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David A. Shallow

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PEPTIDE TRANSPORT IN <u>CANDIDA</u> <u>ALBICANS</u>

AND SYNTHETIC ANTIFUNGAL AGENTS

by

David A. Shallow, B.A. (York)

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ABSTRACT

These studies have characterized the peptide transport systems of Candida albicans, with a view to the rational design of peptide antifungal agents exploiting the 'smugglin' concept.

In initial studies, a series of polyoxin complexes (peptide-nucleoside antibiotics) and individual components, were isolated from a batch of agricultural fungicide (Polyoxin Z). Isolated fractions were toxic to a particulate chitin synthetase preparation from Candida albicans.

Different strains of Candida albicans exhibited varied sensitivities to a series of peptide analogues. From a sensitive strain, B2630, spontaneous mutants were selected for resistance to each analogue; certain mutants showed cross-resistance to other analogues and associated defects in peptide transport. A bacilysin-resistant mutant was cross-resistant to the other analogues and to m-fluorophenylalanylalanine but retained sensitivity to m-fluorophenylalanylalanylalanine. This mutant showed defective dipeptide transport but normal oligopeptide transport, and was unable to utilize Ala-Ala as a sole nitrogen source. Thus, Candida albicans has distinguishable mechanisms for dipeptide and oligopeptide transport which can be exploited for uptake of peptide-drug adducts.

Peptide transport was shown to be stimulated by the presence of peptides (peptone) in the growth medium. On transferring cells from minimal to peptone medium, this stimulatory effect was shown to be rapid, independent of protein synthesis and to override ammonia regulation of peptide transport. The reduction of transport activity on transferring cells from peptone to minimal medium was also rapid. It was speculated that regulation of peptide transport is achieved by a rapid, reversible activation of preformed transport components, or a mechanism of exocytotic insertion and endocytic retrieval of preformed transporters.

The effect of protein-modification reagents on transport activity was also examined. Dipeptide transport was specifically inhibited by N-ethyl-5-phenylisoxazolium-3'-sulphonate (Woodwards Reagent K), offerping potential for the specific labelling of the component(s) of this system. Peptide transport was shown not to be sensitive to osmotic shock though a series of uncharacterized polypeptides was released by the shock treatment.

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TABLES

CHAPTER   I

INTRODUCTION

1.1 Introduction

Both prokaryotic and eukaryotic organisms possess many specific transport systems 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, and the peptides and amino acids released by hydrolytic action are then transported into the cell.

Peptides have long been recognised as important nutrients for microorganisms, though characterization of those systems involved in peptide uptake and utilisation has only recently been undertaken. The broad specificity exhibited by those peptide transport systems characterized to date, has prompted research into the use of these systems for rationally designed peptide mimetic antibiotics, using the illicit transport concept of Ames et al., (1973).

Although peptide transport has been demonstrated in many organisms, the uptake systems have been well characterized in only very few, in particular the bacteria Escherichia coli and Streptococcus faecalis, the yeast Saccharomyces cerevisiae, and in germinating barley embryos. In the present work, the peptide transport systems of the pathogenic yeast Candida albicans have been characterized, and a series of peptide drug-resistant mutants described.

In this introductory chapter, a survey of the literature summarising the current knowledge of microbial nitrogen metabolism and peptide-antimicrobial agents is presented.

1.2 Candida albicans

Together with Crytococcus, Candida is one of the two most important genera of pathogenic yeasts that have been found in association with man. The genus Candida has been found to contain a number of pathogens of medical importance, including Candida albicans, guilliermondii, krusei, parapsilosis, stellatoidea and tropicalis, of which Candida albicans is the one most frequently encountered and the most pathogenic (Odds, 1979).

Candida albicans is a normal commensal of the human gut, and is an opportunistic pathogen which can become pathogenic if the hosts defence mechanisms or metabolism are impaired. Certain conditions predispose humans to candidosis, including disorders such as diabetes melitus, cancers, stress, mechanical factors such as burns, wearing of dentures etc. It has long been argued that the increasing use in recent years of broad spectrum antibiotics has led to a greater incidence of candidosis: these antibiotics have been shown to alter the hosts microflora, enabling opportunistic fungi such as Candida albicans to gain prevalence. However, it is uncertain whether the drugs bring about the invasion of Candida albicans, since the patient is often debilitated before administration and is therefore predisposed to candidosis (Odds, 1979). Candida metabolism, infections and treatment have been comprehensively reviewed by Odds (1979), and thus will not be discussed in any great detail in this review. Superficial Candida infections are very common, whereas systemic infections are less common, though far more serious, proving fatal in isolated cases. Systemic infections may involve single organs or multiple organ sites in which the organism has spread through the blood stream (Candida septicaemia). Systemic candidosis is sometimes fatal, recovery being

achieved by surgical removal of the site of infection in conjunction with the use of antifungal agents. The recent increased incidence of organ transplants, acquired immune deficiency syndrome and space travel have all been associated with increased Candida infections. There are a limited number of antifungal drugs available for the treatment of candidosis, these are reviewed in Section 1.2.1.

Candida albicans is a dimorphic fungus, possessing both yeast and mycelial forms. The yeast replicates primarily by the production of buds from blastospores (yeast cells). The formation of pseudohyphae and true hyphae has also been demonstrated. A number of morphological factors (germ tube production and chlamydospore formation) have been used for the presumptive identification of Candida albicans from clinical isolates. Factors inducing filamentous growth have been discussed by Odds (1979), they include low oxygen tension, polysaccharide carbon source, pH greater than 7, presence of N-acetylglucosamine and a low medium sulphydryl concentration. Blastospores are formed at 25 - 30°C, whereas filamentous growth occurs at 35 - 40°C. Serum at 37°C has been found to induce filamentous growth.

Dimorphism in Candida albicans is thus influenced by available nutrients, temperature and the growth stage of the cells. Early studies investigated the relationship between dimorphism and metabolism by studying cell wall composition, enzymes for carbohydrate metabolism, and intracellular metabolite concentrations in the yeast and mycelial forms (Chattaway et al., 1968; 1973). The chitin content in the mycelial form is three times higher than in the yeast form, whereas yeast cell walls have a higher protein content (22%), compared with the mycelial form (9%).

More recent studies have focussed on germ tube formation, the transition stage between the two morphological forms. Near synchronous germ tube formation may be induced when starved or stationary-phase cells are incubated at 37°C with N-acetylglucosamine (Simonetti, et al., 1974), other acetylhexosamine derivatives (Sullivan & Sheperd, 1982), or glucose plus glucosamine (Shepherd et al., 1980a). During germ tube formation there is extensive RNA and protein synthesis but no DNA synthesis (Shepherd et al., 1980a), changes in lipid composition (Sundaram et al., 1981), a three fold activation of chitin synthetase (Chiew et al., 1980) and an associated induction of the pathway for N-acetylglucosamine catabolism (Sheperd et al., 1980b).

The controlling mechanism for the yeast-mycelial transition is still not fully understood, but is recognised to be a complex interaction of several factors. Cyclic adenosine 3',5'-monosphosphate (cAMP) has been claimed to be one of the controlling factors in the morphological transition (Niimi et al., 1980; Chattaway et al., 1981), though this has been disputed (Sullivan et al., 1983). A central regulatory role for magnesium ions has also been proposed (Walker et al., 1984), the intracellular magnesium concentration increases during germ tube production, and magnesium has been shown to affect the uptake and incorporation of N-acetylglucosamine. Differential protein synthesis during blastospore formation at 24.5°C, and during germ tube formation at 37°C has been shown (Ahrens et al., 1983), though these proteins were not identified. Germ tube formation has been shown to be induced by immobilized N-acetylglucosamine (Sheperd & Sullivan, 1983), the N-acetylglucosamine being covalently bound to agarose beads. It was suggested that N-acetylglucosamine may trigger germ tube formation via an external receptor.

An extensive analysis of the metabolism and cell wall composition of <u>Candida albicans</u> during germ tube formation has been reported (Sullivan <u>et al</u>., 1983). Methods for the <u>in situ</u> assay of <u>Candida albicans</u> enzymes in permeabilized cells (Ram <u>et al</u>., 1983) and for single cell analysis of the yeast-mycelial transition using a perfusion chamber (Soll & Herman, 1983) have recently been described. Spheroplasts have been used extensively to study cell wall biogenesis; glucan synthesis and the metabolism of $[^{14}C]$-labelled glucose in regenerating spheroplasts of <u>Candida albicans</u> has been described (Gopal <u>et al</u>., 1984).

Physiological and ultrastructural studies of the yeast-mycelial transition have also been described. (Cassone <u>et al</u>., 1973; Scherwitz <u>et al</u>., 1978; Mitchell & Soll, 1979; Chaffin, 1984). In the yeast phase the septum is deposited at the intersection between the mother cell and bud, and mycelial formation begins at a time point equivalent to the start of evagination. Mycelial formation commences with germ tube formation, the septum being produced at a variable point but seldom at the intersection between the two cells (Mitchell & Soll, 1979).

The shape of the cell is determined by the cell wall, and thus several studies have concentrated on the influence of the cell wall on the yeast-mycelial transition. The cell wall of <u>Candida albicans</u> has been shown to be composed of five distinguishable layers (Djaczenko & Cassone, 1971; Cassone <u>et al</u>., 1973). The cell wall is composed of mannan, glucan, chitin, protein and lipid. Chitin is restricted to the inner regions of the cell wall and to the primary septa and bud scars (Tronchin <u>et al</u>., 1981). The specific activity of chitin synthetase is greater in mycelia than in the blastospore form (Braun & Calderone,

1978; Chiew et al, 1980), whereas the specific activity of β-(1,3) -glucan synthetase is the same in both morphological forms (Orlean, 1982). Thus, chitin has a central role in the yeast-mycelial transition. The properties of chitin synthetase are discussed in detail elsewhere (Section 1.4.4.1) in relation to targeting of antifungal drugs. Cell wall biosynthesis has been reviewed in detail elsewhere (Gooday, 1977; Gooday & Trinci, 1979; Farkas, 1979).

Candida albicans is a member of the fungi imperfecta and as such has no sexual cycle, thus conventional genetic analysis is not feasible. The ploidy of Candida albicans was unknown until recently, several reports claimed that the yeast was haploid (Poulter et al., 1981; Sarachek et al., 1981), whereas others claimed that yeast was diploid (Olaiya & Sogin, 1979; Whelan et al., 1980; Whelan & Magee, 1981). However, a recent detailed study proved conclusively that Candida albicans is diploid (Riggsby et al., 1982), through DNA reassociation experiments. Candida albicans has been shown to contain repetitive DNA (Wills et al., 1984), and to be naturally heterozygous for several genetic markers (Whelan et al., 1980; 1981; Whelan & Soll, 1982; Crandall, 1983; Kakar et al., 1983). By induced mitotic segregation, several clinical isolates have been shown to be heterozygous for auxotrophic markers. Similarly 5-fluorocytosine-resistant strains have been shown to be heterozygous for 5-fluorocytosine resistance (Whelan et al., 1981), and consequently capable of giving rise to resistant variants at high frequency by segregation.

Recent advances in the biology, genetics and pathogenicity of Candida albicans have been extensively reviewed (Shepherd et al., 1985).

There are a limited number of antifungal drugs active against Candida albicans including 5-fluorocytosine, polyene antibiotics, azole derivatives and allylamine derivatives, the properties and side effects of which have been recently reviewed (Sheperd et al., 1985), and will not be dealt with here.

Candida albicans is thus a pathogen of considerable medical importance. The increasing incidence of systemic candidosis and the lack of effective drugs necessitates the development of a new generation of antifungal agents. Targeting of inhibitory compounds to specific enzyme systems present only in yeast could in principle lead to the development of drugs without side-effects. However, this rational approach to chemotherapeutic development must take into consideration the natural permeability of the yeast cell membrane.

To this end, this project seeks to characterize the peptide transport systems of Candida albicans, with a view to using these systems for facilitated entry of inhibitory compounds in peptide-linked form. During the period of this study, several peptide drugs (both natural products and synthetic compounds) became available, and studies of their mode of uptake were undertaken. In the following sections, the important aspects of relevant membrane transport systems in Candida albicans are reviewed.

1.3 Microbial Nitrogen Metabolism

1.3.1 The Transport of Ammonium Ions

Bacteria and fungi are both able to accumulate ammonium ions against a concentration gradient (reviewed by Brown, 1980), though this topic has received far less attention that that of amino acid or peptide transport.

Since the pK of the dissociation of ammonium to ammonia is 9.25 at $24^{\circ}C$, it is evident that at physiological pH values, the ammonium ion will be the predominant species. The use of the substrate analogue $[^{14}C]$methylammonium has permitted kinetic studies of ammonium transport. Pateman et al., (1974) demonstrated accumulation of $[^{14}C]$methylammonium against a concentration gradient, and described mutants with reduced uptake and poor growth on ammonium. Similarly, Broach et al., (1976) described mutants of Salmonella typhimurium that were defective in ammonium uptake, though they were not defective in ammonia assimilation. Methylammonium accumulation in Sacc. cerevisiae has been shown to be inhibited by ammonium (Roon et al., 1975), mutants defective in methylammonium transport simultaneously lose the ability to take up and utilize ammonium.

