence for a plasma membrane ATPase distinguishable from that of the mitochondrial membrane in both *Schizosaccharomyces pombe* (Delhez, *et al.*, 1977; Foury, *et al.*, 1977) and *Candida albicans* (Marriott, 1975) in addition to other microorganisms. By analogy with *N.crassa* (Bowman and Slayman, 1977) the plasma membrane ATPase has been implicated in proton pumping. Triton X-100 was found to inhibit peptide uptake: At least two mechanisms

of action are possible. Firstly, Triton X-100 may solubilize membrane proteins, including components of the permeases and the ATPases, and hence reduce the activity of the transport system. This surfactant has been used to solubilize the mitochondrial ATPase from *N.crassa* and *S.cerevisiae* (Sebald and Wild, 1979; Tzagoloff, 1979) while membrane fragments (containing complexes of the respiratory chain) were released from vesicles of *Mycobacterium phlei* (Brodie, *et al.*, 1979). It is therefore a possible mode of action for the inhibition of the permease. Secondly, Triton X-100 may interact with the cell membrane and lead to an increase in proton-permeability. The proton permeability of the rat liver mitochondrial membrane has been shown to be increased in the presence of Triton X-100 (Carefou, *et al.*, 1968). A similar effect has been found for Amphotericin B (Palacios and Serrano, 1978). Proton leakage would prevent the maintenance of a p.m.f. and hence halt peptide uptake. The importance of the p.m.f. in peptide transport has been demonstrated in both *S.cerevisiae* (Payne and Nisbet, 1981) and *C.albicans* (Davies, 1980) through the inhibition of the system by CCCP. The p.m.f. would therefore appear to provide the driving force for the uptake of peptides in addition to those of the amino acids.

Peptide transport was found to be dependent upon a supply of D-glucose as the presence of 2-deoxy-D-glucose prevented accumulation. Both 4-deoxy-2-glucose and 6-deoxy-D-glucose have been shown to be transported via the galactose carrier of *S.cerevisiae* although they do not serve as a substrate for yeast hexokinase (Kotyk, *et al.*, 1975). 2-deoxy-D-glucose has been shown to markedly decrease the intracellular ATP levels of yeast

(Reinhard, *et al.*, 1976) again presumably because it is not a substate of hexokinase. The abolition of ala_2 transport in the presence of 2-deoxy-D-glucose implies that there may be only a very low intracellular ATP pool which cannot be replenished from the deoxysugar. The cells used were in mid-exponential phase of growth at which point glucose turnover is rapid, the intracellular glucose pool would therefore be expected to be low and hence rapid inhibition of energy-dependent peptide transport is observed in the presence of 2-deoxy-D-glucose.

The evidence available suggests that exogenous glucose is transported into *C.albicans* and serves as a substrate for glycolysis. Subsequent ATP production and ATPase action produces a proton-motive force across the plasma membrane which is coupled to the peptide permeases (in addition to those for other substrates). The accumulation of intact peptides has not been demonstrated in this study; however the system is active in the sense that it required metabolic energy. Both gly phe and alafosfalin were accumulated by *C.albicans* 6406 (Davies, 1980) indicating that transport is active in this organism. The accumulation of sarcosine-containing peptides by *S.cerevisiae* has been demonstrated (Nisbet and Payne, 1979a) and by analogy such an accumulation would be expected in *C.albicans* 124.

Molecular determinants of transport

If the peptide transport system is to be used as an illicit uptake system for drugs it is necessary that the structural limitations of transportable peptides should be determined.

Peptide transport was found to be highly specific for L-isomers. For example, only L-ala-L-ala showed a significant rate of transport while L-ala-D-ala was taken up at a very low rate. D-ala-D-ala and D-ala-L-ala were not transported. Davies (1980) found that none of the stereoisomers of ala_2 except L-ala-L-ala interacted with the uptake of radiolabelled peptides. This suggests the presence of a highly stereospecific binding site associated with the transport system which prevents the entry of potentially toxic amino acid residues. Thus, stereochemical selection takes place at the level of binding rather than transport. Although L-ala-D-ala was transported at a low rate in the present study no interaction was found by Davies (1980), implying that this peptide may have only very low affinity for binding and hence cannot compete with ala_2 uptake at any level. Logan, *et al.*, (1979) showed that D-met-L-met-L-met but not L-met-L-met-D-met competed with the uptake of radiolabelled met_3. Some tolerance of D-residues at the oligopeptide binding site does therefore exist. This cannot be directly related to transport since binding is not always accompanied by subsequent uptake and may reflect the situation found with leu_2 in this study.

Peptide transport systems of other microorganisms have also been found to be highly sterospecific: the dpp of *E.coli* (Payne, 1980) and the peptide permease of *S.cerevisiae* (Nisbet and Payne, 1979b) accept only L-isomers. However, the opp of *E.coli* does show some toleration of D-residues at the C-terminal position, a parallel with the low degree of toleration found in *C.albicans* (Logan, *et al.*, 1979). The amino acid transport systems of both bacteria and fungi are also highly specific for

L-isomers (only the gap of *S.cerevisiae* has been shown to tolerate D-isomers (Rytka, 1975)). Stereochemical specificity therefore appears to be a general property of substrate-transport systems.

Amidation ofthe carboxyl terminus of ala_2 prevented its uptake into *C.albicans* 124. Similarly Logan *et al.* (1979) found that esterification of met_3 eliminated transport. These data are in agreement with earlier nutritional studies which showed that the methyl esters of met_3, met_4 and met_5 did not support growth of a *C.albicans* methionine auxotroph (Lichliter, *et al.*, 1976). The requirement for transport is for a free acidic function as the carboxyl moiety could be replaced by phosphonate, sulphonate or tetrazole groups without eliminating their competitive effect towards uptake of radiolabelled peptides (Davies, 1980). Furthermore the intracellular accumulation of alafosfalin has been reported (Davies, 1980) confirming that the interaction observed was not simply at the binding site. Esterification of these unusual acidic functions reduced the affinity of the peptide derivatives for their permease. A similar requirement for a free acidic terminal has been described for *E.coli* (Allen, *et al.*, 1978). However, the carboxyl group of met_3, met_4, or met_5 could be esterifiedwithout eliminating transport by the permease of *S.cerevisiae* (Naider, *et al.*, 1974). The difference in the effect of C-terminal blocks on the rate of transport into *C.albicans* and *S.cerevisiae* suggests that species selective toxic agnets might be developed from peptide substrates.

Acetylation of the amino terminal of ala_2 did not remove its ability to inhibit ala_2 transport in *C.albicans* 124. Although this may indicate transport of the acetylated derivative into the cell it could also be competition at the level of binding without subsequent translocation. Davies (1980) found no competition between N-acetyl ala_3, N-acetyl ala gly ala or N-acetyl ala pro ala for ala_3 transport in *C.albicans* 6406 and concluded that the N-acetyl derivatives were not taken into cells. N-acetyl met_3 could act as a growth substrate for *C.albicans* WD18-4 (Lichliter *et al.*, 1976) and competed with radio-labelled met_3 transport (Logan, *et al.*, 1979). Conclusive evidence that N-terminal blocked peptides are transported into *C.albicans* came from the finding that N^4(Succinyl ala leu)5-FC was transported intact into *C.albicans* WD18-4 (Ti, *et al.*, 1980). Chmara *et al.*, (1980) confirmed this finding by showing that N-acylated epoxypeptides were toxic for *C.albicans*. The present study agrees with the transport of N-terminal blocked peptides. The lack of apparent transport of N-acylated peptides by strain 6406 (Davies, 1980) could again be attributed to a strain difference. Further discussion of possible strain differences will be reserved until later. N-acylated peptides were not transported by *E.coli* (Gilvarg and Katchalski, 1965). The requirement was for an N-terminal positive charge (Payne, 1971) since N-alkyl but not N,N-dialkyl peptides were taken up. Fungal systems differ in their specificity. N-acylated peptides were tolerated by the permease of *S.cerevisiae* (Marder, *et al.*, 1977) but not by that of *N.crassa* (Wolfinbarger and Marzluf, 1975b). Substitution of the amino terminal abolished peptide transport in the mammalian gut (Rubino, *et al.*, 1971,

Das and Radhakrishnan, 1975) and, although other mammalian tissues have not be studied in depth, this may provide the basis for peptides toxic to certain microorganisms such as *C.albicans* and yet not toxic to the host.

The rate of transport of a number of dipeptides and oligopeptides was determined to demonstrate the dependence of transport upon side chain residues and chain length. The series of ala-x peptides showed that hydrophobic residues at the C-terminal enhanced the rate of transport when compared with polar or hydrophilic residues. A similar variation of the N-terminal in the series x-ala showed a different specificity of the order methyl$\geq$polar>hydrophobic>hydrophilic. The presence of an N-terminal glycine residue reduced the rates of transport of the equivalent ala-x peptides indicating a requirement for a side-chain residue other than -H and hence a defined stereochemistry. Davies (1979,1980) also studied the dependence upon side-chain residues using variations in ala-x and x-ala peptides competing for [^{14}C] ala_2 transport. In general the affinity for the permease was of the order neutral and hydrophobic>basic>acidic for both the N- and C-terminals. This is in agreement with the findings of the present study. *S.cerevisiae* showed similar specificity in which basic peptides were transported at a greater rate than those with acidic side-chains (Nisbet and Payne, 1979b).

Although ala_3 and ala_4 were competitors for dipeptide transport in *C.albicans* 6406 (Davies, 1980) the uptake of radiolabelled ala_4 could not be detected. This is contrary to the present study in which

both ala_3 and ala_4 were shown to be transported at a high rate (ala_2=38.0 nmol/min/mg; ala_3=30.3 nmol/min/mg ; ala_4=52.6 nmol/min/mg) with uptake of ala_4 occuring at the highest rate of any peptide tested. The properties of the peptide permeases of strain 6406 (Davies, 1979,1980) and strain 124 used in the present study are therefore very different in both chain length specificity and the effect of an N-terminal block. *S.cerevisiae* showed a similar dependence upon chain length as that observed for *C.albicans* 6406: ala_3 was transported while uptake of ala_4 could not be detected (Nisbet and Payne, 1979b).

The nikkomycin used in this study contained mainly the dipeptide nikkomycins X and Z which differ in the base moiety present (Figure 1.2. Zahner *et al.*, 1982). The peptides of both are identical and contain an unusual polar-aromatic residue at the N-terminus [2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-methylbutyric acid]. Although the C-terminal side chain is coupled to a nucleoside, nikkomycin is transported via the peptide permease (Chapter 3) indicating the large side-chain variation permissable for this uptake system. Nikkomycins are highly active against *C.albicans*. In contrast the polyoxins, which are closely related peptide nucleosides, while highly active against cell-free *C.albicans* chitin synthase generally have low activity against the intact organism (Chiew, *et al.*, 1980). All amino acid residues of both the polyoxins and nikkomycins are in the L-configuration (Isono and Suzuki, 1979; Konig, *et al.*, 1980). Previous studies have shown the polyoxins to enter the fungal cell via the peptide permeases (Mitani and Inoue, 1968; Hori, *et al.*, 1976). In the present study a mixture containing 10% polyoxins A,B, K and L was able to competitively inhibit the transport of [$U^{14}C$] ala ala into *C.albicans*. The K_i for this effect

was found to be 195 $\mu g\ ml^{-1}$, a far higher value than that found for nikkomycin (4.85 $\mu g\ ml^{-1}$). If the polyoxins were the only components competing for transport this would give an apparent K_i of 19.5 $\mu g\ ml^{-1}$ which is still far higher than that found for nikkomycin. As insufficient pure polyoxin D was available further interpretation of the results was not possible. Recent work has indicated that *C.albicans* is sensitive to one of the polyoxins (polyxin B, Mehta *et al.*, 1982). The effect of polyoxin B was found to be medium-dependent such that the MIC varied between 1000 $\mu g\ ml^{-1}$ on complex media and 0.1 $\mu g\ ml^{-1}$ on a more defined medium. Further data state that the activity of polyoxin B was antagonized by certain dipeptides and oligopeptides suggesting that the drug enters the cell via the peptide permease. Peptide transport capacity may be activated by the medium change. Alternatively the uptake of polyoxin B may be pH dependent in wich case alteration of the pH of the growth medium will greatly influence the apparent MIC . An equivalent pH effect has been found for the activity of nikkomycin (Dr. P. Troke, personal communication).

A comparison of the structures of the polyoxins and nikkomycins indicates a significant difference in the N-terminal residue which is polar and aromatic for the nikkomycins but hydrophilic for the polyoxins (Figures 1.1 and 1.2). Therefore there is the possibility that the N-terminal residue significantly alters the affinity of the drug for the permease. A comparison with transported dipeptides confirms this since polar N-terminal residues from the sequence x-ala have a greater affinity than hydrophilic residues. This provides one possible explanation for the low toxicity of most polyoxins, however further work is necessary to confirm this suggestion.

The molecular determinants of transport therefore appear to be:

1. Dipeptides must be in the L-L configuration. D-residues at the C-terminal might be tolerated but the resultant rate of transport is low.
2. The C-terminal cannot be amidated or esterified. Other acidic functional groups can replace the carboxyl moiety without eliminating transport.
3. The N-terminal can be acylated
4. The side-chain specificity is very broad, linkages to other molecules do not eliminate transport.

Multiplicity of transport systems

A comparison of those peptides able to antagonize the action of nikkomycin and their rates of transport (Tables 3.1 and 4.3) reveals that transported dipeptides (ala_2 and L-leu gly) compete with nikkomycin uptake while non-transported dipeptides (gly tyr, D-leu gly and gly_2 show no such effect. This indicates that competition between peptides and nikkomycin occurs at the level of transport as has been confirmed by the previously described competition studies between [U^{14}C] ala ala and the drug.

However, there are two exceptions. Firstly, leu_2 was not transported at a significant rate and yet competed with the uptake of nikkomycin. Secondly, the oligopeptides, ala_3 and ala_4, were transported at a high rate and yet failed to interact with the uptake of the drug.

For leu_2 to antagonize nikkomycin action there must be an interaction between this peptide and the permease although leu_2 does not enter the cell. Davies (1980) found leu_2 to have a high affinity for the peptide uptake system and interpreted the data as indicating the rapid transport of the peptide. This competitive effect was again found in the present study: the presence of leu_2 at an equimolar concentration greatly reduced the rate of $[U^{14}C]$ ala ala incorporation. Therefore, it appears possible for peptides to bind to the transport system, or to a component of that system, without their subsequent translocation. Binding proteins have been identified for many bacterial transport systems including those for the amino acids (for example Anraku, 1968) where they appear to increase the substrate availability for the permeases. Amino acid binding proteins have also been found in the filamentous fungus *N. crassa* (Wiley, 1970) while a maltose-binding protein has been identified in *S. cerevisiae* (Singh, *et al.*, 1981). *C. albicans* has been shown to possess an inducible N-acetyl-D-glucosamine binding protein (Singh, *et al.*, 1980) and so there is a considerable precedent for the involvement of such proteins in permease systems. Thus, it is possible that the interaction between leu_2 and nikkomycin occurs at a peptide-binding site on a specific protein producing an interaction with the transport system at a level other than translocation. Therefore the results of competition studies btween radiolabelled and non-labelled peptides cannot necessarily be directly related to the rate of transport of the non-labelled substrate.

The observation that ala_3 and ala_4 (both of which are transported into the cell) do not antagonize nikkomycin action is susceptible to two

explanations: firstly, oligopeptides may have a far lower affinity for transport than nikkomycin and hence do not compete with it. However, this is improbable since oligopeptides were transported at a higher rate than dipeptides, at an equivalent concentration, and would be unlikely to have reduced affinity for a single permease. Secondly, the transport of oligopeptides and dipeptides may occur via separate transport systems. If this is the case it will be the first evidence of multiple peptide transport systems in a fungal species.

The evidence presented by Davies (1980) indicated that di- and tripeptides competed for transport. This implied the presence of a single permease. Logan, *et al.*, (1979), studying radiolabelled met_3 uptake found that met_2 and N-acetyl-met_3 showed only a low affinity for the permease and concluded that these peptides were either transported at a low rate or could be transported via a separate system.