At least two ammonium ion transport systems are present in Saccharomyces cerevisiae (Dubois & Grenson, 1979), whereas a single system has been reported for Aspergillus nidulans (Cook & Anthony, 1978a). However, the number of transport systems in other fungi is unclear.

The control of ammonium ion transport was shown to occur by two mechanisms : repression of synthesis of the transport system and inhibition of the activity of the preformed system.

In Aspergillus nidulans uptake rates were high in organisms grown on poor nitrogen sources (alanine, proline and nitrate) and low on those nitrogen sources known to produce a high intracellular concentration of ammonium (urea, casamino acids and ammonia), (Pateman et al., 1974). Development of the transport system during nitrogen starvation was inhibited by cycloheximide, indicating the involvement of protein synthesis in the regulation of this permease. Thus, the

intracellular ammonium concentration regulates ammonium uptake by a repression of the synthesis of some protein component. A derepression mechanism was shown not to occur in Sacc. cerevisiae (Roon et al., 1975). Specific metabolites have been implicated in the control of the ammonium ion permease : levels of glutamine and asparagine control the activity in Aspergillus nidulans (Cook & Anthony, 1978b), and glutamine in Sacc. cerevisiae (Dubois & Grenson, 1979).

1.3.2   Amino Acid Transport

1.3.2.1   Introduction

Amino acids have long been known to act as nitrogen sources for microorganisms, having been shown to be incorporated directly into protein and also to serve as a general nitrogen source via transamination pathways. The transport of amino acids is therefore coupled to an efficient metabolic system to utilize and store the assimilated nitrogen. Studies of the uptake and subsequent metabolism of amino acids are clearly relevant to work on peptide transport, and hence a brief survey of microbial amino acid uptake is included here.

1.3.2.2   Bacterial Amino Acid Transport

The transport and utilization of amino acids by bacteria has recently been reviewed (Anraku, 1980). E.coli has been shown to possess twelve kinetically-defined transport systems for groups of structurally related amino acids, and systems for cysteine and histidine which have yet to be kinetically defined. The substrate specificities of the transport systems of related bacteria appear to be analagous to those found in E.coli.

The genetics of bacterial amino acid transport have been reviewed Iaccarino et al (1980).

1.3.2.3 Amino Acid Transport in Yeast

Aspects of yeast amino acid transport have recently been reviewed (Eddy, 1980; Oxender et al., 1980; Cooper, 1982b).

Multiple amino acid transport systems have been demonstrated in Sacc. cerevisiae. Work by Grenson and her co-workers has shown the existence of one general amino acid permease (Grenson et al., 1970), which transports a range of L-amino acids but not proline (an imino acid). This general amino acid permease (gap) is subject to ammonium repression, activity of this permease being low when organisms are grown with ammonia as the sole nitrogen source. This gap system can also transport D-stereoisomers of the amino acids, which are toxic to the cell, facilitating the selection of gap-deficient mutants (Rytka, 1975). Specific transport systems exist for the transport of histidine (Crabeel & Grenson, 1970), methionine (Gitts & Grenson, 1967), lysine (Grenson, 1966), arginine and lysine (Grenson et al., 1966; Chan & Cossins, 1976; Seaston et al., 1973), glutamate (Joiris & Grenson, 1969; Darte & Grenson, 1975), leucine (Kotliar & Ramos, 1983) and serine (Shukla et al., 1982; Verma et al., 1984).

A proline transport system has been demonstrated in Sacc. chevalieri (Magana-Schwencke & Schwencke, 1969; Schwencke & Magana-Schwencke, 1969).

Amino acids accumulated by Sacc. cerevisiae are not freely exchangeable with those in the medium, in marked contrast to some bacterial amino acid permeases. Thus, yeast amino acid transport is subject to regulation by transinhibition. Cells preloaded with labelled histidine do not lose label when incubated in a medium containing unlabelled histidine (Crabeel & Grenson, 1970). Preloading with lysine or arginine reduced the activity of the lysine and lysine-arginine permeases (Morrison & Lichstein, 1976). Similarly, when cells

are preloaded with unlabelled valine, arginine, lysine or α-amino isobutyric acid, uptake of the corresponding labelled amino acid is severely inhibited (Woodward & Cirillo, 1977). Thus, transinhibition is a mechanism of feedback regulation in which the intracellular concentration of a substrate regulates the activity of its own specific permease without affecting the activity of other specific transport systems. This transinhibition mechanism could explain the apparent inhibition of amino acid uptake by peptides; accumulation of amino acids released by intracellular peptide hydrolysis may result in transinhibition of subsequent uptake of free amino acids from the medium.

Amino acid uptake by yeast has also been shown to be subject to ammonia repression. When Sacc. cerevisiae is grown on a poor nitrogen source such as proline, all the amino acid transport systems are fully active. However, when cells are grown on ammonia, conditions in which the cells are utilizing ammonia as a sole nitrogen source, activity of the gap system is inhibited (Grenson et al., 1970). Ammonia repression has also been shown for transaminase activity (Woodward & Cirillo, 1977), the synthesis of a number of catabolic enzymes (Dubois et al., 1973), in Sacc. chevalieri, for the proline permease (Schwencke & Magana-Schwencke, 1969), and for peptide transport in Sacc. cerevisiae (Becker & Naider, 1977; Nisbet & Payne, 1979a).

Ammonium ions have been shown to be incorporated into a carbon skeleton via the following reaction :-

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADPH \overset{GDH}{\rightleftharpoons} \text{glutamate} + NADP^+ + H_2O$$
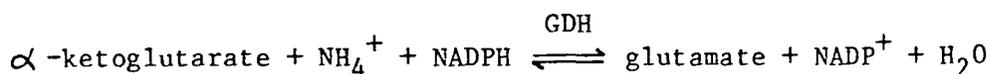
The NADP-dependent glutamate dehydrogenase (NADP-GDH) was shown to be derepressed in ammonia-limited cultures of Sacc. cerevisiae and Candida utilis (Brown, 1976); thus enabling the yeast to assimilate effectively

ammonia at low concentrations by synthesizing large amounts of a rather inefficient enzyme. NADP-GDH has been shown to play a central role in the control of fungal nitrogen metabolism (reviewed by Brown, 1980). Mutants lacking the structural gene for NADP-GDH (gdhA) were shown to grow only slowly on ammonium ions and lacked ammonium repression of the gap system (Grenson & Hou, 1972). Similarly, synthesis of arginase, urea amidolyase and allantoinase is released in mutants lacking a functional NADP-GDH (Dubois et al., 1973).

The precise mechanism of ammonia regulation of nitrogen metabolism in yeasts and the role of NADP-GDH is unclear. However, it seems likely that NADP-GDH may act in an analàgous regulatory fashion to that proposed for the enzyme in the filamentous fungus Aspergillus nidulans (Pateman & Kinghorn, 1976). In this model, NADP-GDH monitors both the intracellular and extracellular ammonia concentrations, and a complex of NADP-GDH and extracellular ammonia represses the synthesis of many enzymes and uptake systems involved in the utilization of alternative nitrogen sources (e.g. nitrate reductase; urea, purine and glutamate uptake etc.). NADP-GDH can also complex with intracellular ammonia and modulates ammonia uptake.

Experimental confirmation of this proposed mechanism is still required to show whether this model is operational in yeast, though this model does propose a mechanism to explain the complex interrelationships involved in ammonia repression.

Two distinct regulatory mechanisms have recently been proposed to explain the control of ammonia-sensitive transport systems (Grenson, 1983a,b); based on the study of the gap system in Sacc. cerevisiae. One is a reversible-inactivation process which progressively develops upon addition of ammonium ions to a proline-grown culture, completely

suppressing transport activity within one hour. In certain mutants, repression of the formation of active permease may be observed in the presence of ammonium ions (Grenson, 1983a). Further aspects of nitrogen regulation are discussed in detail in Chapter 4.

Regulation of ammonia assimilation and the control of nitrogen metabolism in bacteria, via the glutamine synthetase (GS) and glutamine synthase (GOGAT) model has been reviewed (Brown, 1980).

Amino acids have been found to act as sole nitrogen source for yeast by undergoing transamination. When different amino acids are supplied as nitrogen sources, the levels of the intracellular amino acid pools were shown to vary considerably (Watson, 1976), due to the regulatory effect of the supplied amino acid on the closely related amino acid biosynthetic pathways, and the efficiency of transamination to release α-amino nitrogen for biosynthesis. A cell free extract of Sacc. cerevisiae has been shown to catalyse the transfer of amino groups from a range of amino acids to α-ketoglutarate forming glutamate and a keto acid (Sentheshanmuganathan, 1960). Woodward and Cirillo (1977) demonstrated transaminase activity against aspartate, glutamate, leucine, isoleucine, valine, methionine, phenylalanine and tyrosine with α-ketoglutarate as an acceptor, and against alanine with pyruvate as an acceptor. Most amino acids have been found to be rapidly degraded with little being incorporated directly into proteins. Thus, α-amino nitrogen is readily incorporated into glutamic acid a metabolite central in the nitrogen metabolism of yeast. α-keto acids formed by transamination are decarboxylated to the corresponding aldehyde, and then usually reduced to form fusel alcohols which are rapidly released from the cell. The decarboxylation and subsequent exodus of fusel alcohols is of relevance to the use of radioactively-

labelled substrates for the study of amino acid and peptide transport (Section 4.3.8).

The vacuole has a key role in yeast amino acid metabolism, Wiemken & Durr (1974) showed that 60% of the total amino acid pool was localized in the vacuolar fraction, which contained a high proportion of metabolically inactive basic amino acids, which were not freely exchangeable with the cytoplasm. Metabolically active amino acids, such as glutamate, were located principally in the cytoplasm and were more freely exchangeable. A similar situation exists in Candida utilis (Wiemken & Nurse, 1973).

A specific arginine permease has been demonstrated in isolated vacuoles (Boller et al., 1975), which allows vacuolar accumulation of arginine which is compatible with the reported high vacuolar arginine pools found previously (Wiemken & Durr, 1974). 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. Thus, the vacuole acts as a storage organelle for nitrogen metabolites. Basic amino acids are suitable storage compounds, as their metabolism is closely linked to that of glutamate (Jauniaux et al., 1978).

1.3.2.4 Amino Acid Transport in Filamentous Fungi

The transport and utilization of amino acids by fungi has recently been reviewed (Wolfinbarger, 1980a).

Filamentous fungi (Neurospora crassa is taken as representative of this group) have been shown to possess multiple amino acid transport systems, analagous to the situation in yeast. Neurospora crassa has been shown to possess only five genetically and biochemically distinct

amino acid transport systems : a neutral and aromatic system (Wiley & 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 mechanism of ammonia repression of amino acid transport is found in filamentous fungi, having already been discussed with respect to Aspergillus nidulans (see Section 1.3.2.3). A further effect of ammonium ions on the plasma membrane of Neurospora crassa has been reported (Slayman & Goodman, 1975). Extracellularly supplied ammonium transiently depolarizes the membrane potential and hence a decrease in the proton motive force, resulting in a decrease in metabolite transport.

The participation of an extracellular deaminase in amino acid utilization by Neurospora crassa has been reported (DeBusk & Ogilvie, 1984). This deaminase, produced by strains defective in amino acid transport when grown on ammonium as a sole nitrogen source, converts amino acids to their respective keto acids plus ammonium.

Thus, ammonia regulation of nitrogen metabolizing systems appears to be very complex, being variable with individual systems, and care should be taken when extrapolating conclusions from one system to another.

1.3.3 Peptide Transport in Microorganisms

1.3.3.1 Introduction

The importance of peptides in the nutrition of microorganisms has long been recognized, though it is only relatively recently that the availability of defined synthetic peptides has permitted the elucidation of those mechanisms involved in peptide utilization. It has since become apparent that peptides act as amino acid sources for

many organisms, and that their utilization involves transport into the cell and cleavage to their constituent amino acids. It has been established that small peptides are transported intact into a range of microorganisms, many of which inhabit environments where small peptides are readily available (e.g. gut bacteria), so possession of peptide transport systems is clearly advantageous.

The recent availability of synthetic peptides has led to a proliferation of research into peptide transport in microorganisms. The volume of literature precludes an exhaustive survey in the space available here, so this review concentrates on areas of particular relevance to the work undertaken in this thesis. For further information on some of the topics not covered in detail here, the reader is referred to the reviews published over the last fifteen years (Barak & Gilvarg, 1975a; Matthews & Payne, 1975a,b, 1980; Payne, 1972a, 1975, 1976, 1977, 1980a,b, 1983; Payne & Nisbet, 1980a; Nisbet, 1980; Sussman & Gilvarg, 1971; Becker & Naider, 1980; Wolfinbarger, 1980b).

The properties of peptide transport systems in Candida albicans are not discussed in this section, being specifically dealt with in Section 1.3.6.