Although *N.crassa* and *S.cerevisiae* appear to have only single peptide transport systems (Wolfinbarger and Marzluf, 1975b; Marder, *et al.*, 1978; Nisbet and Payne, 1979b) bacteria have been shown to possess two (*E.coli*, Payne, 1968; Gilvarg and Levin, 1972) or even three (*S.faecalis*, Nisbet and Payne, 1982; Payne, *et al.*, 1982) permeases. Each permease has high specificity, one for dipeptides (the dpp) and the second for oligopeptides (the opp) while the third is highly specific for anionic peptides (the App). Multiple amino acid permeases occur in *S.cerevisiae*, *N.crassa* and *C.albicans* and so the existance of multiple peptide permeases cannot be excluded in the present study.

Multiple permeases in bacteria have been demonstrated by the use of toxic peptides such as tri-L-ornithine and alafosfalin which can be used to select for opp^- and dpp^- mutants respectively. However, bacilysin resistance in S.cerevisiae removed all capacity for peptide transport (Nisbet and Payne, 1979b) indicating the presence of a single permease. Equally, resistance to gly leu tyr in *N.crassa* reduced the peptide transport capacity by >90% (Wolfinbarger and Marzluf, 1975b) demonstrating the presence of a single oligopeptide permease). Thus, resistance to nikkomycin (which enters the cell via a system which also transports dipeptides) resulting from a defect in transport gives further information concerning multiple peptide permeases in *C.albicans*.

The *C.albicans* mutant NIK5 which was resistant to nikkomycin also had a general inability to transport dipeptides. Of those assayed only ala_2 and ala met were taken into cells but at a rate considerably lower than that found in the wild type. This suggests that dipeptides and nikkomycin share a common transport system. Thus, resistance to the drug, through a defect in transport, caused a concomitant reduction in the rate of dipeptide uptake.

In contrast the oligopeptide transport rates were increased by as much as 253% (met ala ser) when compared with the wild type. This observation, in conjunction with the data concerning the absence of antagonism between oligopeptides and nikkomycin, provides strong evidence that two peptide transport systems are operational in *C.albicans* one

handling dipeptides including the nikkomycins and the other transporting oligopeptides.

A comparison of the sensitivity of the wild type and NIK5 to toxic di- and tri-peptides indicates the effect of the mutation on the activity of such drugs. Cross resistance with nikkomycin was demonstrated for the dipeptide bacilysin and crude polyoxin, although the latter prouced only a small zone of inhibition against the wild type. The tripeptide m-F-phe ala ala did not show clear cross-resistance, although NIK5 was less sensitive than the wild type. This drug is a good example of the concept of illicit transport (Ames, *et al.*, 1973) via the peptide transport system. The toxic moiety m-F-phenylalanine (which is active due to its incorporation into cellular protein) is not transported by the amino acid permeases of *C.albicans* (Davies, 1979). It was also found to be non-toxic in the present study. However, the tripeptide m-F-phe ala ala is transported and, following its hydrolysis, releases m-F-phenyl alanine intracellularly. If tripeptide transport was separate from dipeptide uptake, resistance to nikkomycin and hence loss of dipeptide transport capacity would have no effect upon the sensitivity to m-F-phe ala ala. The rates of oligopeptide uptake by NIK5 were greater than those of the wild type (YNBP-grown cells) and on this basis NIK5 should be more sensitive than the wild type to the tripeptide drug. However, mutant cells were less sensitive to m-F-phe ala ala although total cross resistance was not observed. Hence the tripeptide was still entering the cell. The activity of the drug was assayed on MX and the rate of uptake of m-F-phe ala ala by the wild type and NIK5 grown on MX reflected the sensitivity observed in NIK5 (rate of uptake by

wild type=18.0 nol/min/mg; rate of uptake by NIK5=7.0 nmol/min/mg).

This raises two important points: firstly, partial loss of the ability to transport m-F-phe ala ala occurs upon gaining resistance to nikkomycin. Secondly, the rates of peptide uptake by MX-grown cells are considerably different from those observed in YNBP-grown cells: For YNBP-grown cells the rates of oligopeptide transport by NIK5 were greater than those observed in the wild type while for MX-grown cells the converse was true.

The partial loss of ability to transport oligopeptides in addition to dipeptides (MX-grown cells) indicates that these share a common permease. However, this system (System I) must have high affinity for dipeptides (and nikkomycins) but low affinity for oligopeptides which show no inhibition of nikkomycin action. An analogous situation is found with the bacterial oligopeptide permease which has high affinity for oligopeptides but low affinity for dipeptides (Alves and payne, 1980). That m-F-phe ala ala still enters the cell in the absence of System I reinforces the theory that a second system with high affinity for oligopeptides (System II) is also operational.

Previous studies have shown the growth medium to have a marked effect upon the subsequent ability of the cells to transport peptides. Cells grown on isoleucine as opposed to ammonium salts as the sole nitrogen source showed a greater ability to transport met_3 (Logan, *et al.*, 1979) while gly phe uptake in peptone-grown cells was more rapid than in proline-grown cells (Davies, 1980). This may be attributed either to permease induction or to a reduction in ammonium repression

brought about by the presence of another nitrogen source. Certain ammonium-repressable amino acid transport systems, including the gap of *S.cerevisiae* (Greson and Hou, 1972) have been well characterized and serve to regulate the nitrogen source available for metabolism. Inducible transport systems are equally common and include the proline permease of *C.albicans* which is induced by the presence of proline as the sole nitrogen source (Jayakumar, *et al.*, 1979). The induced carrier is then operational in addition to a constitutive proline permease. Not only permeases are inducible in *C.albicans*: an extracellular protease is induced when proteins serve as the nitrogen source (Staib, 1965). The uptake of nitrogen-containing metabolites is therefore strictly controlled by a system of induction and /or derepression which allows growth on a wide range of substrates. The present study found that the presence of peptone in the growth medium brought about an increased ability to transport peptides in both the wild type and NIK5 whereas the rates of uptake on MX or the ammonium-based YNB were equivalent. Peptone may therefore act as an inducer for the peptide transport systems. A further observation was the lag period before growth which occured when NIK5 cells were transfered from YNB or MX to YNBP. This amounted to 4 hours after which point the growth rate became equivalent to that observed for the wild type on YNBP. The latter showed no such lag period. It is possible that activation or induction of peptide transport capacity might play a role in this lag period. The initial low rate of transport by MX-grown cells could be a limiting factor for cell growth with peptides as the sole nitrogen source. It was found that NIK5 cells grown on MX rapidly increased in their ability to transport ala_3 on transfer to YNBP. The increase occured over a period of 3 hours, which is within the lag period observed on transfer between the two media.

Either derepression or induction could account for this observation. To distinguish between these possibilities a protein synthesis inhibitor was used to block any synthesis occuring during the period of increase in peptide permease activity. Cycloheximide could not be used since it is inactive against *C.albicans* (Table 3.3 and Speller, 1980). With an alternative inhibitor blasticidin S (which has been shown to inhibit *C.albicans* protein synthesis, Yamaguchi and Iwata, 1970) no increase was evident in the ability of cells to transport ala_3 on transfer to YNBP. Equally no increase was observed on transfer from MX to fresh MX indicating that the increase was not simply due to a supply of fresh growth medium.

Therefore it would appear that NIK5 either synthesizes the complete transport system (SystemII) or a component, such as a binding protein, on transfer to peptone containing media. The possible presence of binding proteins has already been discussed and further work is necessary to confirm their association with the peptide permease.

There was a less pronounced increase in peptide transport capacity when the YNBP-grown wild-type was compared with MX-grown cells. However, the induction observed with NIK5 again appeared to be operational. The presence of multiple permeases may account for both the absence of a lag period on transfer of the wild type from MX to YNBP and also the lower degree of induction which was apparent. The wild type is able to take in peptides via Systems I and II and therefore does not require such a marked induction as NIK5 (which possesses only System II) to gain a sufficient nitrogen source for the support of growth.

Kinetics of peptide transport

Comparison of YNBP grown wild type and NIK5 showed a marked difference in ala_2 transport. In the former uptake was through a low affinity (K_m=1.225 mM) but high capacity (V_m=179 nmol/min/mg) system. The latter transported ala_2 via a system with higher affinity (K_m=440 μM) but lower capacity. In both isolates ala_3 and ala_4 had higher affinities for the permease than ala_2. Therefore it is not possible to explain the absence of antagonism between oligopeptides and nikkomycin in terms of a single permease. Thus, the presence of at least two permeases is again indicated. However, the kinetic values were obtained for induced cells whereas all studies of antagonism were conducted using non-induced cells. Further work must be carried out to determine the kinetics of transport for cells grown on YNB and MX to show the properties of the constitutive peptide permeases, and hence give data which may normally be masked by the presence of an inducible system. This will be aided by the recent development of a continuous monitoring system based upon the fluorescamine technique (Payne and Nisbet, 1981). Studies with many other peptides are also necessary before any understanding of the process is possible.

Nikkomycin was found to be a competitive inhibitor of ala_2 transport into induced wild type cells (K_i=4.85 μg ml^{-1}) and even though NIK5 shows a defect in its ability to transport dipeptides the competition between nikkomycin and ala_2 was also evident in these induced cells (K_i=5.65 μg ml^{-1}). Therefore there is very little difference in the competitive effect. Two possible explanations exist: nikkomcyin may be transported into NIK5 and the wild type at different rates while the interaction at the binding site is unaltered. Alternatively, nikkomycin may not be transported via the constitutive

permease of NIK5 and yet enter through the induced permease. In the case of induced cells the rates of uptake may be equivalent in both the wild type and NIK5 thus explaining the similarity of K_i values obtained. Again, further work is necessary to clarify the situation using both induced and constitutive cells.

A comparison of the kinetic values obtained in this study and those found by previous workers showed considerable difference in both K_m values and the maximal velocities attainable. The K_m values previously described were for met_3 33 µM (Logan, *et al.*, 1979), gly phe 3.50 µM , ala_2 350 µM , and ala_3 115 µM (Davies, 1980). The values obtained in the present study were higher. However, the most noticable difference was in the K_m values for ala_2 transport (wild type=1.225 mM , NIK5=440 µM). This can be attributed either to the difference in the assay methods or to a considerable variation between strains of *C.albicans*. To differentiate between these possibilities, the kinetics of $[U^{14}C]$ ala ala incorporation were studied for both the wild type and NIK5 . The K_m values of 110 µM (wild type) and 62 µM (MIK5) were of the same order as those described by Davies (1980). Therefore the variation in the kinetic parameters obtained by can be attributed to the assay method. No further kinetic data have been published for the fluorescamine technique. However, the differences between this and the radiolabelled peptide method can be explained by a number of interacting events:

1. The fluorescamine method assays peptide uptake and is unaffected by subsequent metabolism or efflux of metabolites.
2. Radiolabelled peptides are subjected to transport followed by hydrolysis. The rate of incorporation of radiolabel is hence influenced by both the kinetic parameters of the permease, those of the peptidases and any subsequent metabolism. This may lead to efflux of either amino acids, carbon skeletons or volatile ^{14}C (CO_2).

This effect will be considered further in Chapter 5 after a discussion of the peptidases of *C.albicans* and the metabolism of peptides.

Summary

It is suggested that *C.albicans* possesses two peptide transport systems. The systems have overlapping specificities. However their affinities towards peptides of differing chain length show converse properties. System I has higher affinity for dipeptides (including dipeptide nikkomycins) than for oligopeptides, whereas System II has higher affinity for oligopeptides than for dipeptides. It is System I which is absent in NIK5 . Both systems would appear to be operational in YNB and MX as is witnessed by the sensitivity of both the wild type and NIK5 to m-F-phe ala ala . On these media (YNB and MX) the systems are present at a constitutive level. In the presence of peptone as the sole nitrogen source there is an additional, inducible level of peptone transport which results in increased activity of System II and possibly also System I.

This model is in partial agreement with the results of both Davies (1980) and Logan *et al.* (1979). Davies, using strain 6406 , showed mutual competition between ala_2 and ala_3 and concluded the presence of a single transport system. It was also stated that acetylated tripeptides could not compete for the permease. The model presented here suggests that ala_2 and ala_3 are transported by both Systems I and II although the specificites of each differ. This could give the mutually competitive effect for transport. Davies (1980) also found that ala_4 competed with transport of ala_2 and ala_3 which again agrees with this model. Transport of ala_4 was not observed by Davies (1980). This leads to the speculation that strain 6406 possesses the System I permease in the absence of System II. In System I the rate of ala_2 uptake is higher than that of ala_3 or ala_4 and so the rate of accumulation of ala_4 may not have been detected in strain 6406. The maximal velocities described by Davies (1980) were 5.1 nmol/min/mg for ala_2 and 4.7 nmol/min/mg for ala_3 and would therefore agree with this supposition. It is equally possible that N-acetylated peptides enter the cell via System II and therefore Davies (1980) did not see a competitive effect.

Logan *et al.* (1979) studied the transport of met_3 into *C.albicans* WD18-4 and found that met_2 competed only slightly with the uptake of this peptide (16% inhibition at 20-fold excess). No data are available concerning the transport of met_2 into WD18-4 , however it is possible that met_2 and met_3 are entering cells via separate transport systems.

Multiple permeases have been described in the bacteria *E.coli* (Payne, 1968) and *S.faecalis* (Nisbet and Payne, 1982; Payne, *et al.*, 1982). In both cases the oligopeptide permease can transport dipeptides but the dipeptide permease is highly specific. The third system of *S.faecalis* is specific for anionic peptides.

The fungal systems studied have shown the presence of a single general peptide permease in both *S.cerevisiae* (Marder, *et al.*, 1978; Nisbet and Payne, 1979b) and *N.crassa* (Wolfinbarger and Marzluf, 1975b); however, the specificity of the two systems differ. This is therefore the first description of multiple peptide permeases in a fungal species. It is interesting to note that the *C.albicans* System I permease resembles that of *S.cerevisiae* (Higher affinity for dipeptides) while System II is similar to that of *N.crassa* (higher affinity for oligopeptides).

CHAPTER 5: PEPTIDE HYDROLYSIS AND METABOLISM

Introduction

The preceding chapters have described the transport of peptides into *C.albicans* via two specific permeases. Hydrolysis of the peptide bond must occur to release the constituent amino acids if the peptides are to serve as a nutrient source for the cell. Only then can subsequent metabolsim of these amino acids take place through the established metabolic pathways.

Very little information is available concerning the peptide hydrolytic system of *C.albicans*. An inducible extracellular protease, produced when albumin serves as the nitrogen source, has been identified (Remold, *et al.*, 1968). The lack of competition for transport between methionine and [^{14}C] met$_3$ (Logan, *et al.*, 1979) demonstrated that no extracellular peptidase activity was present under the growth conditions used. If extracellular hydrolysis of [^{14}C] met$_3$ had been occuring [^{14}C] methionine would have been released thus reducing the rate of ^{14}C incorporation in the presence of added methionine.

With the exception of a single leucine aminopeptidase (Kim, *et al.*, 1962) the intracellular peptidases have not been described, although Logan, *et al.* (1979) found that [^{14}C] met$_3$ was cleaved rapidly by *C.albicans* WD18-4 . Following 5 mins incubation with met$_3$ only a small proportion of the radiolabel could be recovered as the intact peptide while the majority was present as free methionine. Davies (1980) also

found rapid intracellular hydrolysis of peptides. However, multiple intracellular peptidases have been reported in *S.cerevisiae* (Rose, *et al.*, 1979) and in *N.crassa* (Tan and Marzluf, 1979).

The objects of this section were firstly to describe the peptidases of *C.albicans* and to relate the rate of peptide hydrolysis to the rate of transport and secondly, to determine whether the hydrolysis and metabolism of radiolabelled peptides could have any effect upon the apparent rate of transport.

Results. Section 1: Peptide hydrolysis

Peptide isozymes

Cells frequently possess a considerable number of peptidase isozymes: 11 in *N.crassa* (Tan and Marzluf, 1979); 6 in human erythrocytes (Lewis ad Harris, 1967) and as many as 14 in *E.coli* (reveiwed by Hermsdorf and Simmonds, 1980). In some instances they were recognized in crude cell extracts and partially purified preparations by the use of appropriate substrates. However, because of the considerable overlap in the specificites of the enzymes, characterization was difficult until they were purified. This problem was largely overcome by the use of starch gel and polyacrylamide gel techniques, in which a crude cell extract was separated electrophoretically according to the charge and molecular weight of the protein. The gels were then stained with a suitable substrate-dye mixture. Lewis and Harris (1967) described a starch-gel technique in which the gel was incubated with L-amino acid oxidase, peroxidase and o-dianisidine in addition to a peptide substrate. Areas of

peptidase activity were stained with the released brown dye. This method was modified for use with polyacrylamide disc electrophoresis (PAGE) (Miller and Mackinnon, 1974) and was used by Rose *et al.* (1979) to define four bands with peptidase activity in a crude cell extract of *S.cerevisiae*. However, in neither case were the bands very sharp due to diffusion of the dye deposited at the site of peptidase activity.