1.3.3.2 Models for Peptide Uptake

The evidence of, and theoretical basis for the concept of, intact peptide uptake followed by post-transport intracellular cleavage has been reviewed elsewhere (Payne, 1980a; Nisbet, 1980) and will not be discussed in detail here.

The utilisation of a peptide as an amino acid source in a microorganism may occur in one of three ways :-

1) Uptake of intact peptide followed by cleavage

2) Cleavage of peptide during translocation across the cytoplasmic membrane

3)   Cleavage extracellularly and uptake as free amino acids.

The situation is more complex in eukaryotes where intracellular cleavage may occur in intracellular organelles such as the vacuole.

If peptide transport does not involve extracellular cleavage, then peptide and amino acid transport should be distinguishable and the following features demonstrable :-

1)   Lack of inhibition of peptide by free amino acids.

2)   Uptake in peptide-linked form of amino acids impermeant in the free form.

3)   Uptake of peptides in amino acid-permease-deficient strains.

4)   Uptake of amino acids by peptide-permease-deficient strains.

5)   Intracellular accumulation of intact peptides.

Lack of competition by free amino acids for the uptake of radioactively-labelled peptides has been demonstrated for a range of microorganisms (for a review see Nisbet, 1980). Of particular relevance is the lack of competition demonstrated in Sacc. cerevisiae (Becker & Naider, 1977) and Candida albicans (Logan et al., 1979; Davies, 1980). Lack of competition has also been shown by direct fluorescence assays of transport in E.coli (Payne & Bell, 1977a) and Salmonella typhimurium (J.W. Payne, personal communication).

The uptake of impermeant amino acid derivatives in peptide form is of particular relevance in the development of novel antibacterial agents; several examples for a range of microorganisms are presented in Section 1.4.2.

Amino acid transport-deficient mutants have been shown to retain peptide transport capacity in E.coli (De Felice et al., 1973; Guardiola & Iaccarino, 1971; Guardiola et al., 1974a,b), Salmonella typhimurium (Kustu & Ames, 1973) and Lactobacillus casei (Peters et al., 1953). Conversley, peptide transport-deficient mutants have been

shown to retain amino acid transport capacity in E.coli (Payne &
Gilvarg, 1978; Alves & Payne, 1980), Salmonella typhimurium (Ames et
al., 1973); Staphylococcus aureus (Perry & Abraham, 1979); Sacc.
cerevisiae (Marder et al., 1979; Nisbet & Payne, 1979b) and Neurospora
crassa (Wolfinbarger & Marzluf, 1975a,b).

The demonstration of intact peptide accumulation would rule out
the possibility of cleavage being an obligate feature of uptake.
However, many organisms have very high intracellular peptidase activity
making the demonstration of intact accumulation difficult. However,
certain peptides such as triornithine and glycylsarcosine are resistant
to peptidase action, whilst retaining sufficient affinity for
transport, thus allowing intact accumulation to be demonstrated.
Intact accumulation by Escherichia coli has been shown for peptidase-
resistant,ϵ-derivatized lysine peptides (Payne, 1972a); triornithine
(Payne 1968; Gilvarg & Levin, 1972; Payne & Bell, 1977b, 1979) and
glycylsarcosine (Payne 1972b, Payne & Bell, 1977b, 1979). Intact
accumulation of Ala-Pro-Gly (Jackson et al., 1976) and Gly-Pro (Yang et
al., 1977) by peptidase-deficient strains of Salmonella typhimurium has
been demonstrated. Similarly, intact accumulation of glycylsarcosine
against a concentration gradient has been demonstrated for Sacc.
cerevisiae (Nisbet & Payne, 1979a) and Streptococcus faecalis (Nisbet &
Payne, 1982). Thus, cytoplasmic cleavage is implicated in the
utilization of peptides in these strains. Of particular relevance to
this section is the report that peptide transport in Sacc. cerevisiae
is distinct from peptide hydrolysis (Parker et al., 1980). Peptidase
activity towards trimethionine was shown to be localized in the soluble
fraction of the yeast, an extracellular aminopeptidase II-like activity
did not metabolise this peptide. Thus, it was concluded that the
tripeptide is taken up intact and rapidly hydrolyzed inside the cell.

However, cleavage during translocation cannot be excluded in other organisms. There is some evidence that cleavage is by a membrane bound peptidase in Pseudomonas aeruginosa (Miller & Becker, 1978); although this does not appear to be the case in Pseudomonas maltophilia and Pseudomonas putida (Cascieri & Mallette, 1976a,b).

Thus, peptide transport is distinct from amino acid transport in a range of microorganisms, and extracellular peptidase action does not play a significant role in peptide utilisation in these species. Secretion of peptidases capable of cleaving small peptides is rare, and has been reviewed by Hermsdorf & Simmonds (1980) and Nisbet (1980). Peptidases associated with the external surface of the cell have been found in Streptococcus lactis (Law, 1977), Streptococcus cremoris (Exterkate, 1975, 1976), and Sacc. cerevisiae (Matile et al., 1971). In Neurospora crassa dipeptide utilization is inhibited by amino acids, presumably an extracellular dipeptidase is involved (Wolfinbarger, 1980b).

In bacteria, rapid exodus of peptide-derived and metabolically related amino acids is a widespread phenomenon, and is a logical mechanism of regulating the amino acid pools during peptide utilization (Payne, 1975). In a natural environment such as the gut, microorganisms are presented with a wide range of peptide substrates, whereas in uptake assays in vitro large amounts of only a limited number of amino acids are introduced into the cell after uptake and cleavage of a particular peptide. Rapid exodus has been shown to occur in E.coli (Payne & Bell, 1977a, 1979), Salmonella typhimurium (J.W. Payne, personal communication), Staphlococcus aureus (Perry & Abraham, 1979), and Streptococcus faecalis (Nisbet, 1980). Release of excess

amino acids during peptide utilization is a more efficient response than inhibiting peptide uptake, which would compromise intake of potentially useful amino acids.

Exodus of peptide-derived amino acids has been shown not to occur in Sacc. cerevisiae (Nisbet & Payne, 1979a), even though intracellular peptide hydrolysis is rapid. This was attributed in part to the greater capacity of yeast for accumulating amino acids (Indge et al., 1977), and to the possible release of deaminated derivatives (Woodward & Cirillo, 1977; see Section 1.3.2.3) which would not have been detected by the fluorogenic reagents employed in these studies. The lack of exodus of peptide-derived amino acids in the yeast Candida albicans was suggested by Davies (1980), who reported the time-dependent loss of radioactivity from whole cells following uptake of radioactively-labelled peptide. Amino acid analysis of the supernatant-fluid failed to reveal the presence of ninhydrin-positive material. The effluxed radioactive material was preferentially partitioned into an organic solvent. Davies (1980) suggested that this material was the metabolized by-products of peptide-derived amino acids, similar to those described by Woodward & Cirillo (1977). Thus, the use of radioactively-labelled substrates may lead to miscalculation of the true rate of peptide transport and consequently produce misleading data on the kinetics of uptake (Payne & Nisbet, 1980b).

Thus, the principle of intact uptake of peptides followed by the intracellular hydrolysis by peptidase action has been established.

1.3.3.3 Peptide Transport in Bacteria

Peptide transport studies in bacteria have been conducted principally with Esherichia coli and Streptococcus faecalis. A series of reviews on bacterial peptide transport has been published (see Section 1.3.3.1), and thus the early work will not be reviewed here.

The isolation and characterization of mutants resistant to toxic peptides has proved valuable in determining the number and nature of the peptide transport systems in bacteria. Escherichia coli has been shown to possess three peptide transport systems :- one specific for dipeptides (the dpp), and the other for oligopeptides (the opp) (Payne, 1968; Gilvarg & Levin, 1972). Mutants defective in both di- and oligopeptide transport have been shown to possess some residual transport activity of broad specificity (Alves & Payne, 1980), which may be identical to a third 'specialized' system reported by Barak & Gilvarg (1975b) and Naider & Becker (1975). Similar di- and oligopeptide transport systems have been demonstrated in Streptococcus faecalis (Nisbet & Payne, 1980, 1982). A third peptide transport system specific for anionic peptides with N-terminal aspartyl or glutamyl residues (the app) has also been described in this organism (Payne et al., 1982). Separate di- and oligopeptide transport systems have also been reported in Salmonella typhimurium (Ames et al., 1973; Jackson et al., 1976).

The upper size limit for peptide transport may be a property of the permease per se, or a result of a diffusion barrier in the cell wall or outer membrane. In Escherichia coli the upper size limit from peptide transport was shown to be dependent upon the hydrodynamic volume of the molecule (Stokes radius) rather than the number of residues in the peptide (Payne & Gilvarg, 1968). The exclusion limit of about 650 daltons was attributed to a sieving effect of the cell wall (Payne & Gilvarg, 1968). The role of outer membrane proteins in peptide uptake by E.coli has been examined, peptide uptake kinetic parameters were determined for a series of wild type and outer membrane protein-deficient strains (Alves et al., 1985). In porin-deficient

mutants, Km values were changed more markedly than V_{max} values reflecting an alteration in diffusion through the envelope, indicating that porins are involved in facilitated peptide penetration.

The structural specificities exhibited by bacterial peptide transport systems are broad. Bacteria can take up a diverse range of peptides, have a general requirement for L-stereoisomers and an α-peptide linkage, a requirement for a positive charge at the N-terminal amino group and broad C-terminal specificity (reviewed by Payne, 1980a).

1.3.3.4 Peptide Transport in Yeast

The peptide transport systems of the yeasts Saccharomyces cerevisiae and Candida albicans have been studied in some detail, the properties of the peptide transport system(s) in Candida albicans will be discussed elsewhere (Section 1.3.6.2).

Peptide uptake has been shown to be distinct from amino acid transport through the use of amino acid auxotrophs (Becker et al., 1973; Naider et al., 1974; Marder et al., 1977). The uptake of Met-Met-[^{14}C]Met, although subject to inhibition by a range of peptides is insensitive to inhibition by free methionine (Becker & Naider, 1977). A peptide transport mutant of a leucine-lysine auxotroph of Sacc. cerevisiae isolated on the basis of resistance to L-ethionyl-L-alanine, grew normally on free leucine but not on leucine-containing peptides although the mutant possesses peptidases against these peptides (Marder et al., 1978). This mutant did not take up [^{14}C]glycyl-L-leucine under conditions in which the parent took up this dipeptide, and was therefore designated as peptide transport-deficient. The rate of peptide transport was not diminished in amino acid permease-deficient strains relative to the parent (Nisbet & Payne, 1979a), and similarly amino acid accumulation was not diminished in a peptide transport-deficient mutant (isolated as resistant to bacilysin) relative to the

parent (Nisbet & Payne, 1979b). All the available evidence, therefore, indicates that amino acid and peptide transport are mediated by separate systems.

The isolation of peptide transport-deficient mutants has shown conclusively the existence of a single peptide permease (the general peptide permease, the gpp) in Sacc. cerevisiae (Marder et al., 1978; Nisbet & Payne, 1979b). Initial studies had suggested the existence of separate di- and oligopeptide transport systems, Becker & Naider (1977) had reported that Met-Met was a poor inhibitor of Met-Met-[^{14}C] Met uptake. However, as peptide transport systems may be expected to have broad specificity, a wide range of affinities for the diverse range of substrates may be anticipated, thus the lack of competition between a di- and tripeptide is not good evidence for separate systems. However, evidence for a single transport system was provided by Marder et al., (1977), using a leucine-lycine auxotroph. Prolonged lags in the growth response to peptides supplying an essential amino acid were seen on the addition of competing peptides i.e. a prolonged lag in the growth response to Leu-Leu-Leu was seen on addition of Met-Met or Ala-Ala, and also in the response to Leu-Leu, Lys-Gly or Lys-Leu on addition of Met-Met-Met. These lags were possibly caused by competition at the transport step. The peptide transport-deficient mutant isolated as resistant to L-ethionyl-L-alanine was shown to be defective in the uptake of [^{14}C]Gly-Leu, cross resistant to L-Leu-L-Leu-L-ethionine, and to have lost the ability to utilize Leu-Leu and Leu-Leu-Leu as leucine sources (Marder et al., 1978). The peptide transport-deficient mutant isolated as resistant to bacilysin was defective in both di- and tripeptide transport activity (Nisbet & Payne, 1979b). Thus, the evidence confirms the existence of a single peptide transport system in Sacc. cerevisiae.

.The structural specificities of the peptide transport system are discussed in the following section.

1) Size limit for peptide transport   There is evidence that the size limit for peptide transport may be strain dependent. One methionine auxotroph was able to grow on pentamethionine (Naider et al., 1974).   However, a different strain was shown not to utilize pentamethionine and grew poorly on tetrapeptides (Becker & Naider, 1980). Nisbet & Payne (1979b) showed the upper size limit to occur at the tripeptide (trialanine), uptake of tetrapeptides not being detected.

The size limit for peptide transport may arise from the specificity of the permease or from a sieving effect of the cell wall. An apparent exclusion limit of 700 daltons has been demonstrated for the yeast cell wall (Scherrer et al., 1974), which would account for the exclusion of pentamethionine but not of tetramethionine. Experiments with spheroplasts could presumably resolve whether the size limit is a property of the cell wall or the permease per se.

2) Side chain specificity   Side chain specificity appears to be very broad. The first systematic study by Nisbet & Payne (1979b), showed that basic peptides were transported at a greater rate than acidic peptides, dipeptides of the sequence Ala-X were generally transported at a greater rate than those of the sequence X-Ala, and that the presence of N- or C-terminal glycine residues reduced the rate of peptide transport.

3) Stereospecificity   Nisbet & Payne (1979b) have shown that the transport of di- and tripeptides is highly stereospecific. Peptides containing D-residues were shown not to support growth,

and that these peptides were poor inhibitors of uptake of other peptides (Marder et al., 1977). However, in another strain both L-Met-L-Met-D-Met and L-Met-D-Met-L-Met were inhibitory of Met-Met-[^{14}C]Met uptake (Becker & Naider, 1977). Thus, there is some evidence that D-residues can be 'tolerated' at the C-terminus of peptides.

4) <u>N-terminal amino group</u> Auxotrophic growth tests have indicated a tolerance of N-acylated methionine-containing peptides (Naider et al., 1974), N-acetyl-Met-Met-Met was transported in one strain (Becker & Naider, 1977) but N-acetyl peptides were not utilized in another (Marder et al., 1977). Thus, substitution of the N-terminal-α-amino group can be tolerated in some strains, though the affinity and/or the rate of uptake is reduced.

5) <u>C-terminal carboxyl-group</u> Derivitization of the peptide C-terminal-α-carboxyl group is also tolerated. Esterification of the C-terminus of a number of peptides did not affect their utilization (Naider et al., 1974; Marder et al., 1977). The methyl ester of Met-Met-Met was shown to compete with the uptake of Met-Met-[^{14}C]Met (Becker & Naider, 1977). Addition of a poly-(ethylene glycol) group at the C-terminus of Met-Met-Met has been reported (Naider et al., 1980). This peptide [(Met)$_3$-OPEG] competitively inhibited the uptake of Met-Met-[^{14}C]Met, though this peptide was not transported. Derivitization of the N-terminus of this peptide abolished its ability to inhibit peptide transport, indicating that the peptide followed the structural requirements of the permease.

6) <u>The peptide bond</u> Accumulation of sarcosine-containing di- and tripeptides has been demonstrated in <u>Sacc. cerevisiae</u> (Nisbet & Payne, 1979a); thus methylation of the peptide bond nitrogen

does not abolish transport of these peptides. The resistance of these bonds to peptidase action allowed the demonstration of intact accumulation of these peptides.

Organisms grown on proline as a sole nitrogen source are able to transport peptides at about ten times the rate of ammonia-grown organisms (Becker & Naider, 1977; Nisbet & Payne, 1979a). This demonstration of ammonia regulation of peptide transport is similar to the regulation of amino acid transport (Section 1.3.2.3).

Kinetic parameters for peptide transport have been reported. Km values of the order 10^{-5}M for Leu-Leu and 10^{-4}M for Ala-Ala-Ala have been derived from Lineweaver-Burke plots of initial uptake rates measured by direct fluorescence transport assays (Nisbet & Payne, 1979a). A Km value of 10^{-5}M for Met-Met-[^{14}C]Met was obtained from uptake data obtained from measuring the accumulation of radioactively-labelled peptide (Becker & Naider, 1977). However, this value may be an underestimate because the use of radioactively-labelled substrates may lead to a severe miscalculation of kinetic parameters, as initial transport rates may be underestimated (Payne & Nisbet, 1980b).

1.3.3.5 Peptide Transport in Filamentous Fungi

Studies of peptide transport in filamentous fungi have been limited to Neurospora crassa, and have recently been reviewed (Wolfinbarger, 1980b).

Peptide transport has been shown to be distinct from amino acid transport (Wolfinbarger & Marzluf, 1974). The optimal chain length for uptake is 3 - 5 amino acid residues (Wolfinbarger & Marzluf, 1975a). The peptide Gly-Leu-Tyr has been shown to be toxic to a tyrosine-sensitive strain of Neurospora crassa. A peptide transport-defective mutant (glt-r) isolated in the basis of resistance to Gly-Leu-Tyr, was

shown to have lost over 90% of the transport capacity for Gly-Leu-[^{3}H] Tyr. This glt-r strain was crossed with a leucine auxotroph to produce a double mutant which was unable to grow on a number of peptides as a leucine source, indicating the probability of a single oligopeptide transport system in Neurospora (Wolfinbarger & Marzluf, 1975a, 1976). This single system is constitutive in nature, the growth medium having no effect on peptide transport (Wolfinbarger & Marzluf, 1975b).

1.3.4   The γ-Glutamyl Cycle - Its Possible Involvement in the

Transport of Amino Acids and Peptides

The γ-glutamyl cycle is a metabolic pathway that degrades and synthesises glutathione (γ-glutamyl-cysteinyl-glycine). A mechanism of group translocation has been proposed (Meister, 1973); one of the key enzymes of the cycle, the membrane-bound γ-glutamyltranspeptidase, mediates the translocation of one mole of amino acid per mole of glutathione degraded. There is strong evidence for such a cycle in a range of mammalian tissues (Meister & Tate, 1976). The γ-glutamyl cycle has recently been reviewed (Meister, 1980, 1983; Meister & Anderson, 1983).

γ-glutamyltransferase (γ-glutamyltranspeptidase) catalyses the conversion of glutathione and an amino acid to the γ-glutamyl-amino acid and cysteinyl-glycine, and has been found in a range of microorganisms. However, only in Sacc. cerevisiae have all the enzymes of the cycle been found (Mooz & Wrigglesworth, 1976). Mooz & Wrigglesworth (1976) reported that γ-glutamyltransferase activity was increased in the presence of Met, Ala, Glu, Pro, Cys, Gly and the peptide Gly-Gly.

Osuji (1979a,b, 1980) reported that increased glutathione turnover occurs during amino acid uptake in Candida utilis, however

other reports have indicated that methodological problems in these studies invalidate these results (Penninckx et al., 1980; Jaspers & Penninckx, 1981; Robins & Davies, 1980, 1981). Penninckx et al (1980) reported a similar activity for the soluble form of γ-glutamyltransferase in a wild type strain of Sacc. cerevisiae and a gap mutant; however, a mutant which had low activities of all amino acid permeases also had reduced transferase activity. γ-glutamyltransferase was shown to be subject to ammonia repression (Penninckx et al., 1980), therefore it seems likely that the enzyme is controlled by the same regulatory system as the amino acid permeases (Section 1.3.2.3). Payne & Payne (1984) reported that γ-glutamyltransferase activity (located principally with the membrane fraction) was similar in a wild type strain and in gap and gpp mutants (defective in the general amino acid and peptide permeases, respectively). The activity in whole cells was inactivated by covalent modification with glutamine analogues applied from outside.

Inhibition of this activity had no effect on the uptake of D- and L-amino acids, dipeptides or γ-glutamyl-amino acids. Thus, they concluded that these results precluded a major, direct role for γ-glutamyltransferase in the transport of these substrates, and speculated that the γ-glutamyl cycle could play a co-ordinating role in transmembrane regulation by monitoring extracellular nutrient sources. Studies of Robins & Davies (1981) endorsed this conclusion, they reported that the turnover of glutathione was too slow for its stoichiometric involvement in the amino acid uptake observed in the yeast Candida utilis.

These results preclude a major, direct role for the γ-glutamyl cycle in the bulk uptake of amino acids or peptides in yeast.

1.3.5 <u>Fungal Peptide Hydrolysis</u>

A diverse range of fungal proteases and peptides has been isolated from fungi, the properties of which have been recently reviewed (Wolf & Holzer, 1980). The following discussion will be limited to the hydrolysis of peptides in relation to their transport.

1)   <u>Sacc. cerevisiae</u>   The intracellular peptidases characterized to

date are two endopeptidases, proteinases A and B, two

carboxypeptidases, Y and S, and at least five aminopeptidases.

Early studies showed that many peptidases are hydrolysed by

cell extracts of <u>Sacc. cerevisiae</u> (Becker <u>et al.</u>, 1973). Marder

<u>et al.</u>, (1977) demonstrated that some non-growth substrates were

hydrolysed at only a low rate. Becker & Naider (1977)

demonstrated that acetylation of Met-Met-Met reduced its rate of

hydrolysis. Sarcosine-containing peptides, resistant to

intracellular peptidase activity were shown to be accumulated

within the cell (Nisbet & Payne, 1979a). Thus, intracellular

peptidases have been shown to possess side chain, peptide bond

and stereospecificity.

Protease A did not cleave a range of dipeptides (Hata <u>et</u>

<u>al.</u>, 1967). Protease B failed to hydrolyse a range of

benzoyloxycarbonyl dipeptides (Lenney & Dalbec, 1967), though it

did cleave some amino acid p-nitrophenyl esters (Ulane & Cabib,

1976).

Carboxypeptidase Y removes C-terminal amino acids from

peptides, though activity against free dipeptides is negligible

(Hayashi, 1976). Carboxypeptidase S was detected in mutants

lacking carboxypeptidase Y activity, and has a narrower substrate

specificity (Wolf & Weiser, 1977). Activities of both

carboxypeptidases were increased when cells were grown on N-Cbz-Gly-Leu as a sole nitrogen source (Wolf & Ehmann, 1978; Wolf et al., 1979). However, a mutant auxotroph for leucine, lacking both carboxypeptidases was unable to grow on N-Cbz-Gly-Leu as a leucine source, suggesting at least one carboxypeptidase may be involved in peptide utilization (Wolf et al., 1979).

The number and specificity of the aminopeptidases is less clear, being complicated by the different methods of identification. Matile et al., (1971), detected four aminopeptidases with activity on leucine β-naphthylamide (LBNA) after separating crude extracts from the yeast by starch gel electrophoresis. Four aminopeptidases capable of hydrolysing tripeptides were detected by Rose et al., (1979) on polyacrylamide gels, together with a specific dipeptidase. Two aminopeptidases, aminopeptidase I (Metz & Rohm, 1976) and aminopeptidase II (Frey & Rohm, 1978) and a dipeptidase (Rohm, 1974) have been purified and characterized. An additional cobalt-dependent aminopeptidase specific for basic amino acids has recently been detected (Achstetter et al., 1982). Isolation and characterization of aminopeptidase mutants of Sacc. cerevisiae has recently been described (Trumbly & Bradley, 1983). An understanding of the roles in vivo of the peptidases will require further genetic and biochemical analysis that takes into account the multitude of enzymes with overlapping substrate specificities.

Loss of peptide transport activity in a peptide transport deficient mutant was not associated with a reduction in peptidase activity, indicating that there is no interdependence between the two systems (Marder et al., 1978).

Proteases A and B, carboxypeptidase Y and aminopeptidase I are located within the vacuole while their respective inhibitors are located in the cytoplasm together with a single dipeptidase (Lenney et al., 1974; Matern et al., 1974; Frey & Rohm, 1978; Wiemken et al., 1979), though Cabib et al., (1973) were unable to detect protease A activity in a vacuolar fraction. Of the aminopeptidases, one is localized in the vacuole (Matile et al., 1971) whereas the dipeptidase and one aminopeptidase are localized in the ctyoplasm (Wolf & Holzer, 1980).

There is also evidence of extracellular peptidase activity. One of the aminopeptidase activities described by Matile et al., (1971) was lost on the production of spheroplasts indicating that it may be associated with the cell wall or external surface of the plasma membrane. Frey & Rohm (1979) demonstrated external and internal forms of aminopeptidase II; though the extacellular form of the enzyme exhibits only limited activity against peptides and greater activity against amino acid-p-nitroanilides. A highly specific extracellular peptidase involved in the cleavage of the yeast α-mating factor does not appear to affect peptide uptake (Ciejek & Thorner, 1979). Thus, it was concluded that there is no significant peptidase activity associated with peptide utilization (Parker et al., 1980).

The dual location of peptidase activity (cytoplasm and vacuole) does not suggest the probable site of hydrolysis following peptide transport. Vacuolar peptide hyrolysis would presumably necessitate peptide translocation across the vacuolar membrane.

2) <u>Candida albicans</u>   There have been relatively few reports concerning the peptidase and protease systems in <u>Candida albicans</u>.   Studies to date have demonstrated the absence of extracellular hydrolytic activity against small peptides (Lichliter <u>et</u> <u>al</u>., 1976;   Logan <u>et</u> <u>al</u>., 1979).

The ability of <u>Candida</u> <u>albicans</u> to produce an extracellular protease may be of significance in pathogenicity (Stabib, 1965); a non-proteolytic strain did not produce an extensive infection in contrast to a proteolytic strain (Stabib, 1969).   Similarly, a mutant lacking the protease also exhibited lower pathogenicity (MacDonald & Odds, 1983).   Proteinase activity was also demonstrated in the most virulent isolates of other <u>Candida</u> species (MacDonald, 1984).   Secretion of this protease enabled <u>Candida</u> <u>albicans</u> to utilize serum proteins as a sole nitrogen source (Stabib, 1965).   The protease has been purified and partially characterized (Remold <u>et</u> <u>al</u>., 1968), having a pH optimum of 4.6 - 5.5 and a molecular weight of 40,000 daltons. Further characterization (Germaine & Tellefson, 1981) showed that the protease was active below pH 5.0, and inhibited by human saliva.