Tan and Marzluf (1979) developed a technique to separate the peptidases of *N.crassa* using the incorporation of the auxillary enzyme, L-amino acid oxidase, into a polyacrylamide gel. Following electrophoresis of the cell extract the gel was stained with a mixture of peptide substrate, phenazine methosulphate and nitro-blue tetrazolium chloride. The peptides were hydrolyzed to amino acids which were then converted to imino acids by the oxidase. Imino acids underwent spontaneous hydrolysis to ketoacids with the liberation of ammonia. The reduced oxidase transfered hydrogen to phenazine methosulphate which in turn reduced the ditetrazolium salt. This produced an insoluble diformazan dye at the site of peptidase activity.

A new technique was developed in the present study in 3 stages based upon the method of Tan and Marzluf (1979).

(a) Staining of stick gels

Using this method, Tan and Marzluf (1979) demonstrated the presence of eleven peptidases in *N.crassa*. However, several problems were encountered when it was used in the present study. Firstly, the method

was dependent upon the use of stick gels where reproducibility and hence comparison of the bands were difficult. Secondly, diffusion of the bands was apparent although they were sharper than those found by Rose *et al.*, (1979). Thirdly, the method depended upon the diffusion of the substrate and dye to the site of peptidase activity resulting in a delay between separation of the proteins and the supply of substrates for the staining reaction. This allowed further diffusion of the protein bands.

(b) Peptidase staining of polyacrylamide slab gels

A crude cell extract was separated by PAGE using slab gels. This overcame the problems of reproducibility and comparability since electrophoresis followed by subsequent cutting of the slab gels resulted in many, identical gels. However, although the bands were highly reproducible, the problem of diffusion remained.

(c) Peptidase staining of nitro-cellulose blots

Recently a technique for blotting proteins from polyacrylamide gels to nitrocellulose paper has been developed (Towbin, *et al.*, 1979). The electrostatic binding of the proteins to the surface of the paper has two major effects. Firstly the proteins are concentrated into a very thin layer at the surface without any subsequent diffusion and secondly, handling of the paper is easier than that of the gel making further manipulation possible.

All previous work had used denatured proteins in the blotting technique which was originally developed for the detection of specific cellular proteins with antibodies. In the present study native proteins separated by PAGE, were very rapidly transfered to nitrocellulose sheets where they bound to the surface. This permitted th use of an isozyme staining method. That of Tan and Marzluf (1979) was selected since it gave a more intense dye-band than the other methods.

As the peptidases of an *S.cerevisiae* soluble fraction had already been described (Rose, *et al.*, 1979) they were used as a control for the new technique. The nitrocellulose blots showed the separation of the peptidases into four distinct, sharp bands following incubation in the staining medium for 2-3 hours (Figure 5.1). Four bands of activity were also described by Rose *et al.* (1979) who determined the R_f values as 0.18 , 0.42 , 0.52 and 0.74 respectively. The blotting method showed bands with R_f values of 0.14 , 0.27 , 0.60 and 0.65. Band 3 (R_f=0.60) showed very broad specificity while the remaining bands were morehighly specific and showed preferential cleavage of methionyl peptides. This was in general agreement with the findings of Rose *et al.*, (1979).

Thus, as binding tonitrocellulose did not appear to inhibit peptidase activity, the technique was applidd to *C.albicans* (Figure 5.2). Isozyme staining showed the presence of eight bands of peptidase activity. Bands 3-8 had a considerable overlap in their specificities for dipeptides. Bands 1 and 2 were more highly specific and were only shown to cleave the tripeptide met ala ser. Bands 3-8 had no activity

Figure 5.1 Peptidase isozymes of *S.cerevisiae* S7*a*

A soluble fraction of *S.cerevisiae* S7*a* was separated by PAGE under non-denaturing conditions. The proteins were electrophoretically transfered to a nitrocellulose sheet. Protein bands were revealed by staining with amido black (A). Unstained strips of nitrocellulose were incubated in the staining medium (peptide substrate, L-amino acid oxidase, phenazine methosulphate and nitroblue tetrazolium chloride) at 37°C for 2-3 hours until the appearance of bands of peptidase activity. Peptide substrates were as follows:

B	leu_2
C	met_2
D	leu ala
E	gly met
F	gly leu
G	met ala

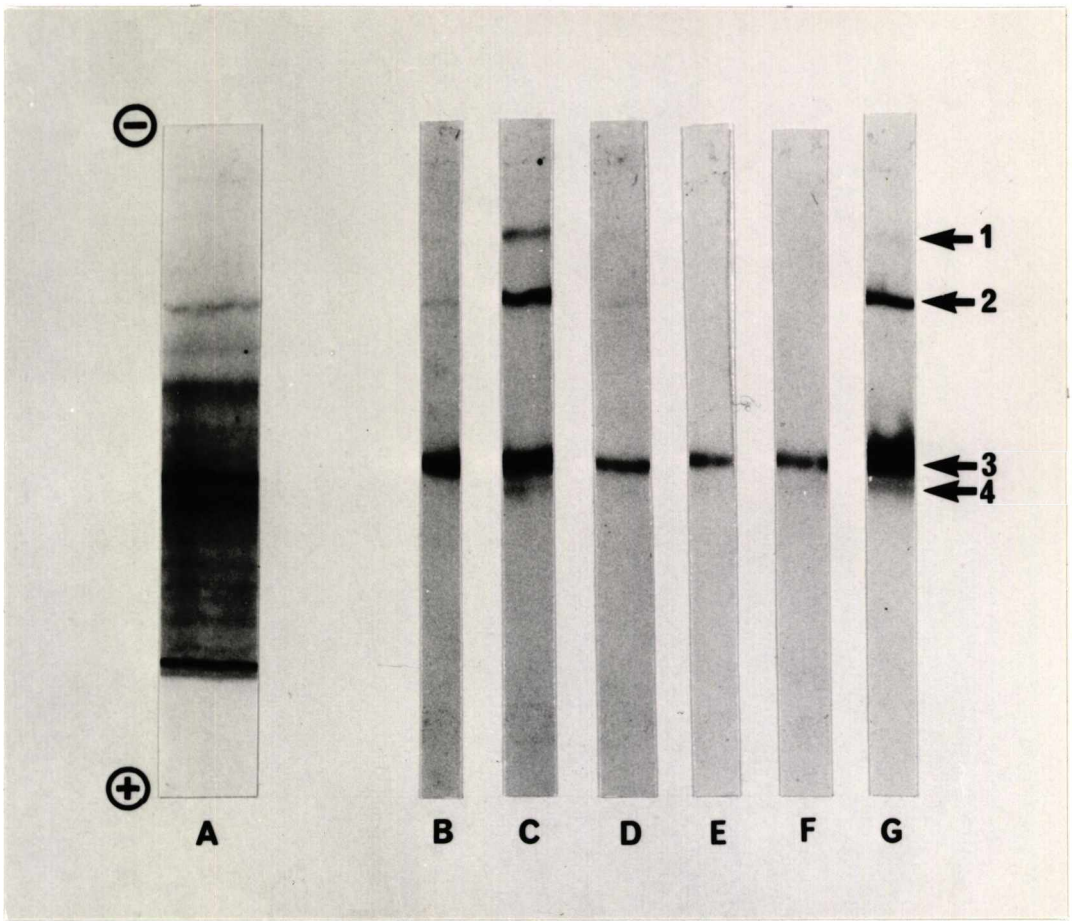
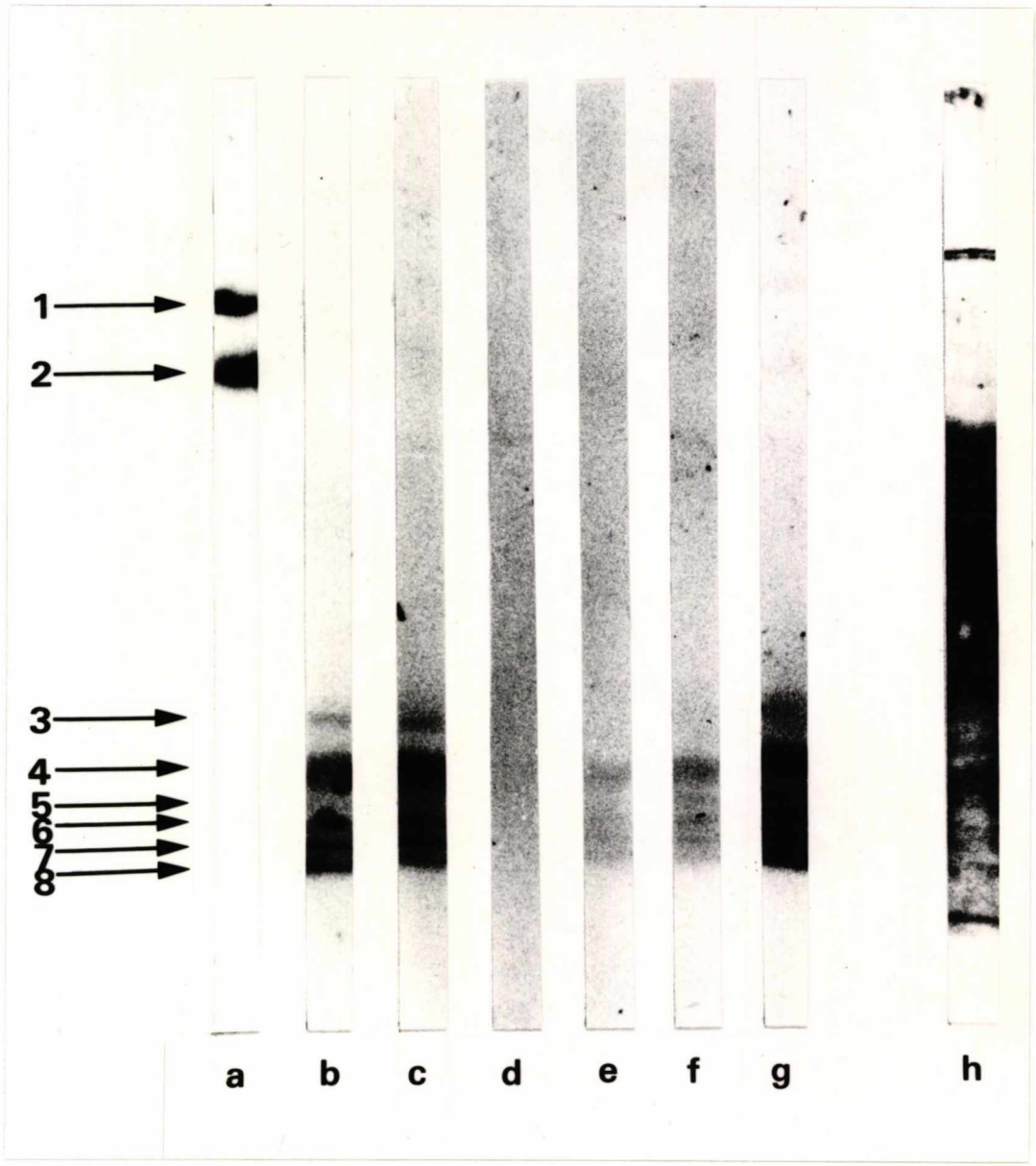

⊖
⊕
A
B
C
D
E
F
G
1
2
3
4

Figure 5.2 Peptidase isozymes of *C.albicans* 124 (wild type)

The method used was identical to that described in Figure 5.1.

A	met ala ser
B	ala leu
C	leu ala
D	ala his
E	gly phe
F	tyr_2
G	met ala
H	protein bands visualized amido black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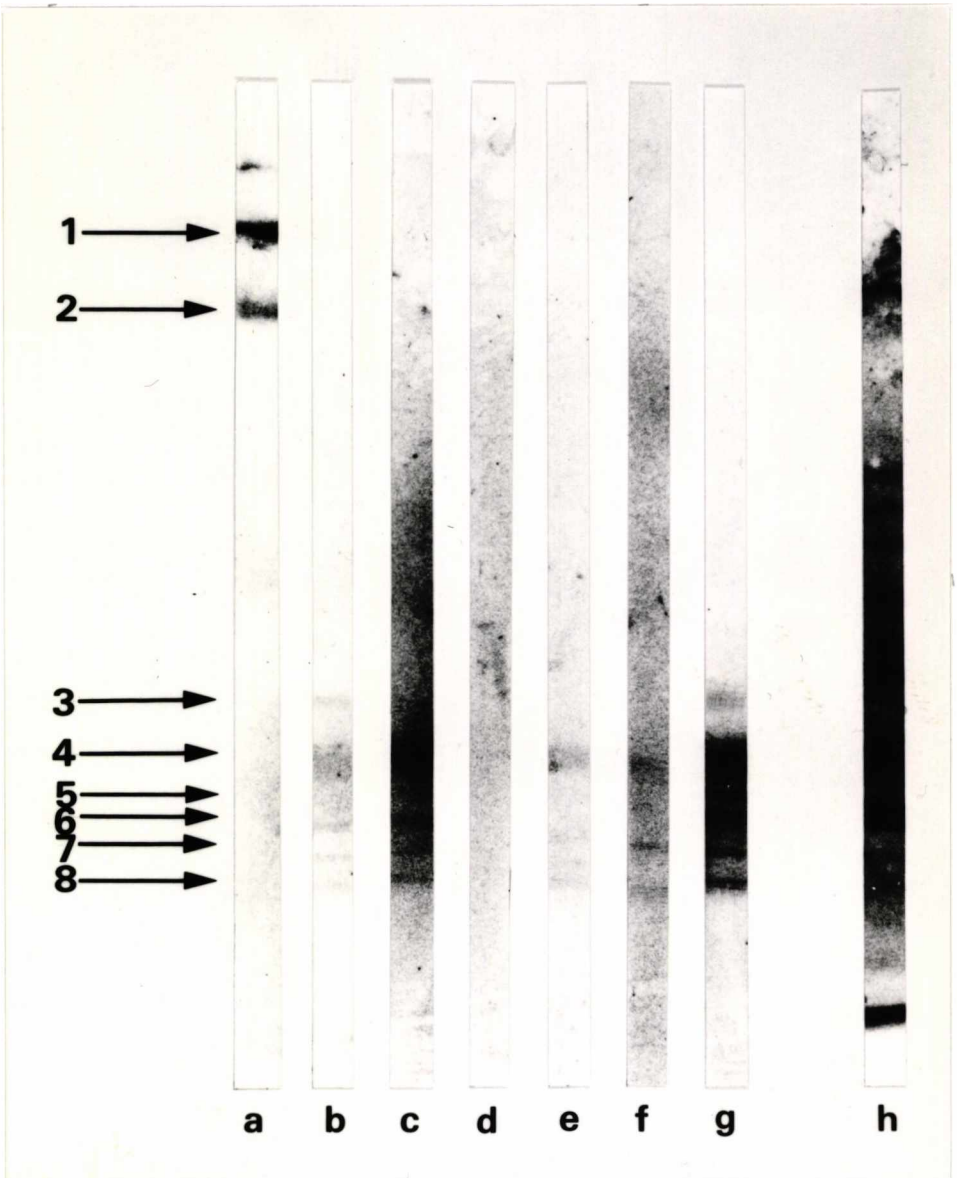
b
c
d
e
f
g
h

Figure 5.3 Peptidase isozymes of *C.albicans* 124 NIK5

The method used was identical to that described in Figure 5.1.

A	met ala ser
B	ala leu
C	leu ala
D	ala his
E	gly phe
F	tyr_2
G	met ala
H	protein bands visualized with amido black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against this tripeptide and it is probable that these are dipeptidases whereas bands 1 and 2 represent amino- and/or carboxypeptidases. This could not be firmly estbalished in the absence of the correct substrates.

The isozymes of *C.albicans* NIK5 (Figure 5.3) were found to be comparable to those of the wild type both in their specificities and R_F values: (wild type)=0.065 , 0.17 , 0.66 , 0.73 , 0.77 , 0.81 , 0.83 , and 0.86; R_F (NIK5)=0.06 , 0.17 , 0.63 , 0.68 , 0.73 , 0.75 , 0.80 and 0.83 . Therefore, the loss of peptide transport capacity does not appear to be reflected in the peptidases of the organism.