Early studies demonstrated an aminopeptidase-like activity in cell homogenates (Chattaway <u>et</u> <u>al</u>., 1971;   Kim <u>et</u> <u>al</u>., 1962). A detailed characterization of the peptidases present in the yeast and filamentous forms of <u>Candida</u> <u>albicans</u> demonstrated a multiplicity of peptidases present in cell extracts, with no major differences between the morphological forms (Logan <u>et</u> <u>al</u>., 1983).   In this study two peptidases (peptidase I and II) and a dipeptidase were purified and partially characterized.

1.3.6 Transport in Candida albicans

1.3.6.1 Amino Acid Transport

There are few reports in the literature of studies on amino acid transport in Candida albicans, despite the fact that amino acids have been shown to induce germination (Odds, 1979). Among those amino acids that induce germ tube formation, those that enter metabolism via conversion to α-ketoglutarate are generally more active (Land et al., 1975) and of these proline is one of the most efficient (Dabrowa et al., 1976). Some characteristics of proline transport in normal and starved cells of Candida albicans have been described (Jayakumar et al., 1978). This activity is normally present at a constitutive level, and additional activity was shown to be induced by the presence of proline in the medium, this increase being dependent on the synthesis of RNA and protein (Jayakumar et al., 1979). However, in contrast to this report Dabrowa & Howard (1981) claimed that the proline permease was not inducible, as the initial rates of proline uptake were the same both for cells grown with glutamic acid or proline as the sole nitrogen source.

Whereas depression of amino acid transport systems is a fairly well established phenomenon in Sacc. cerevisiae (Section 1.3.2.3), it appears that amino acid transport in Candida albicans is not subject to regulation by ammonia repression (Davies, 1979; Verma et al., 1983).

Competition studies have indicated the presence of at least three amino acid transport systems : one for acidic amino acids, one for basic amino acids and a system specific for the transport of

hydrophobic amino acids (Davies, 1979); attempts to demonstrate a system analagous to the general amino acid permease in Sacc. cerevisiae were unsuccessful. Verma et al., (1983) demonstrated that the transport of acidic, basic and neutral amino acids by strains of Candida albicans, Candida krusei and Candida utilis was not subject to ammonia repression, there being no significant difference in the level of accumulation of amino acids, between proline- and ammonium-grown cells. The possibility of a derepressible gap system in Candida species was further excluded by the absence of competition for uptake between L-amino acids and their corresponding D-stereoisomers by proline-grown cells; in Sacc. cerevisiae uptake of D-amino acids has been shown to be mediated via gap (Grenson et al., 1970; Malcolm et al., 1982). The only exception was the enhancement of proline accumulation, which was known to be mediated via an inducible system (Jayakumar et al., 1979).

1.3.6.2 Peptide Transport

Peptide transport in Candida albicans has been studied in some detail with the particular objective of producing novel antifungal agents that are selectively toxic to this fungal pathogen.

The absence of extracellular peptide hydrolysis has been reported for Candida albicans (Lichliter et al., 1976; Logan et al., 1979) allowing growth studies to be related to peptide transport.

The uptake of radioactively-labelled peptides has been studied by two groups : Logan et al., (1979) followed the uptake of Met-Met-[^{14}C] Met by strain WD18-4, and Davies (1979, 1980) followed the uptake of Gly-[U^{14}C]Phe, Ala-[U^{14}C]Phe and Ala-Ala-[U^{14}C]Phe by strain 6406.

Lichliter et al., (1979) demonstrated that Met-Met did not compete with the uptake of Met-Met-[^{14}C]Met, though tripeptides were

effective. N-acetyl-Met-Met-Met also was shown to compete with Met-Met-[^{14}C]Met, confirming the results of the auxotrophic growth studies carried out by Logan et al. (1976). In contrast Davies (1980), reported that di- and tripeptides showed mutual competition implying the existence of a single peptide transport system, and she also reported N-acetylated peptides to be ineffective competitors for transport. The variation in these reports raises the possibility of strain variation in Candida albicans, with respect to the uptake of N-acetylated peptides. However, most studies in the literature based on competition experiments do not take into account the affinity values of the competing substrates, e.g. Davies (1980) measured the uptake of radioactively-labelled peptide (0.3 mM) and examined the competitive effect of other peptides (3.0 mM) without any knowledge of their respective Km's. Also, results based on measurements of the uptake of radioactively-labelled peptides have been shown to give erroneous results due to rapid intracellular metabolism and exodus of peptide-derived secondary metabolites (Section 1.3.3.2). Thus, conclusions derived from competition studies may be subject to misinterpretation.

The pH optimum for transport has been described as pH 3.5 - 4.5 (Logan et al., 1979), pH 4.5 (Davies, 1980) and pH 5 (McCarthy, 1983), with a temperature optimum of 37°C (Logan et al., 1979; Davies, 1980). Kinetic parameters for uptake were determined in these studies both by measuring the uptake of radioactively-labelled substrates, Km values of $3 \times 10^{-6} - 3 \times 10^{-5}$ M (Logan et al., 1979; Davies, 1980) and by fluorescence assays Km values of $1 \times 10^{-4} - 1 \times 10^{-3}$ M (McCarthy, 1983). Certain structural specificities for peptide transport have also been examined and are reviewed below.

1)   N-terminal amino group   Logan et al., (1979) reported that N-
        acetylated tripeptides competed with the uptake of

radioactively-labelled tripeptides whereas Davies (1980) reported that N-acetylated peptides were ineffective competitors. Lichliter et al., (1976) reported that N-acetylation did not prevent utilization of peptides. Similarly, McCarthy (1983) reported that acetylation of the amino terminal of Ala-Ala did not remove its ability to inhibit the uptake of Ala-Ala.

2) C-terminal carboxyl group   Methyl esters of methionyl peptides were shown not to to support growth of a methionine auxotroph (Lichliter et al., 1976). Logan et al., (1979) demonstrated that esterification of Met-Met-[^{14}C]Met prevented transport. Similarly, McCarthy (1983) reported that amidation of the C-terminus eliminated uptake. The carboxyl moiety may be replaced by phosphonate, sulphonate or tetrazole group indicating the requirement for a free acidic function, all of these derivatives competed for uptake with radioactively-labelled peptides (Davies, 1980). The intact intracellular accumulation of alafosfalin (Section 1.4.2.2) has been demonstrated in Candida albicans (Davies, 1980) which contrasts with results from E.coli in which this peptide is hydrolysed intracellularly to release the toxic warhead (Atherton et al., 1979). This result indicated that alafosfalin was not cleaved by the intracellular peptidases, though substitution of the C-terminus carboxyl group did not prevent transport. Esterification of the carboxyl group did not prevent transport in Sacc. cerevisiae (Naider et al., 1974). This species difference may possibly be exploited in the design of selective toxic agents.

3) Stereospecificity   Logan et al., (1979) demonstrated that L-Met-L-Met-D-Met and D-Met-D-Met-D-Met did not compete for transport of Met-Met-[^{14}C]Met but D-Met-L-Met-L-Met was a competitor,

confirming the earlier auxotroph growth response studies (Lichliter et al., 1976). However, Davies (1980) reported that only L-Ala-L-Ala competed for uptake with radioactively-labelled Ala-Ala, other stereoisomers being ineffective. This suggested that stereochemical selection takes place at the level of binding rather than transport, suggesting the presence of a stereospecific binding site associated with the transport system McCarthy (1983) reported that of all the stereoisomers examined only L-Met-D-Met was transported at a very low rate.

Synthetic tripeptides containing D-amino acids in the central position have been reported to be toxic to Candida albicans (see Section 1.4.2.5), implying that the permease may tolerate D-residues in the central position of a tripeptide. However, synthetic D-m-fluorophenylalanyl-L-alanine, D-m-fluorophenylalanyl-L-alanine-L-alanine and D-m-fluorophenylalanyl-L-methionyl-L-methionine were inactive against Candida albicans whereas the corresponding L-m-fluorophenylalanyl-substituted peptides were toxic (Kingsbury et al., 1983). Thus, there appears to be a degree of strain variation for utilization of D-amino acid-substituted peptides.

4)   The peptide bond   There is a general requirement for an α-linkage, γ-Glu-Ala, γ-Glu-Gly-Gly and β-Ala-Ala were poor inhibitors of peptide uptake whereas their corresponding α-linked peptides were much more effective (Davies, 1980).

5)   Size limit for uptake   Pentamethionine was shown to support growth of a methionine auxotroph (Lichliter et al., 1976). However, Davies (1979) reported that Candida albicans was unable to take up radioactively-labelled tetra-alanine although the

unlabelled tetrapeptide was able to compete with the uptake of radioactively-labelled Gly-Phe, Ala-Ala and Ala-Ala-Ala. This suggested that peptides may compete at the level of binding rather than of translocation. McCarthy (1983) demonstrated the uptake of tetra-alanine using direct fluorescent assays. Thus, there may be strain variation of the size limit for uptake. During the course of this work the isolation of a dipeptide transport-deficient mutant isolated as resistant to nikkomycin was reported (McCarthy, 1983; McCarthy et al., 1985a). This mutant retained oligopeptide transport, demonstrating the existence of at least two peptide transport systems. Further evidence for the existence of distinct transport systems has been reported (Yadan et al., 1984; McCarthy et al., 1985b). Further aspects of these studies are discussed in detail in Chapters 4 and 5.

Peptide transport in Candida albicans has been demonstrated to be subject to ammonia repression (Logan et al., 1979). This is analagous to both amino acid (Section 1.3.2.3) and peptide transport (Section 1.3.3.4) regulation in the yeast Sacc. cerevisiae.

A synthetic photoaffinity label, 4-azidobenzoyltrimethionine was shown to inhibit competitively trimethionine uptake in Candida albicans ATCC 26278 (Sarthou et al., 1983). Upon UV irradiation, it irreversibly and specifically blocked oligopeptide uptake. Photoinactivation of peptide transport has also been reported both for Sacc. cerevisiae (Becker et al., 1982) and E.coli (Staros & Knowles, 1978).

1.4 Peptide Drugs

1.4.1 Introduction: The Concept of Illicit Transport

Antibiotics are very important and often chemically complex compounds made principally by microbiological synthesis. Since the discovery of the first antibiotics, more than 6000 natural microbial compounds have been described, which display antibiotic activity. Only about 150 are produced on a large scale, all but two being produced by conventional microbiological fermentation processes. In a few cases post-synthetic modification (achieved by chemical or enzymatically catalysed conversion) is carried out to produce a so-called semisynthetic antibiotic with superior therapeutic properties.

The potential of small peptide mimetics as useful drug molecules lies largely in their ability to act in one of three ways :-

1)   as a carrier or targeting function

2)   as a peptidase inhibitor

3)   as an antagonist/agonist of a natural peptide hormone or
        transmitter.

Examples of small peptides as carriers and targets have been recently reviewed (Ringrose, 1980, 1983, 1985).

This review is principally concerned with the ability of small peptides to act as carriers. The concepts of 'illicit transport' (Ames et al., 1973), 'smugglin' of impermeant molecules (Matthews & Payne, 1975b) and 'warhead delivery systems' (Ringrose, 1980) are of particular relevance to this section and will be discussed in detail. The linear peptides described in the following sections fall into two distinct categories :-

1)   Di- and tripeptides acting purely as disguised delivery systems
        for amino acid mimetics, the active amino acid moiety being
        released by post-transport, intracellular peptidase activity.

2)   Peptides acting as the intact molecule, acting either
      intracellulary or at membrane level. Consequently, enzymic
      hydrolysis will result in inactivation of the peptide.

Examples of each will be discussed in the following sections.

Illicit transport is a mechanism by which normally impermeant moieties are attached to normal nutrient molecules and absorbed via nutrient transport systems. The potential of the mechanism for antibiotic uptake was first suggested by Payne (1972a) and given experimental validation by Ames et al., (1973) for histidinol phosphate, and Fickel & Gilvarg (1973) with homoserine phosphate. Since then other naturally-occurring antimicrobial peptides, known for some time, have been shown to use this mechanism.

Peptide transport systems have a broad specificity, particularly with respect to side-chains and C-terminus (Sections 1.3.3.3 and 1.3.3.4) whereas amino acid transport systems have far more exacting structural requirements for uptake. Thus, it is possible for an amino acid mimetic, which is not transported by the specific amino acid permease, to be taken into the bacterial cell disguised as a peptide, i.e. illicit transport. For antibiotic activity the 'warhead' must be rapidly released from its carrier peptide by intracellular peptidase action, so it can act at its target site. The carrier-warhead complex itself is inactive at the warhead's target site. In order that a peptide may act as an efficient carrier, it is necessary for its stereochemistry, sequence, chain length etc. to conform to the structural specificities of the peptide permeases and petidases of the target organism. The optimal warhead is usually one which is poorly transported into the cell by itself, thus once it has been released intracellularly, it is unlikely to take part in carrier-mediated

efflux, consequently very high intracellular concentrations of the warhead can be achieved.