In both the wild type and NIK5 a small proportion of peptidase activity was observed at the top of the 6% polyacrylamide gel which had been blotted onto nitrocellulose. This only had activity against met-ala ser and may indicate the presence of a membrane-linked isozyme or an enzyme aggregated to anothercytoplasmic constitutent making it too large to enter the gel. Whether it was equivalent to the peptidases of bands 1 and 2 or represented a further isozyme was unclear.

A severe limitation of either the gel or nitrocellulose methods of peptidase elucidation is the staining technique itself. The auxiliary enzyme (L-amino acid oxidase) is highly specific, with many amino acids (such as alanine) not acting as substrates (Lichtenburg and Wellner, 1968). In consequence certain peptides may be hydrolyzed and yet not result in the production of a dye band. A separate method was therefore used to determine the specific activity of the peptidases within the soluble fraction of *C.albicans*.

Specific activity of peptidases

In order to show the relationship between transport and hydrolysis of peptides it was necessary to show the effects of side-chain residues, stereochmistry and N- and C-terminals on peptidase activity. The specific activities of the peptidases present in a cell extract may be determined, irrespective of the amino acid composition of the peptides, by the o-phthaldialdehyde method (Porter, *et al.*, 1982). Rates of hydrolysis of peptides (initial concentration 2 mM), were determined for a cell extract prepared from YNBP-grown wild type cells (Table 5.1).

A comparison of varous dipeptides (Table 5.2) revealed that greater rates of hydrolysis were achieved when hydrophobic side-chains occured in both the amino- and carboxyl-terminal residues.

Dipeptides and tripeptides were hydrolyzed at linear rates while with ala_4 a reproducible sigmoid curve was obtained thus producing two rates of hydrolysis (Figure 5.4).

Over a period of 10 mins none of the stereoisomers of ala_2, apart from L-ala-L-ala, were hydrolyzed. Neither were N-acetyl-ala_2, ala_2 ONH_2 nor the phosphonopeptide alafosfalin hydrolysed (Table 5.1). To show whether hydrolysis was occuring at a low rate the peptides were exposed to the cell extract for a period of 2 hours followed by TLC of a sample. This revealed that D-ala-L-ala., L-ala-D-ala, D-ala-D-ala, N-acetyl-ala_2 and alafosfalin were resistant to hydrolysis (Figures 5.6-5.9) whereas ala_2-ONH_2 was cleaved at a low rate over this period (Figure 5.8).

Table 5.1 Rates of peptide hydrolysis by a soluble fraction of *C.albicans* 124 (wild type)

A 10 µl sample of a *C.albicans* 124 sample fraction was added to a prewarmed peptide solution (2 mM) in phospate buffer pH 7.5 containing 0.5 M sucrose. The solution was incubated at 37°C . Samples (50 µl) were removed at timedintervals and immediately added to 3 mls of buffered OPA reagent. The fluorescence of the solution was assayed after 2 mins at 25°C . Rates of hydrolysis were determined in triplicate and are expressed as mean initial rates with standard deviations from this value.

Peptide (2 mM)	Rate of Hydrolysis (nmol/min/mg protein)	% Rate of ala_2 Hydrolysis
ala met	26.4 ± 0.2	123.5
gly phe	25.2 ± 0.15	117.0
ala ala	21.4 ± 0.11	100.0
ala leu	18.9 ± 0.10	88.2
leu leu	18.9 ± 0.12	88.2
met ala	15.1 ± 0.08	70.5
leu ala	13.2 ± 0.10	61.7
ala_3	13.0 ± 0.09	60.7
leu gly	10.0 ± 0.08	47.0
tyr ala	7.5 ± 0.05	35.3
ala his	7.5 ± 0.10	35.3
tyr tyr	6.3 ± 0.10	29.4
ala glu	5.0 ± 0.08	23.5
leu pro	1.2 ± 0.10	5.8
gly gly	1.2 ± 0.12	5.8
ala_4	V_O = 10.0 ± 0.07	46.7
	V_{AV}= 28.0 ± 0.18	130.9

Peptides not hydrolysed by the extract:

N-acetyl ala ala

ala ala - ONH_2

alafosfalin

D-ala-L-ala

L-ala-D-ala

D-ala-D-ala

Table 5.2 Effect of alteraion of N- and C-terminal residues upon hydrolysis of dipeptides

Results taken from Table 5.1.

Alteration of the N-terminal Residue

Peptide	Rate of Hydrolysis (nmol/min/mg protein)	Nature of the N-terminal Residue
ala_2	21.4	Neutral/Hydrophobic
met ala	15.1	
leu ala	13.2	
tyr ala	7.5	Polar

Alteration of the C-terminal Residue

Peptide	Rate of Hydrolysis (nmol/min/mg protein)	Nature of the C-terminal Residue
ala met	26.4	Neutral/Hydrophobic
ala ala	21.4	
ala leu	18.9	
ala his	7.5	Polar
ala glu	5.0	Hydrophilic

Figure 5.4 Time courses for the hydrolysis of ala_2 (●), ala_3 (□) and ala_4 (○) by a soluble fraction of *C.albicans* 124 (wild type)

The method used was that described in Table 5.1.

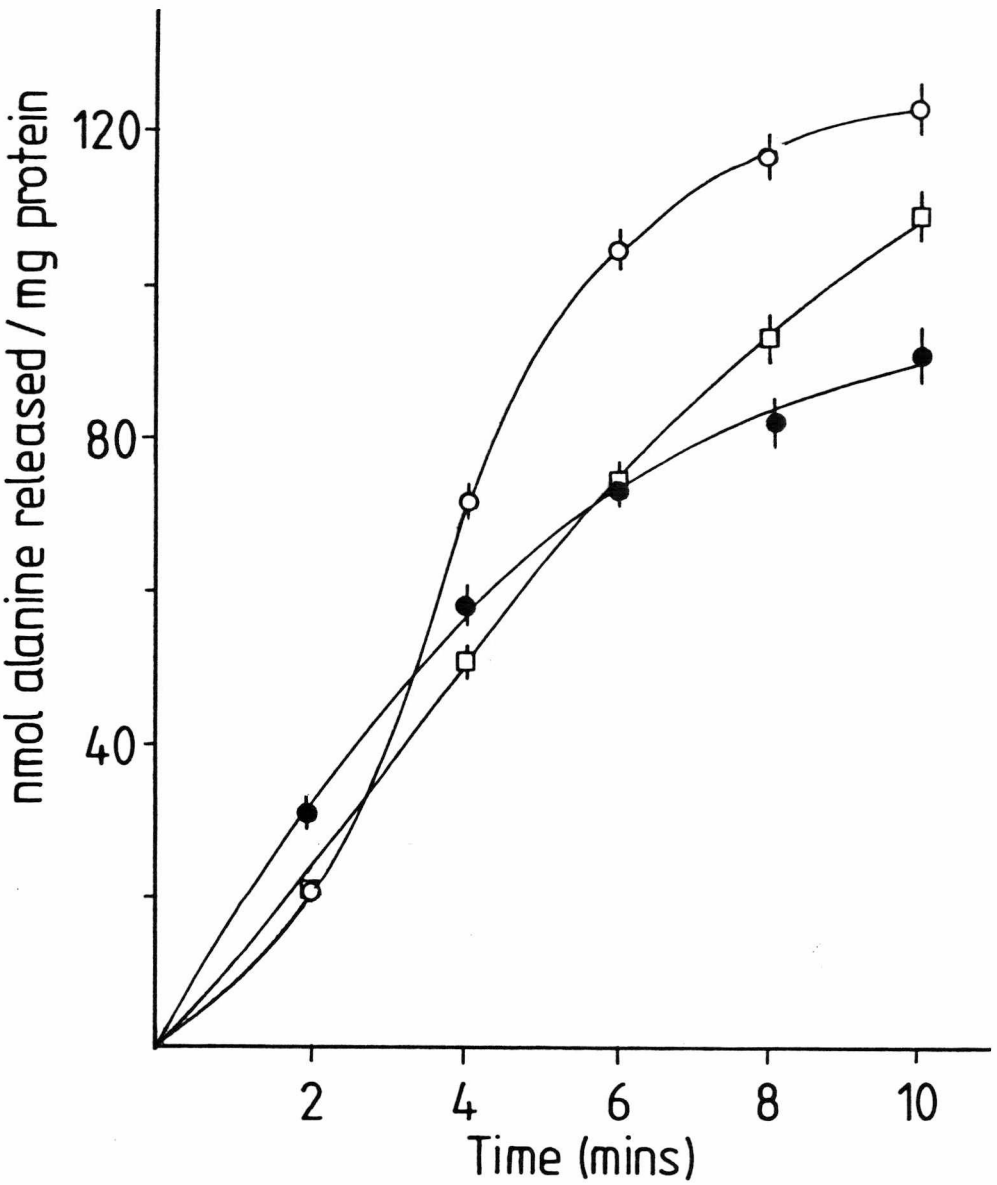

nmol alanine released / mg protein
120
80
40
2
4
6
8
10
Time (mins)

Figure 5.6 Thin layer chromatograms of peptidase assays

A 10 µl sample of a soluble fraction of *C.albicans* 124 wild type was added to a prewarmed peptide solution (2 mM) in phosphate buffer pH 7.5 containing 0.5 M Sucrose. The solution was incubated at 37°C . Samples (10 µl) were removed at hourly intervals and spotted onto cellulose chromatogram sheets which were developed in butanol/acetic acid/water (60:15:25 by volume) by ascending chromatography. The amino acids and peptides were visualized with ninhydrin spray (Merck).

a. L-ala-L-ala standard (incubated for 2 hours)
b. L-ala-L-ala (incubated with cell extract for 1 hour)
c. L-ala-L-ala (incubated with cell extract for 2 hours)
d. L-alanine standard
e. L-ala-D-ala standard (incubated for 2 hours)
f. L-ala-D-ala (incubated with cell extract for 1 hour)
g. L-ala-D-ala (incubated with cell extract for 2 hours)
h. L-alanine standard.

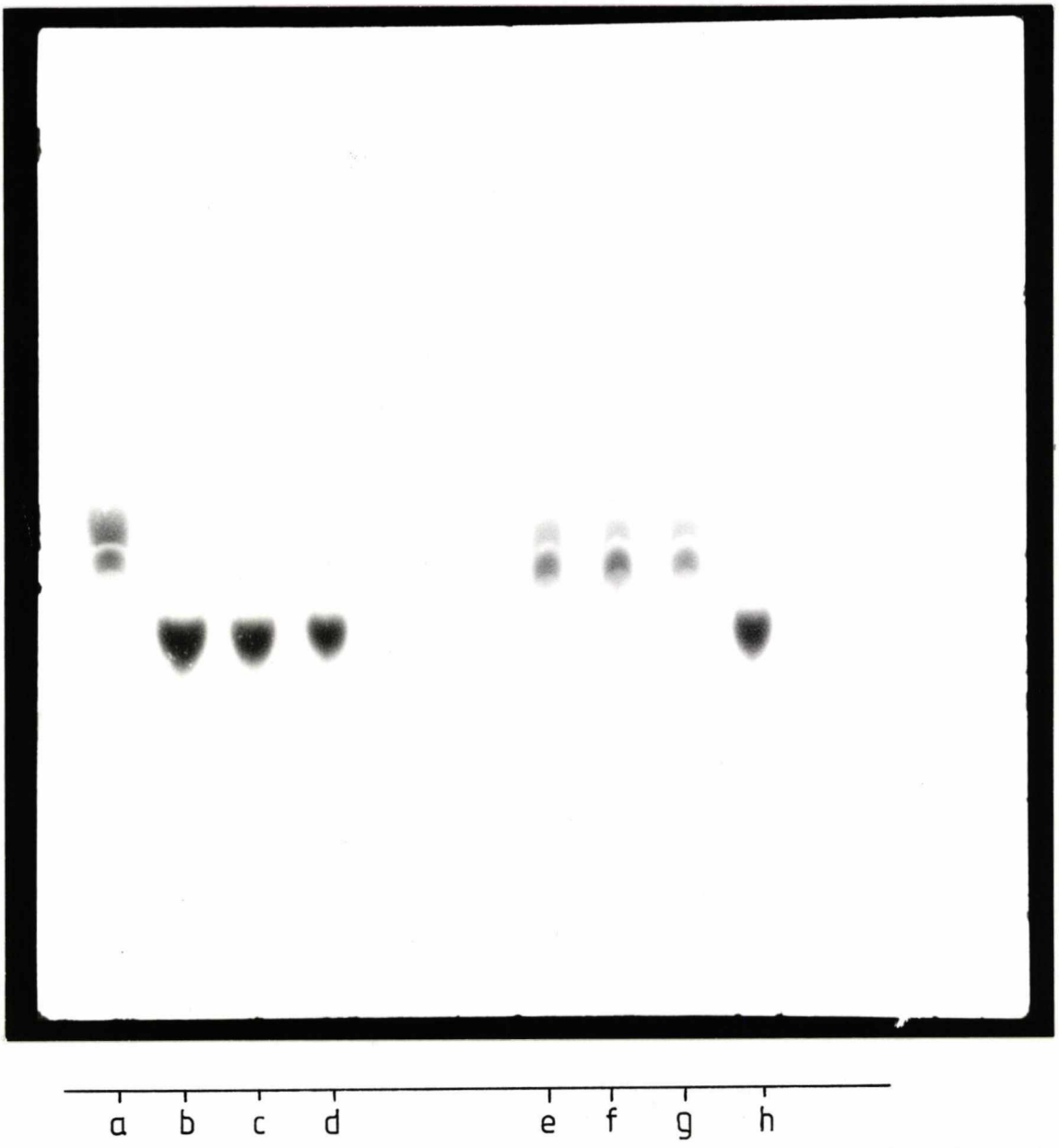

a
b
c
d
e
f
g
h

Figure 5.7 Thin layer chromatograms of peptidase assays

The method used was identical to that described in Figure 5.6.

a. D-ala-L-ala standard (incubated for 2 hours)

b. D-ala-L-ala (incubated with cell extract for 1 hour)

c. D-ala-L-ala (incubated with cell extract for 2 hours)

d. L-alanine standard

e. D-ala-D-ala standard (incubated for 2 hours)

f. D-ala-D-ala (incubated with cell extract for 1 hour)

g. D-ala-D-ala (incubated with cell extract for 2 hours)

h. D-alanine standard

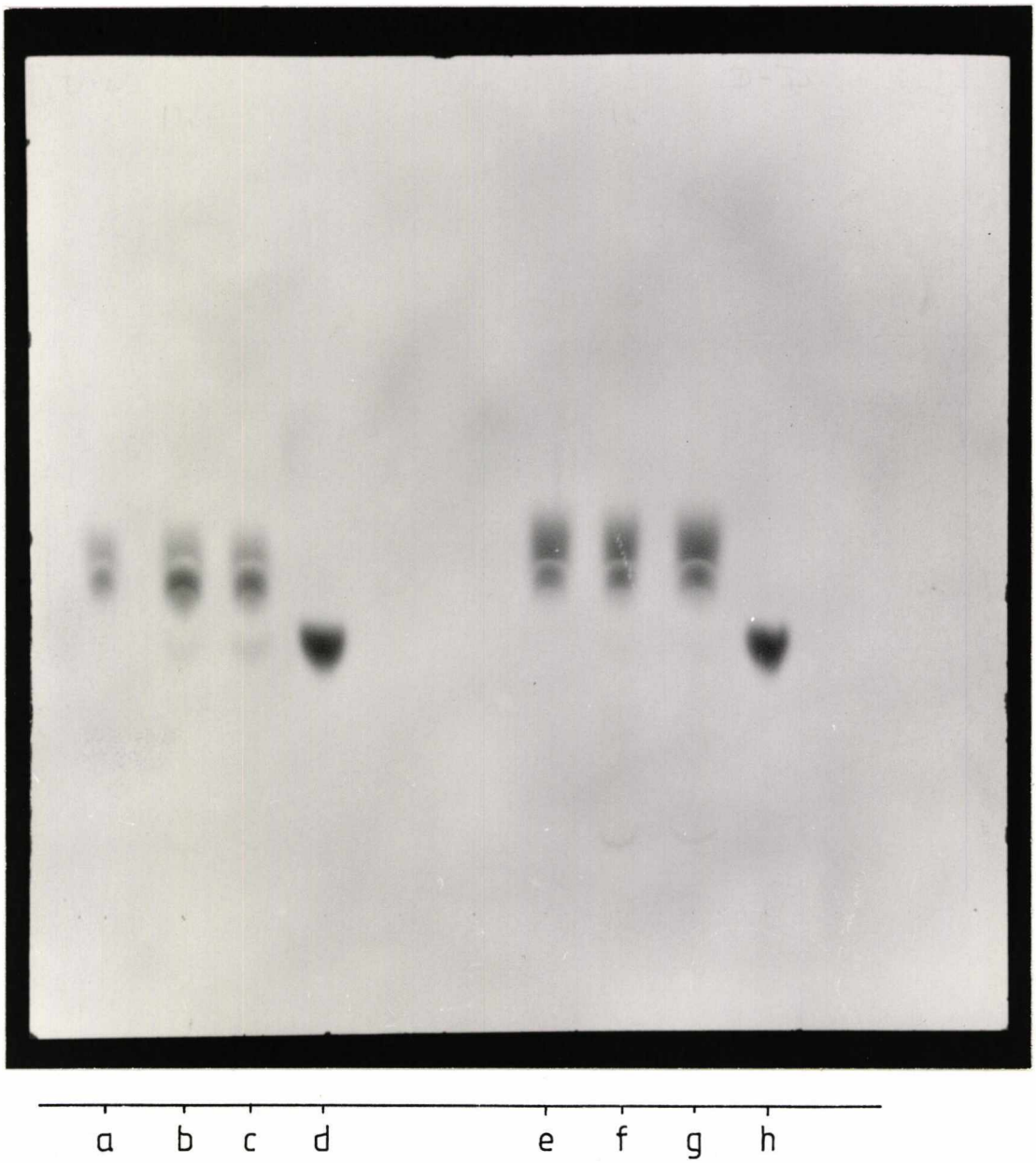

a
b
c
d
e
f
g
h

Figure 5.8 Thin layer chromatograms of peptidase assays

The method used was that described in Figure 5.6.