Antimicrobial peptides of this type may be regarded as being composed of a carrier moiety (one or more amino acids at the C- and/or the N-terminus), and a warhead moiety, linked by a peptidase labile bond. Similar considerations apply to the transport of linear peptides that are active as intact molecules, except that these peptides must be resistant to intracellular peptidase action.

The concept of illicit transport has applications in the rational design of chemotherapeutic agents. Knowledge of the molecular basis for permeation of different metabolites is essential for the rational design of synthetic antibiotics targeted for a specific intracellular site. Characterization of the molecular requirements for metabolite transport for a given cell, may indicate certain metabolites to become prime candidates as carriers to facilitate the translocation of normally-impermeant, toxic moieties into the cell.

For peptide carriers to become effective drugs several other factors should be considered, especially for the treatment of systemic bacterial or fungal infections. The carrier conjugate must ideally not be taken up by the host-tissue, or at least the uptake by the target organism must be many-fold greater than that in the host. Also the carrier conjugate must have a sufficient half-life in the host tissue so that the concentration can be maintained at levels that are toxic to the target organism. Thus, appreciation of the molecular requirements of the host's peptide transport systems and peptidases is important for the design of an efficient peptide-antibiotic. Drugs administered orally would have to traverse the intestinal mucosa epithelial cells to be released into the venous and lymphatic systems. Thus, peptide

carrier drugs would have to be potentially resistant to several different host peptidases before arriving at the target organism. Peptide transport in the animal small intestine has been reviewed (Matthews & Payne, 1980; Ganapathy & Leibach, 1982) and evidence for the passage of intact peptides across the intestinal mucosa recently reviewed (Gardner, 1983).

A series of specific examples of both linear peptides acting as amino acid mimetic delivery systems and linear peptides active as intact molecules is presented in the following sections.

1.4.2 Linear Peptides that Act as Amino Acid Mimetic Delivery Systems

1.4.2.1 Bacilysin

Bacilysin (Fig. 1.1A) is a L,L-dipeptide antibiotic produced by Bacillus subtilis (Walker & Abraham, 1970a,b). Early confusion with what appeared to be a related dipeptide, tetaine was resolved when bacilysin and tetaine were shown to be identical (Kaminski & Sokolowska, 1973). Bacilysin has a broad antibiotic spectrum being active against both Gram-positive and Gram-negative bacteria as well as certain yeasts, including Candida albicans (Kenig & Abraham, 1976 Milewski et al., 1983).

The C-terminal epoxyamino acid, anticapsin, acts as a potent inhibitor of glucosamine synthetase (2-amino-2-deoxy-D-glucose-6-phoshate ketol isomerase, amino-transferring, E.C.5.3.1.19, formerly L-glutamine: D-fructose-6-phosphate $\overset{keto}{amino}$ transferase, E.C.2.6.1.16), (Kenig et al., 1976; Chmara et al., 1980; Chmara & Zahner, 1983). Anticapsin is also produced independently by Streptomyces griseoplanus (Neuss et al., 1970). Anticapsin was inactive against staphylococci, activity being observed only when the epoxyamino acid was coupled in peptide form (Kenig & Abraham, 1976). Anticapsin is a potent inhibitor
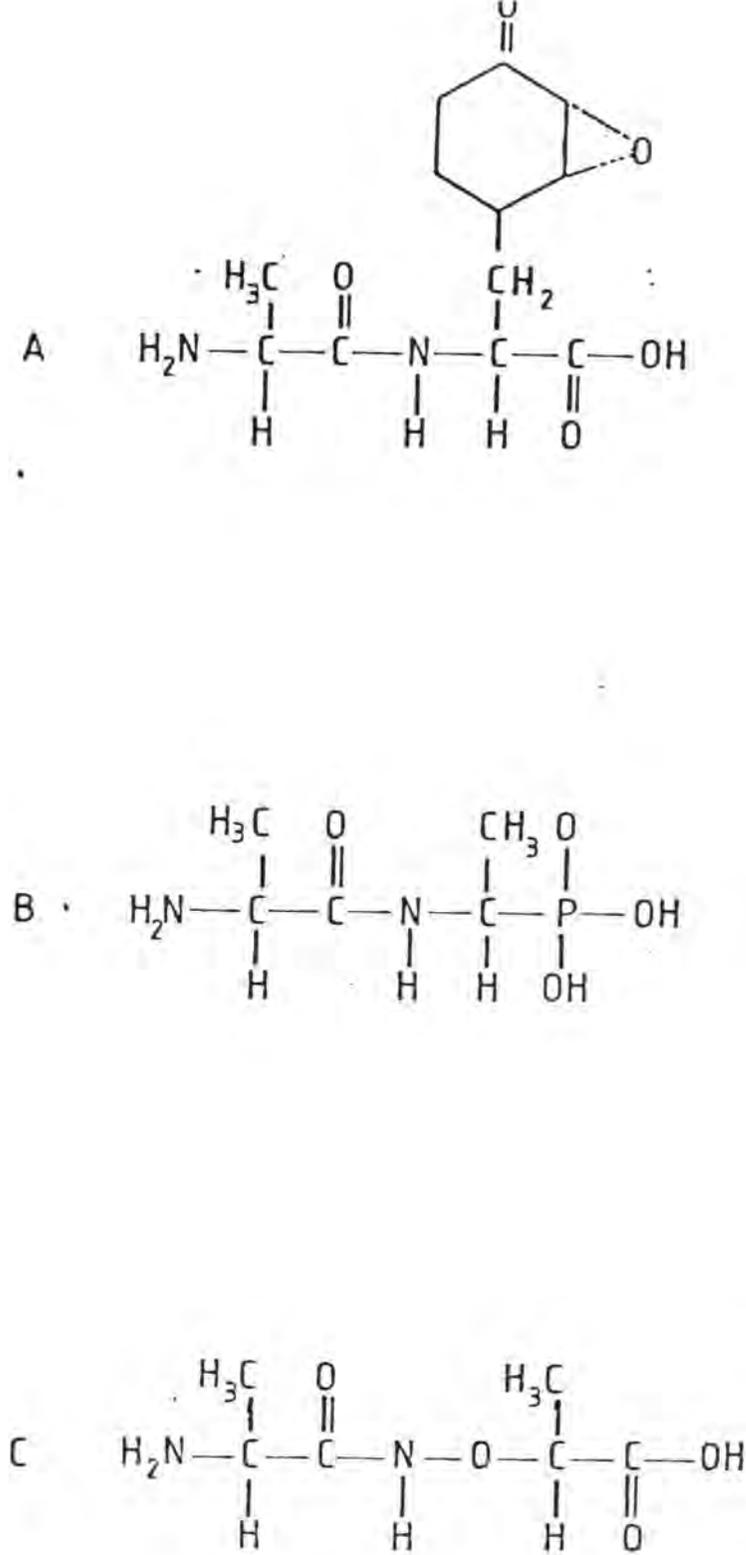
Figure 1.1 Structures of Peptide Antibiotics

Structures of A, Bacilysin; B, Alafosfalin and C, L-alanyl L-α-aminoxypropionic acid.

of glucosamine synthetase activity in cell-free extracts of Gram-positive and Gram-negative bacteria, and yeast including Candida albicans (Kenig et al., 1976; Chmara et al., 1980, 1982), bacilysin per se being inactive as an inhibitor of this enzyme.

The activity of bacilysin is antagonised by various dipeptides and by complex media, and by the presence of glucosamine and N-acetyl glucosamine (Kenig & Abraham, 1976). Cell extracts from bacilysin-resistant strains of Staphylococcus aureus were shown to cleave bacilysin with the same activity as the parental wild-type strain, thus resistance was attributed to a transport defect (Kenig & Abraham, 1976). In bacteria, bacilysin was shown to be transported by a dipeptide permease (Perry & Abraham, 1979; Diddens et al., 1979; Chmara et al., 1981), and in Sacc. cerevisiae, bacilysin-resistant mutants were shown to be defective in peptide transport (Nisbet & Payne, 1979b).

Thus, the mode of action of bacilysin against microbial cells involves transport into the cells via a peptide transport system, rapid intracellular hydrolysis by peptidases to release alanine and the anticapsin warhead. The anticapsin liberated then inhibits glucosamine synthesis resulting in cessation of synthesis of amino-sugar-containing macromolecules; in bacteria the inhibition of peptidoglycan synthesis (Chmara et al., 1981; 1973a,b), and in Candida albicans, the inhibition of mannoprotein and chitin synthesis (Chmara et al., 1980; 1982).

Bacilysin is therefore, a classic example of a natural, microbiologically-produced, broad spectrum antibiotic that utilises the 'illicit transport' and 'warhead delivery system' concepts in its mode of action.

1.4.2.2 Phosphonopeptides

Phosphonopeptides are peptides with a C-terminal phosphonic acid group instead of a carboxylic acid group. The most intensively studied of the group is L-alanyl-L-aminoethylphosphonic acid (alafosfalin, or Ala-Ala (P)), (Fig. 1.1B). This peptide is taken up by many species of bacteria and cleaved intracellularly to release L-aminoethylphosphonic acid (Ala (P)) which is an inhibitor of alanine racemase (Allen et al., 1978; Atherton et al., 1979b), thereby preventing the formation of D-alanine, which is required for bacterial cell wall biosynthesis. The free Ala (P) moiety is essentially impermeant and is inactive if supplied extracellularly, but when coupled in peptide form it can be accumulated intracellularly to concentrations 100 - 1000 fold that of the peptide precursor in the medium (Atherton et al., 1979b).

Phosphonopeptide-resistant mutants have been isolated from E.coli (Alves, 1983) and Streptococcus faecalis (Nisbet & Payne, 1980; 1982; Payne et al., 1982). Resistant mutants were either defective in peptide transport or in intracellular peptidase activity.

Many other phosphonopeptides have been shown to have antibacterial action (Atherton et al., 1979a; Allen et al., 1979a) and other phosphono-related peptides described (Allen et al., 1979a; Atherton et al., 1980). The metabolism and pharmacokinetics of alafosfalin in humans and animals have been described (Allen et al., 1979b).

A species-dependent synergism of alafosfalin and β-lactam antibiotics has been reported (Maruyama et al., 1979). Other aspects of phosphonopeptide biochemistry have been reviewed (Allen et al., 1978; Ringrose, 1980).

1.4.2.3 Aminoxy- and other Backbone Modified Peptides

Aminoxy-peptides (Fig. 1.1C) contain an oxygen atom between the amino group and the carbon skeleton of the C-terminal amino acid, i.e. the normal peptide linkage (-CO-NH-) is replaced by (-CO-NHO-). The antibacterial activity of backbone-modified analogues of small peptides and preliminary evidence on their uptake has been presented (Morley et al., 1983a,b).

Isolation of analogue-resistant mutants and cross-resistance tests with peptide-transport deficient mutants indicate that all three peptide permeases in E.coli can transport these backbone-modified analogues (Payne et al., 1984). A number of peptidase-deficient mutants should reduce sensitivity to a range of these analogues, thus cleavage is essential for their activity. Following uptake and hydrolysis, the aminoxy amino acid analogue rapidly inhibits mRNA and protein synthesis. In crude extracts the liberated analogue inhibits pyridoxal phosphate-dependent transaminase, the peptide analogues per se were not found to be inhibitory (Payne et al., 1984).

1.4.2.4 m-Fluorophenylalanine-Peptide Conjugates

Anticandidal activity of synthetic carrier di-, and tripeptides containing m-fluorophenylalanine has been reported (Kingsbury et al., 1983). The intracellular site of action of L-m-fluorophenylalanine is unclear; several possibilities exist, including intracellular conversion of m-fluorophenylalanine to fluorocitrate, which is a potent inhibitor of aconitase and/or the synthesis of defective proteins

containing L-m-fluorophenylalanine. Incorporation of L-m-fluorophenylalanine (which is inactive by itself) into a peptide results in activity against Candida albicans in vitro. The above authors also showed that peptides containing D-m-fluorphenylalanine had greatly reduced activity, which was attributed to either a lack of transport of D-m-fluorophenylalanyl-peptides, resistance to intracellular peptidases or to the possible inactivity of the free intracellular D-amino acid.

The activity of the dipeptide L-m-fluorophenylalanylalanine (L-m-FPhe-Ala) was antagonized by both a dipeptide (Ala-Ala) and a tripeptide (Ala-Ala-Ala), whereas the activity of the tripeptide L-m-fluorophenylalanylalanylalanine (L-m-FPhe-Ala-Ala) was not affected by the dipeptides Ala-Ala or Gly-Ala but was antagonized by the tripeptide Ala-Ala-Ala. These results were interpreted as evidence for either a different mode of entry for di-, and tripeptides or for them having different binding affinities for a common transport system. The activity of L-m-FPhe-Ala-Ala was antagonized by Gly-Phe, though this probably arose from competition between intracellularly-released Phe and L-m-FPhe for a common enzyme site, rather than competition for uptake via a common transport system. These conclusions were supported by the fact that phenylalanine was the only free amino acid of those tested that was found to antagonize the action of L-m-FPhe-Ala.

Thus, it appears that L-m-fluorophenylalanyl-substituted peptides are transported into Candida albicans via a peptide transport systems(s), and hydrolysed intracellularly to liberate the toxic L-m-fluorophenylalanine.

1.4.2.5 L-arginyl-X-L-phenylalanine Carrier Peptides

Antifungal activity has been reported for the natural peptide L-arginyl-D-allo-threonyl-L-phenylalanine has been reported (Kenig et al., 1973), whereas synthetic L-arginyl-L-threonyl-L-phenylalanine was shown to have no activity. Synthetic L-arginyl-N- α -methyl-L-phenylalanyl-L-phenylalanine (Eisele, 1975) and L-arginyl-D-phenylalanyl-L-phenylalanine (Meyer-Glauner, 1976), showed similar activity to the D-allo-threonyl peptide.

Antifungal activity of the carrier sequence L-arg-X-L-phe containing amino acid antagonists or typical non-biogenic D-amino acids in the central position has also been reported (Meyer-Glauner et al., 1982). L-arginyl-p-fluoro-DL-phenylalanyl-L-phenylalanine was reported to be active against Candida albicans, whereas the fluoro-amino acid analogue showed no activity per se. Thus, it was concluded that the amino acid sequence may serve as a carrier function, enhancing the delivery of toxic amino acid derivatives.

1.4.2.6 Peptide Pyrimidine Conjugates

5-fluorocytosine (5-FC) has been shown to be active against Candida albicans (Shepherd et al., 1985), acting primarily through inhibition of both DNA and RNA synthesis. The possibility of coupling 5-FC into peptide-pyrimidine conjugates was proposed by Becker et al., (1977) with a view to using the peptide transport system for drug delivery. Steinfeld et al., (1979), reported that several 5-FC-peptide conjugates inhibited the growth of several strains of yeast, though these compounds were unstable in solution. Ti et al., (1980) reported the coupling of 5-fluoroorotic acid into peptide-pyrimidine conjugates, which had longer half-lives in solution (though these were highly pH dependent). These conjugates were shown to competitively

inhibit the uptake of radioactively-labelled trimethionine. The relative activity of 5-FC and 5-FC-peptide conjugates was dependent on both the strain and pH of the assay medium. The half life for N^4-(succinyl-ala-leu)-5- FC was 1.7 h at pH 7.2, and 49 h at pH 3. At pH 7.2 the activity of the peptide-conjugate against Sacc. cerevisiae was equivalent to that of free 5-FC, which was attributed to hydrolysis of the conjugate and release of 5-FC into the medium, whereas at pH 5 the activity of the conjugate was less than that of 5-FC, reflecting the low rate of transport of N-acylated peptides. However when tested against Candida albicans, the conjugate and 5-FC showed equivalent toxicities at both pH values, implying that transport of the intact drug occurred at pH 5, followed by the release of 5-FC by intracellular hydrolysis. These results were the first example of peptide-conjugates which can carry pyrimidine analogues into a eukaryotic cell.

In similar studies, peptides conjugated with 5-fluorouracil (5-FU) were shown to have antimicrobial activity equivalent to that of free 5-FU (Kingsbury et al., 1984b), see Section 1.4.2.7.

1.4.2.7 α-Glycine Substituted Peptides

Sythetic peptides containing glycine residues α-substituted with thiophenol, aniline or phenol were shown to be transported by bacteria (Kingsbury et al., 1984a), and hydrolysed by intracellular peptidases. After liberation of its amino group the α-substituted glycine residue is chemically unstable (though it is stable in peptide form) and decomposes, releasing the nucleophilic moiety.

Similarly, synthetic peptides containing α-(5-FU)-glycine were transported by the peptide transport systems of Candida albicans and E.coli, cleaved by intracellular peptidases, and the liberated α-(5-FU)-glycine decomposes to release free 5-FU. (Kingsbury et al., 1984b). The peptide conjugates were shown to have equivalent

antimicrobial activity to 5-FU. Non-inhibitory peptides were shown to antagonize the antimicrobial activities of the peptide conjugates but not of 5-FU, which is consistent with peptide permease mediated entry of the peptide conjugates into the cell.

1.4.3 Peptides that are Active as Intact Molecules

Some toxic linear peptides have been shown to act as the intact molecule, examples of which are reviewed by Ringrose (1980). The growth inhibitory effects of glycyl-leucine (Simmonds, 1970) and triornithine (Payne, 1968) against E.coli have been attributed to the activity of the intact peptide inside the cell.

The properties of the fungal chitin synthetase inhibitors, the polyoxins and nikkomycins, which are active as the intact peptide, are reviewed in detail in the following sections.

1.4.4 Chitin Synthetase - A Target for Peptide Drugs

1.4.4.1 Chitin Synthetase

Chitin, a β-(1-4) polymer of N-acetylglucosamine, is a characteristic component of the cell walls of most filamentous fungi. The enzyme that catalyses the synthesis of chitin is uridine diphosphate-N-acetylglucosamine:chitin-N-acetylglucosaminyl transferase (EC 2.4.1.16), chitin synthetase. This enzyme is found in fungi (and arthropods) but not mammals, and so a specific inhibitor might be expected to prevent fungal growth but lack host toxicity. The polyoxins and nikkomycins which are specific inhibitors of chitin synthetase are discussed in the following sections; this section is restricted to a discussion of the enzyme per se.

The properties of chitin synthetase have been well characterized for particulate preparations from a range of fungi including Sacc. cerevisiae, Candida albicans, Coprinus cinereus, Neurospora crassa, Mucor rouxii etc. The properties of chitin synthetase and its role in

fungal morphogenesis have been reviewed (Gooday 1977, 1979; Farkas, 1979; Gooday & Trinci, 1980; Adams & Gooday, 1983), and will not be discussed in detail here. Chitin synthetase catalyses the incorporation of N-acetylglucosamine (NAG) units from uridine 5'-diphosphate-N-acetylglucosamine (UDP-NAG) into the polymer chitin, the general equation for this reaction being :-

$$\text{UDP-NAG} + [\beta\text{-}(1,4)\text{-NAG}]_n \xrightarrow{\text{Mg}^{2+}} [\beta\text{-}(1,4)\text{-NAG}]_{n+1} + \text{UDP}$$
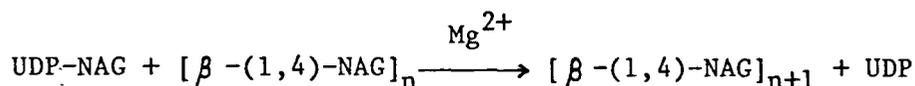
Chitin synthetase is an allosteric enzyme, with Hill number close to 4 at low substrate concentrations (0.1 mM), and about 2 at higher concentrations. There is no evidence for the participation of a 'lipid intermediate' in the reaction (Endo & Misato, 1969; Ohta et al., 1970; McMurrough et al., 1971), though a chitin synthetase preparation from Coprinus cinereus was shown to require phospholipids for activity (Montgomery & Gooday, 1985). Similarly, evidence for a phospholipid requirement for a chitin synthetase preparation from Schizophyllum commune has been reported, delipidification resulting in a decrease in activity (Vermeulen & Wessels, 1983). Chitin synthetase is strongly inhibited by the reaction product, UDP, whereas the monomer and dimer of chitin, NAG and diacetylchitobiose, both activate the enzyme at very low substrate concentrations.

All evidence from fungal systems indicates that the cell membrane is the site of chitin synthesis. Duran et al., (1975) demonstrated that cell membrane ghosts from Sacc. cerevisiae contained nearly all of the chitin synthetase activity recoverable from the cells. Evidence obtained both in vivo and in vitro, indicates that chitin synthetase receives NAG residues from UDP-NAG at the cytoplasmic face of the membrane, and transfers them vectorially to a growing chain of chitin that is concomitantly extruded to the outside (Cabib et al., 1983).

This process does not require a source of energy other than the free energy of hydrolysis of UDP-NAG.

Chitin synthetase is found mainly in a zymogen form which requires proteolytic activation for maximal activity. Activation can be achieved _in vitro_ by the addition of proteases e.g. trypsin, _in vivo_ activation presumably occurs via an endogenous protease. However, it has been recently proposed that in _Candida albicans_ significant differences between activation _in vivo_ and _in vitro_ may occur (Gozalbo et al., 1985). Chitin synthetase zymogen has also been shown to be localized in chitosomes (Ruiz-Herrera et al., 1975). Chitosomes are intracellular microvesicles which probably serve as conveyors of the zymogen from its point of synthesis to its final destination at the plasma membrane (Bartnicki-Garcia et al., 1978). Thus the enzyme occurs in at least three states in the fungal cell :-

1)   as a zymogen in chitosomes.

2)   as a zymogen in the plasma membrane.

3)   as an active enzyme in the plasma membrane.

The relative proportions in these three states will vary with the particular fungus and state of growth (Gooday & Trinci, 1980).

The lytic enzyme, chitinase also plays an important role in the control of fungal cell wall biosynthesis (Gooday & Trinci, 1980). A periplasmic chitinase activity from _Sacc. cerevisiae_ (Elango et al., 1982) and a wall-bound chitinase activity from _Aspergillus nidulans_ (Polachek & Rosenberger, 1978) have been described. A membrane-bound and soluble form of chitinase from _Mucor mucedo_ have been described (Humphreys & Gooday, 1984). The membrane-bound chitinase exists as a zymogen which is activated by treatment with proteases such as trypsin.

On activation the enzyme can efficiently degrade nascent chitin as fast as it is synthesised. Similarly, the chitin synthetase inhibitors from Mucor rouxii (Lopez-Romero et al., 1982) and Neurospora crassa (Zarain-Herzberg & Arroyo-Begovich, 1983) have been shown to be chitinases. The coexistence of chitin synthetase and chitinase may represent a lytic/synthetic complex, with the role of inserting new chitin into pre-existing walls as it is required for branch and septum formation and for hyphal apical extension (Humphreys & Gooday, 1984).

Chitin synthetase from the stɯipes of Coprinus cinereus has been purified and characterized (Montgomery et al., 1984). A nearly pure enzyme preparation had an activity of 4.7 umol substrate incorporated min^{-1} (mg protein)$^{-1}$, and an apparent molecular weight of 6.7 x 10^4. Extensive kinetic analysis of the chitin synthetase from Coprinus cinereus has been undertaken (de Rousset-Hall & Gooday, 1975; Gooday, 1977, 1979). The values of the kinetic parameters for chitin synthetase preparations from a range of fungi are remarkably similar (Gooday, 1977, 1979), Km values being of the order 0.6 - 4.0 mM, and Ki values for inhibition by polyoxin of the order 0.6 - 3.0 uM.

Chitin synthetase is also present in arthropods, being responsible for cuticle formation, in which chitin is a major component. Targeting of chitin synthetase inhibitors against insects is hoped to lead to the development of a new class of insecticides (Marx, 1977).

Chitin synthesis in Candida albicans has been studied extensively, principally with regard to the control of dimorphism. The amounts of chitin in the cell wall of mycelial cells are three times higher than those found in yeast cells (Chattaway et al., 1968; Chiew et al., 1980). In addition, the incorporation of N-acetylglucosamine

into chitin is higher in hyphal cells than in blastospores (Braun &
Calderone, 1978) and there is a five-fold activation of chitin
synthetase during germination (Chiew et al., 1980). Chitin synthetase
preparations from Candida albicans have been described (Hardy & Gooday,
1978; Braun & Calderone, 1979; Chiew et al., 1980; Hardy & Gooday
1983; Gozalbo et al., 1985). A method for the assay in situ of chitin
synthetase (and other membrane-bound and cytoplasmic enzymes) has
recently been described (Ram et al., 1983). Chitinase activity has
been detected in the yeast form of Candida albicans (Barrett-Bee &
Hamilton, 1984).

The properties of the naturally-occurring chitin synthetase
inhibitors, the polyoxins and nikkomycins will be discussed in the
following sections.

1.4.4.2   The Polyoxins

Polyoxins A – M are a class of peptide-nucleoside antibiotics
produced by Streptomyces cacaoi var. asoensis, the structures of
polyoxins A, B and D are shown in Fig. 1.2. The fermentation and
isolation of polyoxin complexes from culture broths, and the chemical
characterization of the components have been reviewed (Isono et al.,
1969; Isono & Suzuki, 1979) In this study the isolation of polyoxins
from a crude batch of Polyoxin Z fungicide by a series of adsorption
steps followed by cellulose column chromatography is described (Section
3.3). Recently, purification of polyoxin D by reversed phase high-
performance liquid chromatography has been described (Shenbagamurthi et
al., 1982). The chemical synthesis, modification and biosynthesis of
polyoxins has been reviewed (Isono & Suzuki, 1979).