a. ala ala ONH_2 standard (incubated for 2 hours)

b. ala ala ONH_2 (incubated with cell extract for 1 hour)

c. ala ala ONH_2 (incubated with cell extract for 2 hours)

d. L-alanine standard

e. ala_2 standard

f. N-acetyl ala_2 (incubated for 2 hours)

g. N-acetyl ala_2 (incubated with cell extract for 1 hour)

h. N-acetyl ala_2 (incubated with cell extract for 2 hours)

i. L-alanine standard

j. ala_2 standard


a b c d e f g h i j

Figure 5.9 Thin layer chromatograms of peptidase assays

The method used was identical to that described in Figure 5.6.

a. alafosfalin standard (incubated for 2 hours)

b. alafosfalin (incubated with cell extract for 1 hour)

c. alafosfalin (incubated with cell extract for 2 hours)

d. L-alanine standard

e. ala_2 standard

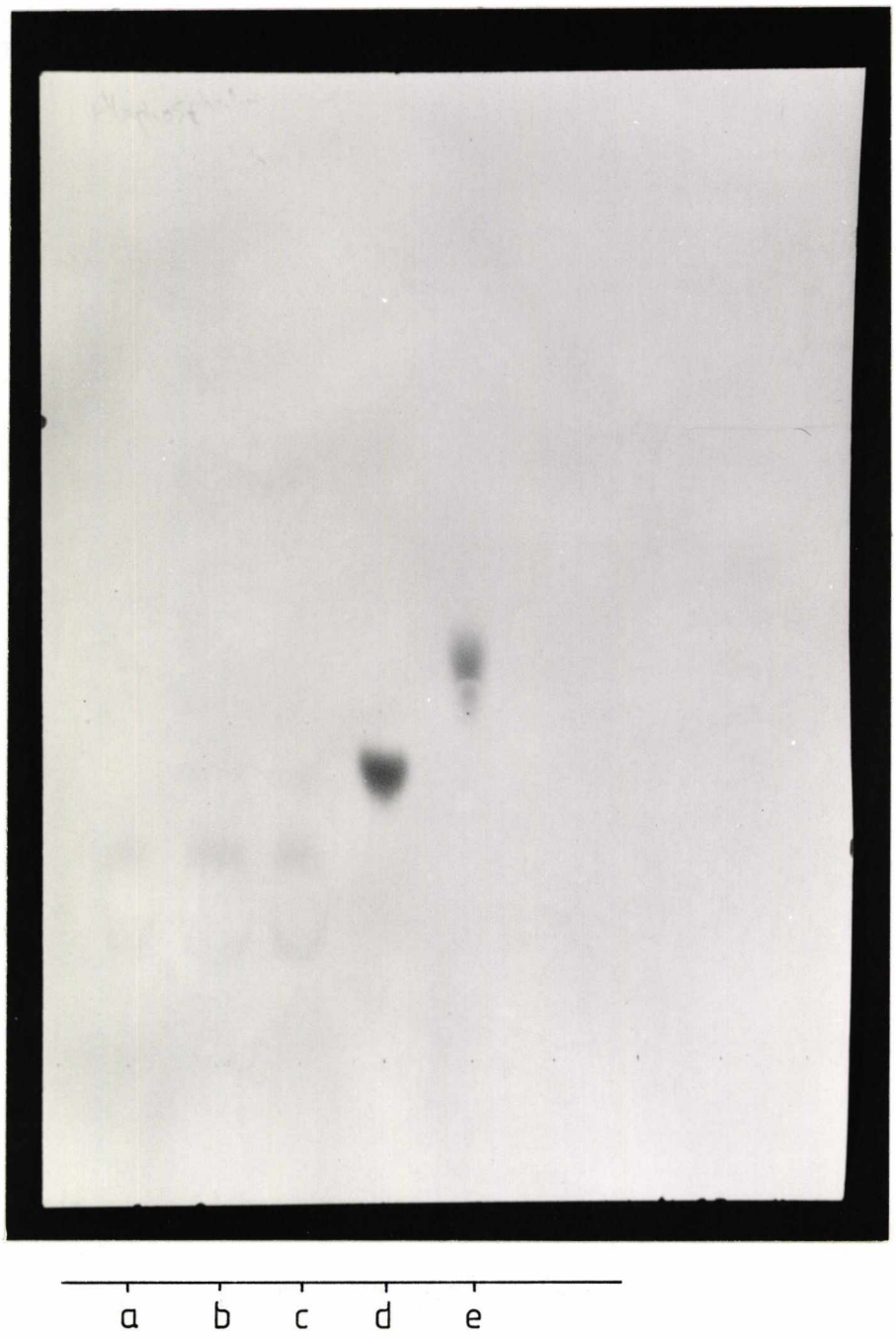

a
b
c
d
e

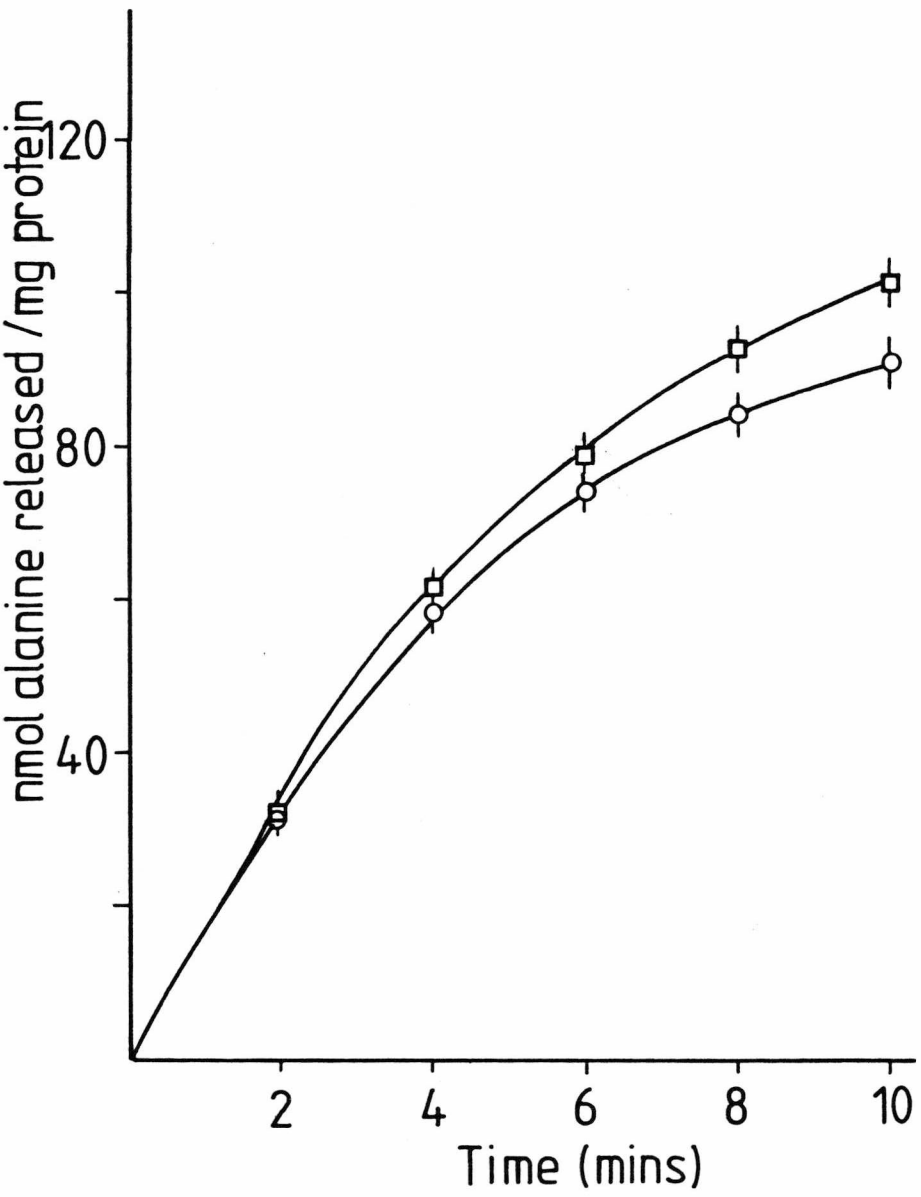
Table 5.3 The rates of hydrolysis of peptide-nucleosides by a *C.albicans* 124 (wild type) soluble fraction

The method used was that described in Table 5.1. The peptide concentrations are contained within the Table.

Peptide	Rate of Hydrolysis (nmol/min/mg protein)
ala_2 (2 mM)	21.4 ± 0.11
Nikkomycin (100 μg ml^{-1})	0
Nikkomycin (1 mg ml^{-1})	0
ala_2(2 mM)+Nikkomycin(1 mg ml^{-1})	21.4 ± 0.1
Polyoxin D	0

Figure 5.5 Time courses for the hydrolysis of ala_2 by a soluble fraction of *C.albicans* 124 wild type (O) and NIK5 (□)

The method used was that described in Table 5.1.


nmol alanine released /mg protein
120
80
40
2
4
6
8
10
Time (mins)

The antifungal agent nikkomycin contains a peptide residue. It was possible that intracellular peptidase activity might alter the drug and hence affect the interaction with the target molecule, chitin synthase. Nikkomycin was not hydrolyzed by a cell extract over a period of 20 mins (OPA assay). Furthermore the rate of hydrolysis of 2mM ala_2 by a cell extract was unaffected by the presence of 1 mg ml^{-1} nikkomycin, indicating that there was no interaction between the peptidase system and this drug (Table 5.3). Polyoxin D was also found to be resistant to hydrolysis by a cell extract.

The rate of hydrolysis of ala_2 by a cell extract prepared from NIK5 was found to be equivalent to that of the wild type (Figure 5.5). This confirms that no alteration of the peptidase system arises from loss of peptide transport capacity.

Extracellular peptidase activity

C.albicans has been shown to possess an inducible extracellular protease (Remold, *et al.*, 1968). No extracellular peptidase activity has been previously reported. However, the present transport study was dependent upon the fluorescamine technique which assays peptides even in the presence of amino acids. A reduction in the peptide concentration in the presence of an organism can therefore be interpreted either as transport or extracellular hydrolysis of the peptide. Three experiments indicated that extracellular hydrolysis was not occuring.

A culture of *C.albicans* (wild type) was grown to late logarithmic phase in YNBP. Cells were removed by filtration and the medium assayed for peptidase activity. No hydrolytic activity was observed againsteither ala_2 or ala_3 . This showed that peptidases were not released fromthe cells under these growth conditions. It was possible that a peptidase was released on transfer of cells from the growth medium to PCG , the buffer used in the transport assay. No activity was found, against ala_2 or ala_3 , in thePCG buffer in which cells grown in YNBP had been incubated for 20 mins.

These two experiments indicate that a peptidase is not released from cells into the medium. There remained the possibility that a cell wall-bound peptidase existed. Cells were incubated in PCG containing 2 mM ala_2 . Samples were taken at timed intervals and the cells removed by filtration. 10 µl samples of the filtrate were separated by TLC (Figure 5.10). Over a period of 45 mins transport of ala_2 was visible as a gradual decrease in the intensity of the ninhydrin positive spot. No free alanine was released during the course of the incubation indicating that no cell wall-bound peptidase activity was present.

Section 2: Peptide metabolism

The studies of peptidase activity showed that peptides are hydrolyzed following transport into *C.albicans*. Previous studies (Davies, 1980; Woodward and Cirillo, 1977; Norkrans and Tunlad-Johansson, o981) have shown that amino acids are metabolized by yeasts and can serve as a nitrogen source for the cell.

Figure 5.10 Thin layer chromatogram of ala_2 uptake by *C.albicans* 124 (wild type)

C.albicans 124 was grown to mid-logarithmic phase in YNBP , harvested, washed and resuspended in PCG . Cells were diluted to 1.0 mg dry wt/ml in PCG and $[U^{14}C]$ ala ala was added to a concentration of 2.0 mM. Samples were taken at timed intervals and the cells removed by filtration. The extracellular medium was collected and 10 µl of this was spotted onto cellulose chromatogram sheets. These were developed in butanol / acetic acid/ water (60:15:25 by volume) by asecnding chromatography. The amino acids and peptides were visualized with ninhydrin spray (Merck).

a. ala_2 standard
b. L-alanine standard
c. Extracellular medium after 5 minutes
d. Extracellular medium after 10 minutes
e. Extracellular medium after 15 minutes
f. Extracellular medium after 30 minutes
g. Extracellular medium after 45 minutes
h. ala_2 standard
i. L-alanine standard

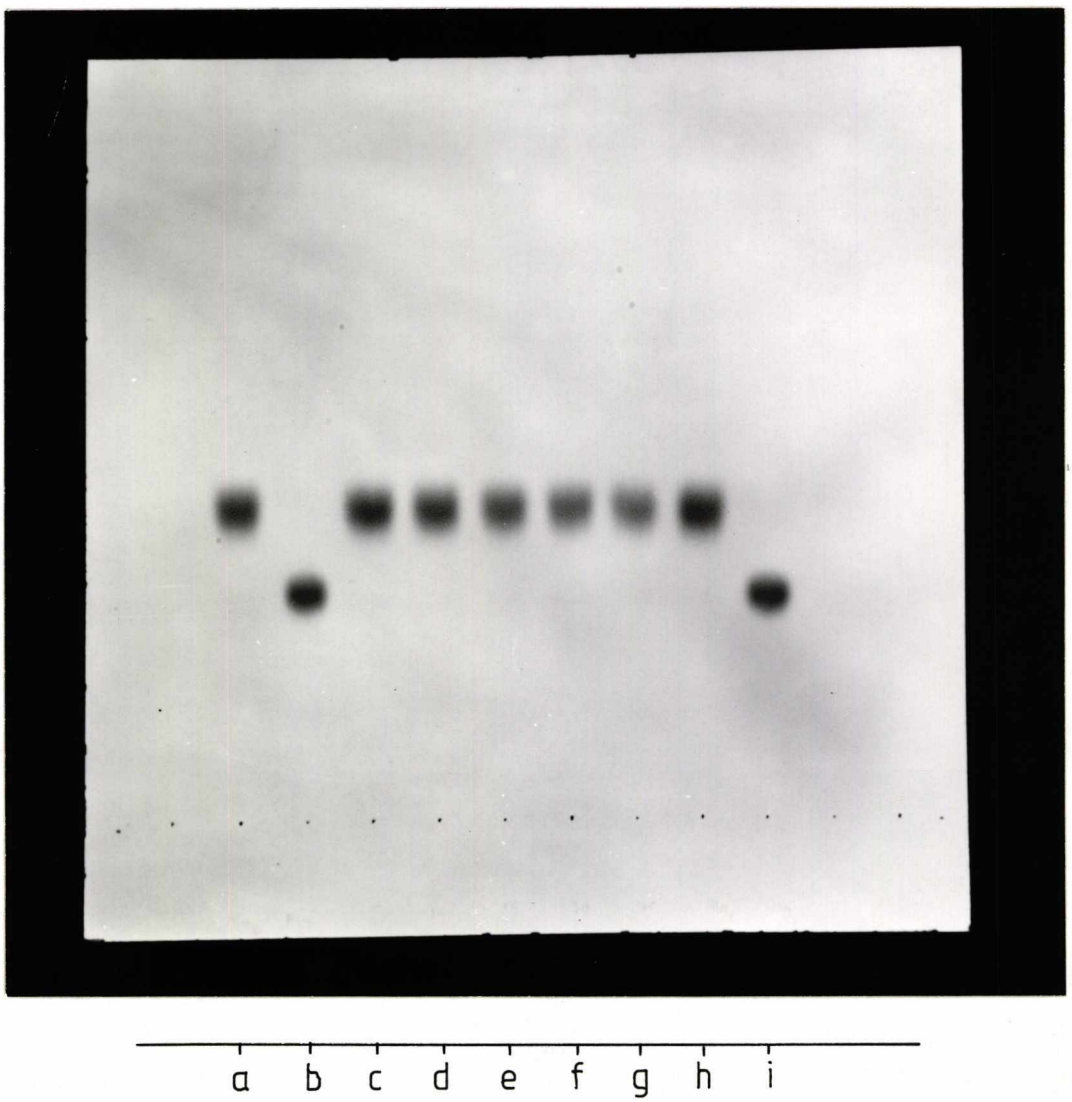
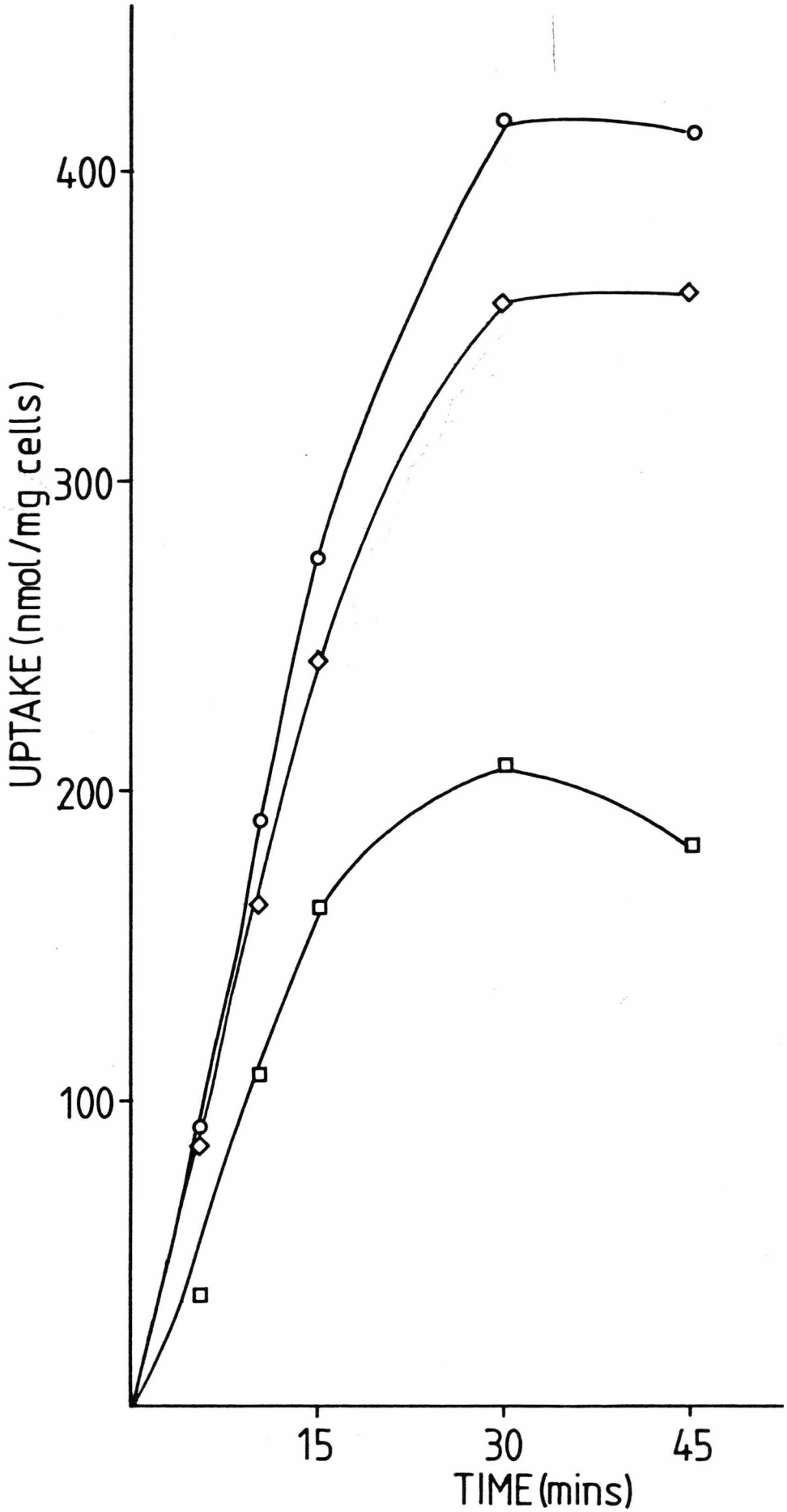

a b c d e f g h i

Figure 5.11 Variation in the apparent rate of ala_2 uptake when assayed by: The fluorescamine method (○); radiolabel remaining in solution correlated with radiolabel incorporated into cells (◇); radiolabel incorporated into cells (□).

The transport assay was carried out as described in Figure 5.10. The extracellular medium was collected and assayed for either peptide concentration by the fluorescamine method or the quantity of radiolabel remaining in solution. The reduction in the concentration of peptide obtained by both method was converted to the apparent increase in the intracellular [$U^{14}C$] ala ala incorporated and plotted as such.

Alternatively 1 ml samples of the cell suspension were collected at timed intervals, washed with buffer and assayed for incorporated radiolabel.


400
300
200
100
UPTAKE (nmol/mg cells)
15
30
45
TIME (mins)

This work was instigated following the observation that the apparent rates of transport of ala_2 differed considerably when assayed by either the fluorescamine or the [U^{14}C] ala ala uptake methods (Figure 5.11). While fluorescamine measured only the transport of ala_2 into the cells, the [U^{14}C] ala ala method partially assayed transport, hydrolysis and subsequent metabolism. It was necessary to show whether the differences between the two methods could be accounted for by loss of radiolabel from the cells and to show in which from the radiolabel was lost.

The fluorescamine method measures the reduction in the concentration of peptide in the incubation medium. Although radiotracers are generally used to measure incorporation of the label into cells they can also be used, as in this case, to measure the reduction in the extracellular concentration. A comparison of the rates of peptide uptake by these two methods revealed that the initial rates were identical (Figure 5.11). However after 10 mins incubation the apparent rate of uptake of the radiolabel was lower than the actual rate of peptide transport implying that ^{14}C was being released from the cell in a form other than [U^{14}C] ala ala.

There is a poor correlation between the [U^{14}C] ala ala incorporated into the cells and the amount removed from solution (Figure 5.11). Radiolabel would appear to be lost from the cells thus giving a low apparent rate of incorporation.

One possible explanation for the loss of label is that it was due to a temperature-sensitive efflux of peptides or amino acids during the

washing of cells, prior to scintillation counting. However it was found that washing cells in buffer at $4^{o}C$, $25^{o}C$ or $37^{o}C$ had no effect upon the apparent rate of transport.

The second possibility was that efflux of radiolabel was occuring during the transport assay in the form of amino acids or as carbon skeletons following deamination. However it was shown that production of amino acids did not occur during a 45 minute incubation period in the presence of ala_2 (Figure 5.10). Therefore loss of label could not be accounted for in this way. The release of carbon skeletons could not be assayed but will be discussed at a later point.

The final possibility for the loss of ^{14}C from the cells was that it occured as the release of $^{14}CO_2$ following decarboxylation of the amino acid carbon skeleton. Peptide transport was assayed by the fluorescamine technique and concurrent release of $^{14}CO_2$ was monitored (Figure 5.12). This revealed a considerable loss of radiolabel from cells as $^{14}CO_2$. Release occured at a linear rate after 10 mins incubation with $U^{14}C$ ala ala (2 mM) when a saturating concentration was achieved in the buffer. Therefore it must be assumed that $^{14}CO_2$ was being produced prior to its release from solution. This suggests that very rapid metabolism of [U^{14} C] ala ala was taking place after its hydrolysis.

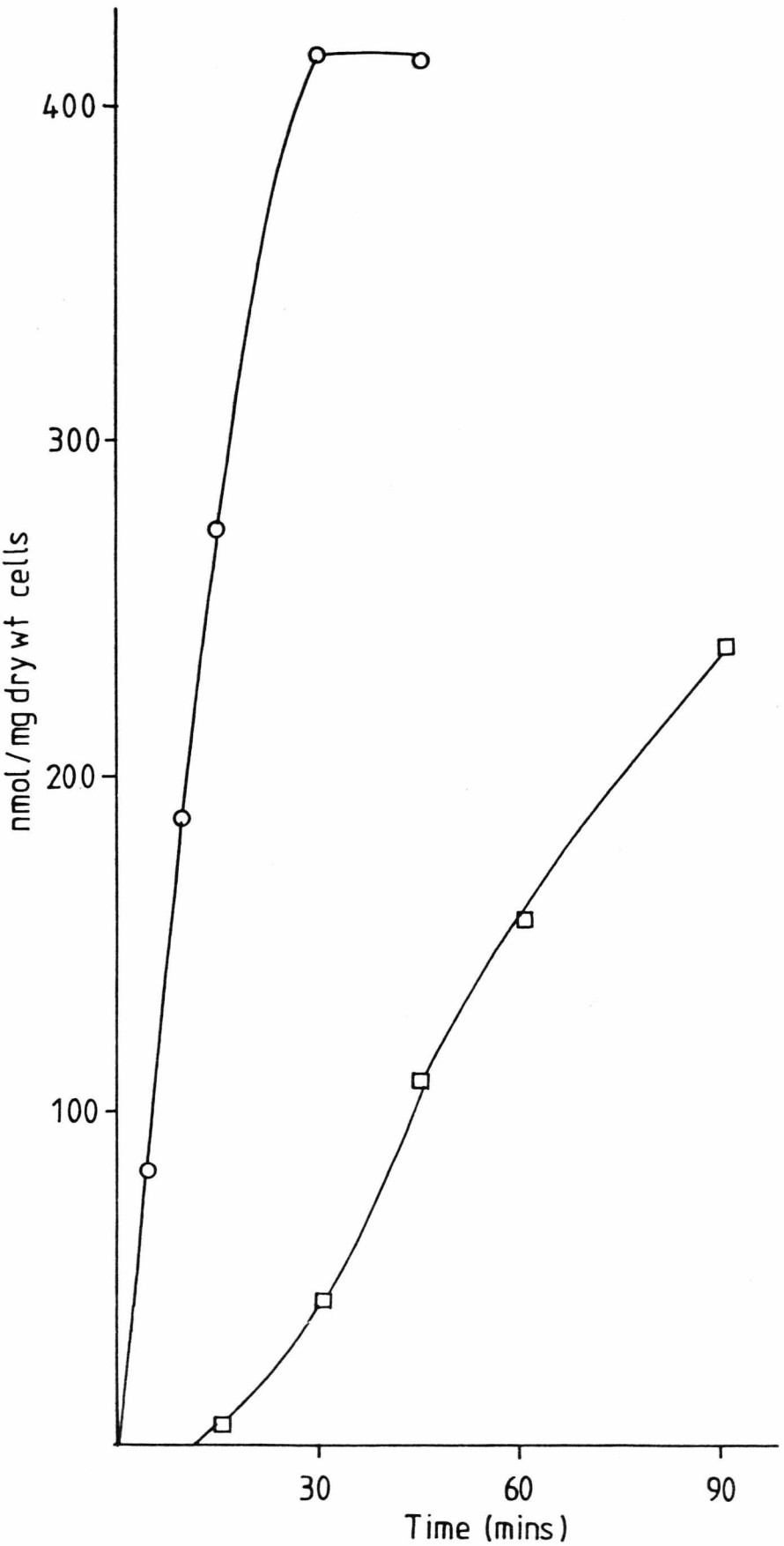
The loss of radiolabel as $^{14}CO_2$ accounted for the difference between the assay methods, since some CO_2 would continue to be released

Figure 5.12 The release of $^{14}CO_2$ from blastospores of *C.albicans* 124 incubated with [U^{14}C] ala ala

The uptake of [U^{14}C] ala ala (O) was assayed by the fluorescamine technique as described in Figures 5.10-5.11.

YNBP grown blastospores were incubated in PCG at 37^{o}C containing 2 mM [U^{14}C] ala ala (cell density=1.0 mg dry wt/ml). The release of $^{14}CO_2$ was monitored in sealed 50 ml flasks containing 1 ml ethanolamine (2:1 (v/v) dilution in ethanol) in the centre-well.

At timed invervals the flasks were opened and the radioactivity of the ethanolamine samples were determined. Three flasks were used for each time point and the radioactivity was correlated with ^{14}C in [U^{14}C] ala ala (□)


400
300
200
100
nmol / mg dry wt cells
30
60
90
Time (mins)

between the harvesting of a cell sample and drying it onto the filter prior to scintillation counting. It also accounts for the decrease in the amount of label incorporated between the 30 min and 45 min time points (Figure 5.11).

The difference between the rates of uptake obtained by the fluorescamine technique and from determining the amount of radiolabel remaining in the medium increased progressively (Figure 5.11). This was due to an apparent excess of radiolabel in solution and can be explained as both a constant, saturating level of $^{14}CO_2$ and the continued efflux of a non-volatile carbon skeleton into the medium.

The previous studies of peptide transport into *Candida albicans* have depended upon the transport of radiolabels into cells. Davies (1980) stated that loss of radiolabel was probably insignificant during the course of competition studies. The present study indicates that this is not the case. A comparison of the kinetic parameters for transport of ala_2 and [U^{14}C] ala ala (Table 4.6) shows that the difference in the apparent rates of uptake had a profound effect upon the K_m and V_{max} values obtained.

Discussion

Section 1: Hydrolysis of peptides

The transport of peptides into *C.albicans* was followed by rapid intracellular cleavage. This was to be expected if the peptides were to serve as a nitrogen source for this organism. The o-phthaldialdehyde

(OPA) assay was used to show the specific activity of the peptidases in a soluble fraction of the cells. OPA reacts with the amine terminus of amino acids and peptides. However, above pH 9.0 the fluorescence of the product is specific for amino acids. It is therefore possible to assay the release of amino acids from peptide substrates (Porter, *et al.*, 1982).

A large range of peptides was found to be hydrolyzed by a soluble fraction from *C.albicans*. Neutral and hydrophobic side-chain residues at both the amino- and carboxyl-terminals of a dipeptide enhanced the rate of hydrolysis. These rates do not represent the specificities of individual enzymes and so they may, in certain instances, be due to a few of the six dipeptidases detected by electrophoretic analysis.

Gly_2 was only slowly hydrolyzed and leu gly was cleaved at a slower rate (10.0 nmol/min/mg protein) than leu_2 (18.9 nmol/min/mg protein). This shows that the presence of a glycine residue greatly reduced the rate of hydrolysis. Glycine residues were also found to reduce the rate of peptide transport. Equally the presence of the imino acid proline reduced the rate of hydrolysis.

The rate of hydrolysis of all peptides, except ala_4, were found to be linear over the first 4 mins of incubation. Ala_4 gave a sigmoid curve for the release of alanine. This may be due to the presence of an endopeptidase in the extract which would cleave ala_4 to ala_2 followed by dipeptidase action releasing alanine. The initial low rate would be due to exopeptidase action producing ala_3 and released alanine.

The peptidase system was found to be highly stereospecific in that none of the stereoisomers of ala_2 other than L-ala-L-ala was hydrolyzed, even upon prolonged exposure to a cell extract. Marder *et al.* (1977) demonstrated that an *S.cerevisiae* cell extract hydrolyzed L-leu-D-leu and D-leu-L-leu at only a low rate, indicating that stereospecificity is a general phenomenon amongst the yeast peptide hydrolytic systems. The peptide transport system of *C.albicans* was also found to be highly stereospecific and hence there are two mechanisms by which D-steroisomers are prevented from entering the cell. The low rate of transport of L-ala-D-ala is not accompanied by cleavage and so D-alanine is not released intracellularly.

N-acetyl ala_2 was found to be resistant to hydrolysis indicating the absence of either acetylase or carboxypeptidase activity. Carboxypeptidase Y has been well characterized for *S.cerevisiae*. The activity of this enzyme is maximal at pH 6-7 (Hayashi, *et al.*, 1975) whereas this assay was carried out at pH 7.5. Thus, the lack of hydrolysis may have been due to the inactivity of the enzyme at this pH. Similarly the peptidases of *S.cerevisiae* were found to be able to hydrolyze acetyl-met_3 by carboxypeptidase action (Naider, *et al.*, 1974). However, no such activity was observed in gels (Rose, *et al.*, 1979).

The amide of ala_2 were susceptible to hydrolysis over a period of 2 hours in the presence of a cell extract. There are two possible mechanisms of cleavage. Firstly aminopeptidase activity may have

released alanine and alanine-ONH_2 , and secondly amidase activity may have released ala_2 followed by rapid hydrolysis of this peptide to alanine. From the TLC plate (Figure 5.8) it would appear that only alanine is produced with a concurrent reduction in the quantity of ala_2ONH_2 present. No alanine-ONH_2 appeared to be released. This suggests that amidase activity was present in the cell extract and constituted the rate-limiting step. Brady (1969) identified an amidase from *C.utilis* with activity against a broad range of aliphatic amides, however K_m values ranged from 2-5 mM . Therefore, if a similar amidase is active in *C.albicans* and has activity against peptide-amides, this rate of hydrolysis may indeed have been the rate-limiting step as has already been indicated. Such a process may again be pH dependent and occur more rapidly at a pH different from that of the assay.

Peptidase activity was not demonstrated against alafosfalin (in which the carboxyl group of ala_2 is replaced by a phosphonate group). Davies (1980) found alafosfalin to interact with the peptide transport system and to be resistant to hydrolysis. This indicates that following transport into cells, it was not cleaved and that there is a difference in the specificities of the uptake and hydrolytic systems (transport required a free acidic function whereas hydrolysis required a free carboxyl terminus). *E.coli* has been found to both transport and hydrolyze alafosfalin (Atherton, *et al.*, 1979) and so there is a considerable difference between the peptide utilization systems of *E.coli* and *C.albicans*.

No hydrolysis of the peptide-nucelosides nikkomycin and polyoxin D was found to occur, indicating that the peptidases possess a narrower side-chain specificity than the transport system. The lack of competition between nikkomycin and ala_2 for hydrolysis confirmed this observation. It showed that nikkomycin is unable to compete with ala_2 for binding to the active site of a dipeptidase and will not therefore undergo hydrolysis even at a low rate.

A comparison of the rates of hydrolysis of peptides (Table 5.1) with their rates of transport (Table 4.3) reveals that all the transported peptides assayed were also hydrolyzed. However, there was one major difference in that the preferred amino terminal side-chain residue for hydrolysis was hydrophobic whereas such peptides were transported at the lowest rate. This raises the important point that the function of intracellular peptidases is not only to hydrolyze transported peptides. If this was their sole function the transport and hydrolytic systems would ideally be of equivalent efficiency. In addition to their function in nitrogen metabolism the peptidases are also active in protein maturation and turnover.

Eukaryotic proteins are initiated with an N-terminal methionine residue (Haselkorn and Rothman-Denes, 1973). This is removed by an aminopeptidase, together with a variable amount of the N-terminal of the nascent protein, before the mature protein is released. Many such proteins have been described such as the carboxypeptidase Y of *S.cerevisiae*, from which a 5000 dalton signal peptide is removed probably as a result of proteolysis (Hasilik and Tanner, 1976).

Another role of peptidases is in protein activation: Cabib and Ulane (1973) have described the proteolytic activation of chitin synthase. This enzyme is believed to be present primarily as an inactive zymogen in yeast and is probably only activated at the point in the cell where chitin synthesis is required. Although the precise mechanism of activation within the cell has not been defined certain proteolytic enzymes, such as trypsin, are able to activate this enzyme (Ryder and Peberdy, 1977). This represents another possible role for a specific intracellular peptidase.

Degradation of proteins occurs during growth of *S.cerevisiae*, with a turnover rate of about 1% per hour (Betz, 1976). This rate increases under conditions of starvation. It is probable that proteolysis is followed by peptidase activity equivalent to that involved in the utilization of transported peptides.

The first of these functions (the cleavage of N-terminal methionyl residues) will require the creation of a complement of peptidases which will preferentially cleave peptides containing a hydrophobic amine terminus. This was observed in the present study and could explain the apparent discrepancy between transport and hydrolysis.

Future work must determine the location of peptide hydrolytic activity within *C.albicans* and correlate this with the presence of transported peptides and their constituent amino acids. In *S.cerevisiae* many of the intracellular hydrolytic enzymes, including peptidases, are located within the vacuole (Wiemken, *et al.*, 1979). A similar situation

could occur in *C.albicans*. Therefore it will be necessary to differentiate between vacuolar, cytoplasmic and mitochondrial peptidases which may perform very different functions. This raises two further points if hydrolysis of transported peptides is occuring within the vacuole. Firstly the mechanism by whch peptides are translocated to the site of peptidase activity must be elucidated and secondly, further peptide permeases could be operational at the vacuolar membrane. Separate arginine permeases have been identified at the plasma- and vacuolar-membranes of *S.cerevisiae* (Grenson, *et al.*, 1966; Chan and Cossins, 1976; Boller, *et al.*, 1975). It is possible that such vacuolar peptide permeases could regulate the supply of peptides for hydrolysis and hence play an important role in the utilization of these substrates.

The peptidase isozymes of *S.cerevisiae* also exhibited preferential cleavage of methionyl peptides. Band 3 showed general dipeptidase activity whereas bands 1,2 and 4 cleaved only met_2 and met ala of the peptides supplied. The bands obtained by the nitrocellulose blotting technique showed slightly different R_f values from those previously described (Rose, *et al.*, 1979: R_F=0.18 , 0.42 , 0.52 , and 0.74; present study: R_F=0.14 , 0.27 , 0.60 and 0.65). Although the electrophoresis buffers were the same in both cases differences may have arisen from either the yeast strain used or from the extract preparation technique. Rose, *et al.*, (1979) described band 3 (R_F=0.52) as a general dipeptidase as was also found in the present study. The remaining bands were described as cleaving tripeptides with preference for those containing an N-terminal methionyl residue. The findings of

the current report were therefore in general agreement with those of Rose *et al.* (1979). No inhibition of peptidase activity was apparent upon binding to nitrocellulose. The bands of activity were far sharper and more intense than those previously described and diffusion was not a problem, even after prolonged incubation in the staining medium. Thus the electrophoretic blotting technique described in this report may have wide applicability for the detection of isozyme activities. A minor modification of the incubation medium (removal of the peptide and L-amino acid oxidase) and inclusion of appropriate substrates (amino acids, alcohols and their respective coenzymes) would allow the elucidation of dehydrogenase isozymes.

A soluble fraction of *C.albicans* wild type cells was subjected to an identical technique. This gave eight bands of peptidase activity which were highly reproducible. Six of these bands were general dipeptidases whereas two were able to cleave only the tripeptide met ala ser. *C.albicans* therefore has a larger peptidase complement than *S.cerevisiae*. It should be stressed that in both cases this represents the minimum number of peptidases present. Further isozymes may become apparent with a broader range of substrates. The large number of dipeptidases present in *C.albicans* may be a reflection of the role of peptidases in the nutrition of this organism: The extracellular protease (Remold, *et al.*, 1968; MacDonald and Odds, 1983) probably plays an important role in pathogenicity. However it exhibits low side-chain specificity which would result in the production of many small peptides. The peptide transport systems together with a large complement of dipeptidases would result in the efficient utilization of proteinaceous nitrogen sources.

NIK5 also possessed eight peptidase isozymes which correlated with those present in the wild type both in R_F values and specificities This indicated that no loss of soluble peptidases occured upon loss of peptide transport capacity. It is possible that peptidase activity could be associated with the peptide transport system. However, if this is membrane bound, it would be lost in the preparation technique.

The specific activity of the NIK5 peptidases towards ala_2 was unaltered when compared with the wild type and so the close proximity of the dipeptidase bands had not resulted in a misinterpretation of the data.

Identification of the vacuolar peptidases by the new isozyme staining technique will be necessary to show whether all eight exo-peptidases are involved in the hydrolysis of transported peptides. This will aid an understanding of the functions of these enzymes.

The present study has shown the peptide transport systems of *C.albicans* to be inducible. The peptidases were prepared from YNBP-grown cells and it is possible that this represents an induced complement of the enzymes in response to peptides serving as the sole nitrogen source. No data are available concerning the effect of the growth medium on the peptidases.

In summary, there appears to be at least nine peptidases present in the soluble fraction of *C.albicans*. Six of these are dipeptidases

and of the remaining three one is an endopeptidase and two have high activity against tripeptides. These latter two may represent amino-peptidases (such as that described by Lee, *et al.*, 1962) or carboxy-peptidases. Further work is necessary to define the enzymes by the use of appropriate substrates.

As all the peptides transported, except L-ala-D-ala , are rapidly hydrolyzed by a cell extract it would appear that peptides may serve as a nitrogen source for the cell.

Section 2: Metabolism of the constituent amino acids

Following peptide transport and hydrolysis the metabolism of the resulting amino acids can provide a nitrogen source. This was found to have a profound effect upon the apparent rates of peptide transport. When monitored by the radiotracer or fluorescamine methods the rates of ^{14}C incorporation were lower than the actual rate of peptide transport. This may be explained in two ways: Either the transport of peptides was followed by their hydrolysis and subsequent exodus of the constituent amino acids from the cell or rapid metabolism of the transported peptides occured resulting in a loss of label from the cell.

Loss of amino acids following peptide transport has been reported for *E.coli* (Payne and Bell, 1977), however no such exodus has been reported for yeasts. In the present study no alanine was released

from cells during a 45 min incubation with ala_2. Equally Davies (1980) showed that phenylalanine was not released from *C.albicans* after incubation of the cells with gly phe and no alanine was effluxed from *S.cerevisiae* in the presence of ala_2 (Nisbet and Payne, 1979a). Thus the retention of amino acids by yeasts would appear to be a general phenomenon, however radiolabel was lost from both *C.albicans* in the present study and *S.cerevisiae* (Nisbet and Payne, 1979a) in a form other than amino acids.

Webb and Ingraham (1963) described the production of fusel oils and keto acids in the medium following growth of *C.albicans* in the presence of amino acids. In addition Narayanan, *et al.*,(1974) identified phenyllactic acid and phenylalcohol following incubation in the presence of phenylalanine. Davies (1979) demonstrated that no deamination products of phenylalanine could be detected within the cytoplasmic pool and concluded that these were rapidly effluxed from the cell and not accumulated.

The principal amino acid present in the intracellular pool of yeasts has been shown to be glutamate. In *S.cerevisiae* this amino acid comprises 50% of the cellular amino acid pool while in *Candida lipolytica* this figure may be as high as 70% (Norkrans, *et al.*,1981). Amino acids which enter the cell are therefore metabolized (with NH_3 being transferred to form the α-amino group) to glutamate, which then acts as a metabolizable nitrogen store. There are two mechanisms by which nitrogen can be transferred to α-ketogluarate: either direct transamination or dehydrogenation releasing NH_3 which is then incorporated into α-ketoglutarate by the anabolic ($NADP^+$) glutamate dehydrogenase ($NADP^+$-gluDH).

1. Transamination: Woodward and Cirillo (1977) have described a scheme for the production of fusel oils and keto acids by *S.cerevisiae* (Figure 5.13) in which the nitrogen of an amino acid is transferred to α-ketoglutarate to form glutamate. Decarboxylation and reduction of the carbon skeleton results in the production of the fusel oil which has been shown to be effluxed from the cell (Narayanan, *et al.*, 1974). Transamination occurs with many naturally occuring amino acids, however this does not take place with alanine or glycine and at only a low rate with methionine (Woodward and Cirillo, 1977). There is a report of transamination of alanine by *N.crassa* (Fincham and Boulter, 1956) however no data are available for *C.albicans*.

2. Dehydrogenation: Tsinberg, *et al.*(1980) have reported that the principal enzymes of alanine, aspartate, and glutamate metabolism in *C.albicans* are the pyrimidine-linked dehydrogenases (ala-DH; asp-DH; glu-DH). Three NAD^+-linked ala-DH isozymes have been identified (Tsinberg, *et al.*, 1980) which oxidatively deaminate alanine to pyruvate (Figure 5.14). Two types of glutamate dehydrogenase are present within the cell: the NAD^+-linked glu-DH performs a catalytic function while the $NADP^+$-gly-DH is anabolic in its action. The latter enzyme therefore incorporates NH_3 released from alanine

Figure 5.13 The pathway of fusel oil production in *S.cerevisiae* (from Woodward and Cirillo, 1977)

αKG	α-ketoglutarate
glu	glutamate
I	deaminated acid
II	fusel oil
(1)	transamination
(2)	decarboxylation
(3)	reduction

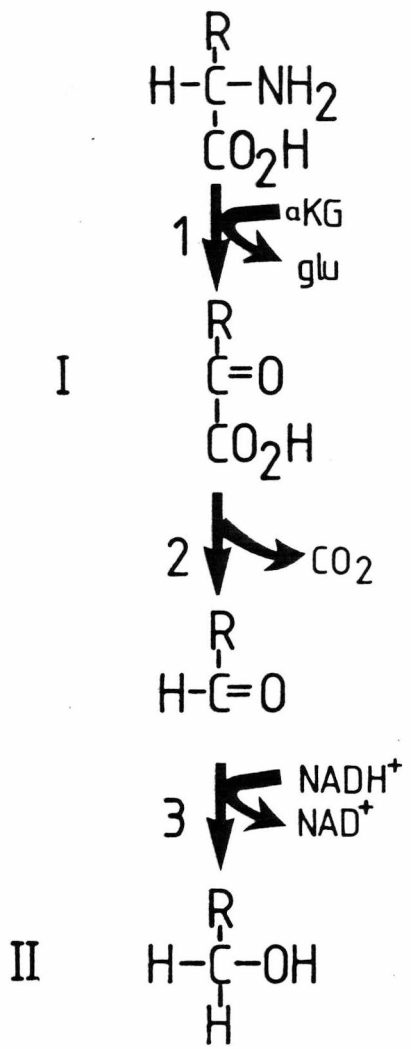
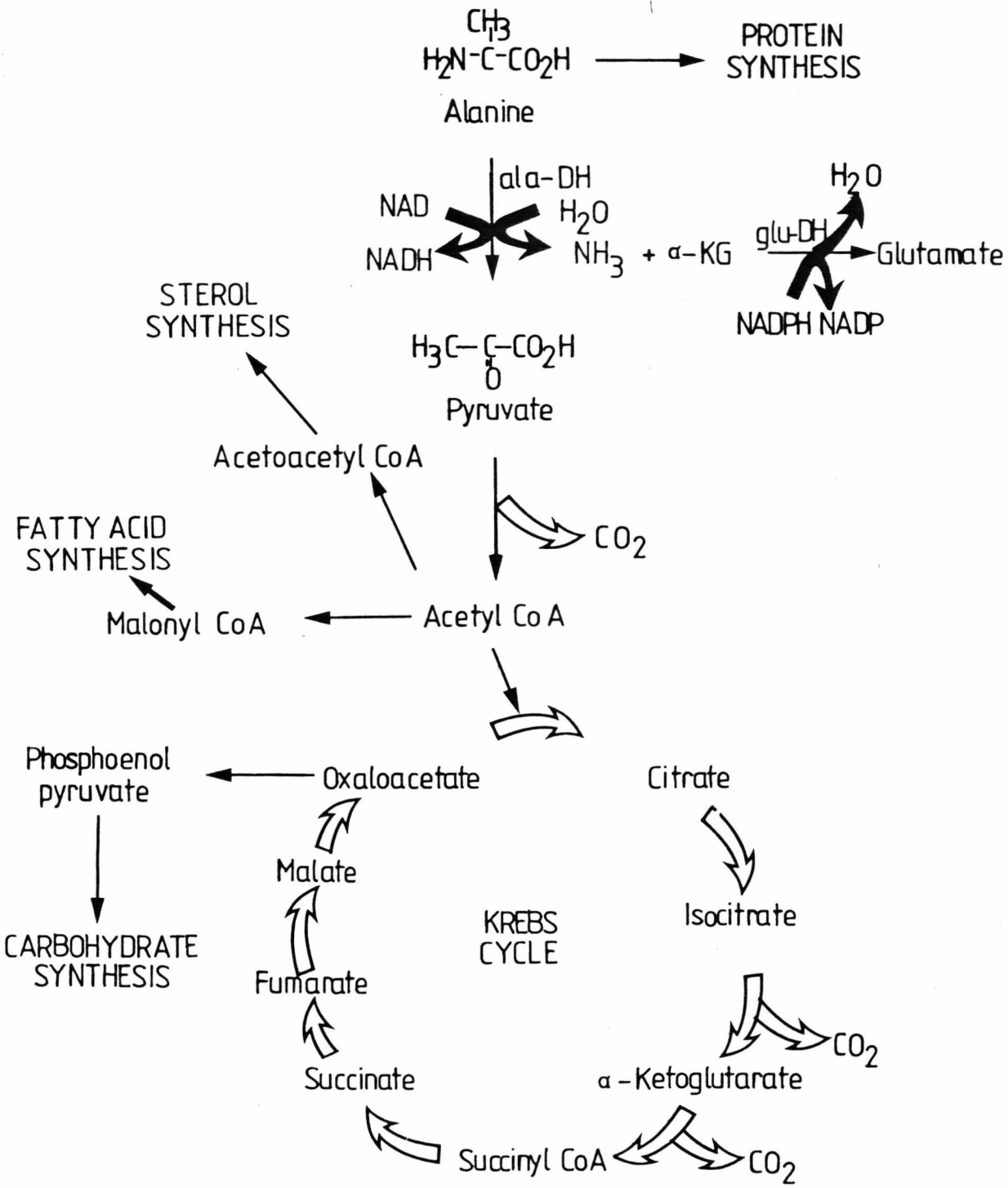

R
H–C–NH2
CO2H
1
αKG
glu
I
R
C=O
CO2H
2
CO2
R
H–C=O
3
NADH+
NAD+
II
R
H–C–OH
H

Figure 5.14 Schematic representation of the pathways of alanine degradation

ala-DH	alanine dehydrogenase
glu-DH	glutamate dehydrogenase
α-KG	α-ketogluatarate


PROTEIN SYNTHESIS
Alanine
ala-DH
NAD
NADH
H_2O
NH_3
+ α-KG
glu-DH
H_2O
Glutamate
NADPH NADP
STEROL SYNTHESIS
Pyruvate
Acetoacetyl CoA
CO_2
FATTY ACID SYNTHESIS
Malonyl CoA
Acetyl Co A
Phosphoenol pyruvate
Oxaloacetate
Citrate
Malate
Isocitrate
CARBOHYDRATE SYNTHESIS
KREBS CYCLE
Fumarate
CO_2
Succinate
α-Ketoglutarate
Succinyl CoA
CO_2

into α-ketoglutarate to produceglutamate. These amino acids (ala, asp, glu) thus provide an important link between nitrogen and carbon metabolism.

Pyruvate produced from alanine is decarboxylated during the production of acetyl-CoA which may then enter the Krebs cycle, although biosynthesis of fatty acids and sterols is also possible from this intermediate. The possible routes of alanine metabolism are outlined in Figure 5.14. The points at which ^{14}C from [U^{14}C] alanine may be found are as follows:

a. [U^{14}C] alanine incorporated into protein

b. [U^{14}C] pyruvate produced by dehydrogenation of alanine

c. $^{14}CO_2$ produced by decarboxylation of pyruvate

d. The [U^{14}C] acetate moiety of acetyl-CoA which may enter
   i. steroid biosynthesis
   ii. fatty acid biosynthesis
   iii. the Krebs cycle

e. The presence of [U^{14}C] acetate in the Krebs cycle will result in the release of $^{14}CO_2$

f. Krebs cycle intermediates may be involved in further metabolism such as carbohydrate biosynthesis, however this is of less importance since glucose served as the carbon source in the incubation buffer.

Therefore, ^{14}C presented in the form of an amino acid such as alanine is not located in a single metabolite but becomes incorporated into many organic molecules of differing complexity.

In the present study, $^{14}CO_2$ was found to be released from cells indicating that much of the $[U^{14}C]$ alanine was deaminated and decarboxylated during the production of acetyl-CoA . Linear release of $^{14}CO_2$ occured for at least 90 mins after the commencement of incubation and therefore much of the label entered the Krebs cycle, eventually being released as $^{14}CO_2$. Some of the label was retained by cells in the form of protein, fatty acids, steroids or carbohydrates.

The rate of release of radiolabel from different amino acid substrates will vary: metabolically active amino acids such as glutamate aspartate, alanine and glycine will under rapid metabolism followed by loss of $^{14}CO_2$ while other such as tyrosine and phenylalanine will enter the pathway leading to the production of fusel oils.

Although $^{14}CO_2$ was released from the incubation medium (*C.albicans*+ ala_2) there was also a progressive increase in the concentration of non-volatile metabolites in the medium. These were not identified and, from the complexity of the metabolic pathways involved, the nature of the released substance cannot be predicted.

The kinetics of transport were greatly influenced by the use of radiotracers and gave both reduced rates of uptake and K_m values when compared with the fluorescamine technique. The kinetic values obtained for different pepides cannot be compared directly since they are dependent upon the rate of loss of label from the cell. Nisbet

and Payne (1980) found the apparent rate of [^{14}C] gly phe uptake by *E.coli* to be lower than that of gly[^{14}C] phe. This can be explained by the rates of metabolism of glycine and phenylalanine and hence the differing rates of loss of ^{14}C from cells.

Therefore the apparent rate of uptake of a radiolabel will be dependent upon the rate of peptide hydrolysis and the rate of subsequent metabolism of the amino acid residues. A peptide which is transported at a low rate and yet hydrolyzed and metabolized slowly may give an apparently higher rate of incorporation than one which is rapidly transported, hydrolyzed and metabolized.

Radiolabelled substrates have gained widespread use in transport studies of metabolites and yet these may not give data appertaining directly to the transport of the molecule.. New methods, such as the fluorescamine technique, are now becoming available. These may provide more satisfactory data which are independent of the subsequent fate of the substrate.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

The present study has demonstrated the existence of at least two peptide permeases in *Candida albicans:* System I has high affinity for dipeptides and also transports oligopeptides; System II is a general oligopeptide permease with low affinity for a few dipeptides. The dipeptide nikkomycins enter the cell via the system I permease and exert their antifungal activity through the inhibition of chitin synthase, thereby interfering with the growth processes of the cell. Resistance to these drugs, through a defect in transport, produced a mutant defective in the system I permease.

A report has been published confirming some of these results (Sarthou, *et al.*, 1983). Photolysis of 4-azidobenzoyl trimethionine, an N-acetylated derivative of met_3, with ultraviolet light photoinactivated the peptide tansport system of *Candida albicans* ATCC 26278. Total inhibition of met_3 transport was not observed. Details of much of the work are not available. However, it is stated that the kinetic studies indicated the presence of at least two oligopeptide permeases. The inactivation of a single permease by an N-acylated peptide confirms the suggestion made earlier that such peptides enter through only one of the permeases, whereas oligopeptides with a free N-terminal are able to utilize both systems. From the data available it is not possible to correlate the transport system for N-acylated peptides with either system I or II. Sarthou, *et al.* (1983) also showed that leu_3 protected the

permease from photoinactivation. Leu_3 is not itself transported (as has been shown in the present study) and this may indicate that the photo-inactivation is taking place at the peptide binding site. Met_3, which is transported, gave less protection than leu_3. Therefore the former may bind with high affinity to the permease, without subsequent transport (in a way equivalent to leu_2), thereby inhibiting binding of the N-acylated derivative. However, met_3 which also binds to the binding site, is then transported. Therefore the competitive effect of the binding site may be less than that of leu_3 and hence met_3 exerts a lower protective effect.

The potential of the technique of photoinactivation of peptide permeases has also been studied by Becker, *et al.* (1982) with the yeast *Saccharomyces cerevisiae*. They used leucyl leucyl-4-azido-2-nitrophenylalanine and leucyl-p-nitronilide (leu-pNA). Leu-pNA, which is readily available, appears to be an effective inhibitor of the *S.cerevisiae* peptide permease. If this is also an inhibitor of *C.albicans* peptide transport it will provide a useful tool for the inactivation of the system I permease. Other peptide analogues such as ala-pNA may be effective in inactivating systems I and II because certain alanyl peptides are able to utilize them both. In the absence of both systems it will be possible to identify any passive diffusion which may be occuring (as suggested by Sarthou, *et al.*, 1983). The photoinactivation of individual systems will also allow a detailed study of the kinetics of transport via thepermeases in the constitutive and induced states without interference from other factors (such as the large degree of induction of one system, as observed in the peptide-transport deficient mutant NIK5).

It is interesting to note that Sarthou *et al.* (1983) obtained kinetic evidence (not published) for the presence of multiple met_3 permeases using cells grown on a defined medium containing amino aicds as the nitrogen source. In contrast, the present study used a peptone-based medium and found no kinetic evidence for the presence of multiple peptide permeases. The observation that peptide permease induction masks the constitutive level would therefore appear to be confirmed.

Further evidence for multiple permeases will become available through the use of toxic oligopeptides. In the present study of m-F-phe ala ala was toxic for the mutant NIK5 , hence a further mutation is possible to produce a variant lacking both systems I and II. The toxicity of m-F-phe ala ala for the strain used was low, and the quantities of the drug needed to create such a mutant were not available. However, since this is a simple tripeptide, it can be readily synthesized and could therefore be used in large amounts. It is also possible that the tri-peptide nikkomycins (I,J,M and N) will become available (Zahner, personal communication). These may be toxic for *C.albicans* and many other fungi, and therefore give further information concerning oligopeptide transport.

C.albicans growing on a simple medium (ammonium salts or amino acids as nitrogen source) possesses constitutive peptide permeases. However when the nitrogen source is composed of peptides there is an inducible level of uptake. The constitutive nature of the permeases provides a mechanism whereby any peptides in the environment may be captured by the cell even in the presence of amino acids. The induction of permeases in the presence of peptone suggests that peptides are acting as inducers

for these systems as does proline for the proline permease of *C.albicans* (Jayakumar, *et al.*, 1979). In the pathogenic state the principle nutrient sources are the host proteins and peptides. Therefore, the induced transport system is likely to be of importance in the invasive state. In these circumstances an extracellular protease is also induced (Staib, *et al.*, 1965) thus providing a highly efficient mechanism for both invasion and utilization of host tissues. The inter-relationship between protease secretion and peptide transport capacity has not been studied and it is not known whether proteins will act as inducers or whether hydrolysis to peptides is necessary before induction can take place. It is possible that a situation similar to that described for *Neurospora crassa* may be in operation (Wolfinbarger, 1980): The secretion of ahydrolytic enzyme takes place in response to peptides larger than those which can be transported. A mutant of *N.crassa (glt-r)*, lacking the peptide permease, secreted this enzyme in response to any added peptide. When mutants of *C.albicans* lacking both permeases become available, it may be found that extracellular protease or peptidase secretion may take place in response to any added peptide. Such a mutant will give further information cocnerning both the regulation of transport of nitrogen-containing metabolites and the role of proteolytic activity in pathogenicity. A mutant producing proteases or peptidases in response to peptides may be of higher pathogenicity than the wild type: MacDonald and Odds (1983) have described a mutant unable to secrete a protease which was of low pathogenicity when compared with the wild type. Hence a mutant which is in effect constitutive for protease secretion may be highly pathogenic.

Although inducible amino acid permeases have been described for many organisms (such as the lysine permease of *Pseudomonas putida*, Miller and Rodwell, 1971; the proline permease of *C.albicans*, Jayakumar, *et al.*, 1979), inducible peptide permeases have not been previously described. Minimal defined media have been used in most instances. However Davies (1979) showed peptone grown cells to have an increased capacity for the transport of peptides than those grown on proline. Further work was not carried out to define the nature of the difference. The present report is therefore the first description of the inducible nature of the peptide permeases.

It must be pointed out that peptides do not only provide a nitrogen source for the cells, they are also able to provide carbon and sulphur sources for growth. Further work must determine whether conditions of carbon- or sulphur-limitation are able to induce peptide transport systems even in the presence of an ammonium salts-based nitrogen source. The interrelationship between nutrient sources and the induction of specific permeases may therefore become highly complex.

N.crassa has been shown to possess amino acid and peptide permeases comparable to those of the non-filamentous fungi. The present study followed peptide transport by blastospores of *C.albicans* with no attention being given to the hyphal form. Although there is similarity between the filamentous and non-filamentous fungi, it is possible that there are important differences between the blastospores and hyphal stages of growth of *C.albicans*. These differences may be reflected in

the activity of the various permeases including those for peptides. *C.albicans* hyphae are however less amenable to study than the blastospres. As has been previously indicated, there is no defined medium which will allow the formation of stable hyphae. There is much work being carried out at present concerning the regulation of dimorphism and this may produce a satisfactory medium for the hyphal induction. The invasive form of *C.albicans* is hyphal, therefore it is important to study this phase in any rational drug-development studies.

The development of peptide-drug conjugates

The purpose of this research was to investigate the potential of the peptide permease as a possible system for the selective introduction of chemotherapeutic agents into the fungal cell. Two major points have to be considered: The potential sites of attachment for toxic agents and the interactions of peptide-drugs with the environment of the cell.

N-acylated peptides can enter via the peptide permease and this is therefore a potential site of attachment of toxic residues. If linked by a peptide bond intracellular hydrolysis would result in the release of the toxic moiety and hence unmask drug action. However, a more important observation is the considerable tolerance for large side-chain residues shown by the system I permease (.e.g. in the uptake of the dipeptide nikkomycins). Although it is not clear whether system II also shows this degree of tolerance there is obviously a considerable scope for linking toxic residues to the side-chains of peptides. If such an attachment is via a peptide or glycoside bond intracellular

hydrolysis will be possible. Alternatively the drug may be active intact as are the nikkomycins. One major advantage of peptide derivatives as drugs is that alteration of N- or C-terminals, will determine their specificity. Reference to Table 1.1 reveals that N-acylated peptides will only enter the yeasts *S.cerevisiae* and *C.albicans* of the organisms so far studied. An N-terminal blocked peptide linked via a side-chain residue to a toxic moiety will therefore produce a highly specific molecule toxic for yeasts. Such peptides appear to be transported by the mammalian gut, the possibility of oral administration therefore exists. It will be necessary to show whether all mammalian tissues show an equivalent specificity. Serum peptidases were not found in the present study, however red blood cells have a defined complement of such enzymes (Lewis and Harris, 1967). If the peptide-drug is unable to enter the host's cells the problem of hydrolysis before reaching the site of action should be minimal. Anticandidal agents of this type could therefore be of considerable importance in the treatment of systemic candidoses.

There is a potential problem with the development of anticandidal peptide-drugs in that nikkomycin, the example used in this study, was found to be inactive in the presence of serum proteins or peptides. This is a problem not encountered with antibacterial agents such as alafosfalin. The inactivity of nikkomycin was not due to hydrolysis within the serum and would therefore appear to be due to an interaction between the proteins and the peptide permease. The yeasts have been shown to be able to interact with large peptides in their environment (such as the yeast mating-factors) even though these are unable to enter the cell. Presumably differences in the cell wall structure of the

fungi and bacteria allow large polypeptides to interact with the cell membrane of fungi, while peptides larger than those which can be transported are unable to enter/affect the bacterial cell. This may be a major factor in determining the practicality of this line of drug development.

Although there are problems associated with the design of anticandidal peptide-drugs there is the potential for selective drug development. Therefore they may eventually provide an alternative, selective, therapy for the treatment of candidoses and other fungal infections, which are becoming of increasing medical importance.

This is especially true in the immunocompromised host occurring through either surgical procedures (transplants) or disease states such as AIDS (acquired immune deficiency syndrome). AIDS appears to be of increasing incidence and death frequently occurs through invasion of host tissues by *C. albicans*.

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