Polyoxins are structural analogues of uridine diphosphate N-
acetylglucosamine, the substrate for chitin synthetase (chitin : UDP-N-
acetyl-glucosaminyl transferase E.C. 2.4.1.16), (Endo et al., 1970).
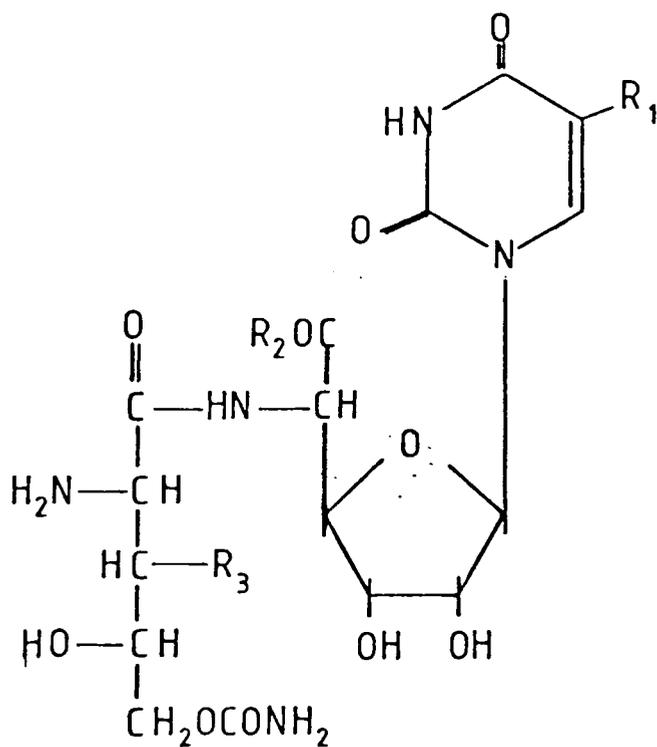
Polyoxin	R_1	R_2	R_3
A	CH_2OH	(azetidine with COOH, methylene)	OH
B	CH_2OH	OH	OH
D	COOH	OH	OH

Figure 1.2   Structures of Polyoxins A, B and D

Chitin is a cell wall polymer and is thus important in the maintenance of fungal cell wall rigidity (Section 1.4.4.1); thus, the inhibition of chitin synthesis by polyoxins has a profound effect on fungal morphology.

Polyoxins have been shown to be active against a wide range of chitin-containing fungi, even Sacc. cerevisiae in which chitin constitutes less than 1% of the cell wall (Gooday, 1977). Polyoxins have been used extensively as agricultural fungicides in Japan, particularly against black spot disease of pears caused by Alternaria kikuchiana Tanaka and rice blast disease caused by Peccicularia filamentosa f. sasakii (Isono & Suzuki, 1979). Morphological effects of polyoxin treatment are easily observed, growing hyphal tips (the site of chitin synthetase action) of many fungi swell and burst when treated with 20 uM polyoxin (Gooday, 1977). Polyoxin inhibits the formation of the chitin-containing ring in the bud scar of Sacc. cerevisiae and thus inhibits budding (Bowers et al., 1974), and induces characteristic bulbous structures on hyphae and germ tubes of Alternaria kikuchiana (Ishizaki et al., 1976). Polyoxins also affect the growth, differentiation and morphology of Coprinus cinereus (Gooday, 1972, 1975) and Achyla radiosa (Dietrich & Campos, 1978).

Polyoxins have been shown to inhibit fungal growth through an effect on chitin synthetase resulting in the formation of defective cell walls (Isono & Suzuki, 1979). In initial studies, polyoxin D was shown to be a competitive inhibitor of chitin synthetase in Neurospora crassa (Endo & Misato, 1969; Endo et al., 1970). Polyoxin D was also shown to inhibit the incorporation of [^{14}C]-glucosamine into cell wall chitin with an associated build up of UDP-N-acetylglucosamine, kinetic parameters were Km 1.4 x 10^{-3}M, Ki for polyoxin D was 1.4 x 10^{-6}M. In

related studies, polyoxin D was shown competitively to inhibit a chitin

synthetase preparation from Piricularia oryzae, and to inhibit the

incorporation of [^{14}C]-glucosamine into the cell wall of Cochliobolus

miyabeanus with an associated build up of UDP-N-acetylglucosamine (Ohta

et al., 1970), whereas polyoxin D did not inhibit the incorporation of

[^{14}C]-labelled glucose, amino acid or acetate into the cell wall.

Thus, it was concluded that polyoxins specifically inhibited the

biosynthesis of fungal cell wall chitin.

Simple peptides (particularly dipeptides) have been shown to

antagonize the action of polyoxin against Pellicularia sasakii and

Alternaria kikuchiana in vivo (Mitani & Inoue, 1968; Hori et al.,

1977), though they have no effect on the activity of chitin synthetase

assayed in vitro (Hori et al., 1974b). Thus, on the assumption that

the active site of chitin synthetase was located on the cytoplasmic

face of the plasma membrane, a situation found in the yeast Sacc.

carlsbergensis (Keller & Cabib, 1971), it was concluded that peptides

may compete with polyoxin for transport through a common permease (Hori

et al., 1974b; Gooday, 1979). This situation could explain some

earlier reports that polyoxins were inactive in media rich in peptides.

Mutant strains of Alternaria kikuchiana Tanaka which showed

varying degrees of resistance to polyoxins have been isolated from

polyoxin-treated orchards (Tanaka & Takanashi, 1975; Nishimura et al.,

1976). The inhibition of [^{14}C]-glucosamine incorporation and

associated build up of UDP-N-acetylglucosamine is reduced in polyoxin-

resistant strains (Hori et al., 1974a), and the chitin synthetase

preparations from both sensitive and resistant strains were sensitive

to inhibition by polyoxin B. Hori et al., (1974a), concluded that

polyoxin resistance was caused by a lower intracellular accumulation of

polyoxin due to reduced transport, and not due to the altered affinity of chitin synthetase for its substrate or for polyoxin. In more detailed studies, Hori et al., (1976) showed that polyoxin-resistance in Alternaria kikuchiana Tanaka was associated with reduced uptake of [^{14}C]-labelled polyoxins A and B, with a concomitant reduction in the inhibition of mycelial growth and [^{14}C]-glucosamine incorporation. Sensitive and resistant strains inactivated polyoxins A and B to a similar extent, inactivation being due to degradation. In related studies, polyoxin-resistance was shown to be associated with a reduction in [^{14}C]-glycylglycine transport, though uptake of radioactively-labelled amino acid, nucleosides and glucosamine were the same in both wild type and polyoxin-resistant strains (Hori et al., 1977). Dipeptides were shown to inhibit competitively the uptake of [^{3}H]-polyoxin A with a concomitant increase in the incorporation of [^{14}C]-glucosamine into cell wall chitin, whereas amino acids and nucleosides had a negligible effect on [^{3}H]-polyoxin A uptake. Thus, polyoxin-resistance was clearly associated with a loss of peptide transport activity demonstrating a common mode of entry via the peptide transport system(s).

Structure-activity relationships of polyoxins and associated analogues have been reviewed (Isono & Suzuki, 1979). Studies on the mode of action of polyoxin (Hori et al., 1971, 1974b,c; Hori & Kakiki, 1977) have concluded that polyoxins bind to the active site of chitin synthetase in an analogous manner to the binding of the substrate UDP-N-acetylglucosamine, the pyrimidine moiety of polyoxin binding at the same position as the uridine moiety of the substrate (Fig. 1.3). Polyoxin is an excellent competitor with the substrate for the active site of chitin synthetase, apparent Ki values are about 1000x less than the apparent Km values for the substrate (Gooday, 1979).
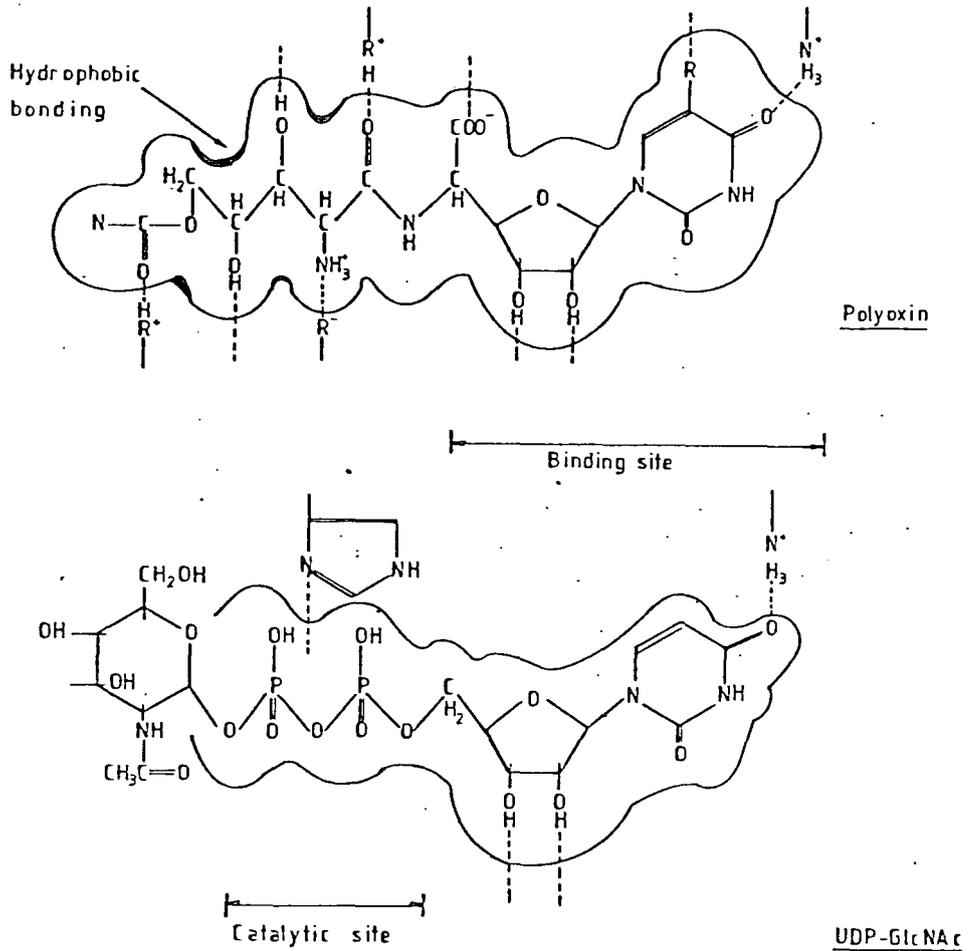
Figure 1.3   Proposed Mechanism of Interaction between Polyoxin

or UDP-N-acetylglucosamine and the Active Centre of Chitin

Synthetase

(Taken from Hori and Kakiki, 1977).

Polyoxins have been shown to inhibit chitin synthetase from a wide range of fungi e.g. <u>Saccharomyces carlsbergensis</u> (Keller & Cabib, 1971), <u>Saccharomyces cerevisiae</u> (Bowers <u>et al</u>., 1974; Guillermet <u>et al</u>., 1982), <u>Mucor rouxii</u> (Bartnicki-Garcia & Lippman, 1972), <u>Aspergillus flavus</u> (Lopez-Romero & Ruiz-Herrera, 1976), <u>Aspergillus fumigatus</u> (Archer, 1977), <u>Trichoderma viride</u> (Benitez <u>et al</u>., 1976), <u>Coccidioides immitis</u> (Hector & Pappagianis, 1983), <u>Coprinus cinereus</u> (Gooday <u>et al</u>., 1976), and <u>Candida albicans</u> (Chiew <u>et al</u>., 1980).

During the course of this study, several reports on the inhibitory effect of polyoxins on <u>Candida albicans</u> were published (Mehta <u>et al</u>., 1982, 1984; Becker <u>et al</u>., 1983; Shenbagamurthi <u>et al</u>., 1983; Naider <u>et al</u>., 1983). The results of these studies are discussed in detail in Chapter 5.

The isolation and characterization of a new polyoxin, produced by <u>Streptomyces piomogenus</u>, polyoxin N, has recently been reported (Uramoto, <u>et al</u>., 1981). Polyoxin N was reported to be active against some phytopathogenic fungi and yeasts, and <u>Candida albicans</u> (MIC 50 ug ml^{-1}) and to be non-toxic to mice.

1.4.4.3 <u>The Nikkomycins</u>

The nikkomycins are a group of peptide-nucleoside antibiotics produced by <u>Streptomyces tendae</u> Tu 901 (Dahn <u>et al</u>., 1976). They are structurally related to the polyoxins (Section 1.4.4.2), and have a similar mode of action, i.e. they are competitive inhibitors of chitin synthetase. Partial inhibition of protein synthesis by nikkomycins has also been reported (Dahn <u>et al</u>., 1976).

The structures of the nikkomycins have been elucidated (Hagenmair <u>et al</u>., 1979; Konig <u>et al</u>., 1980a,b), and their partial synthesis described (Konig <u>et al</u>., 1980b; Hass & Konig, 1982). The structures

of nikkomycins X and Z are shown in Fig. 1.4. The production of nikkomycin by immobilized Streptomyces cells has been described (Veelken & Pape, 1982, 1984). The production of new nikkomycins by mutasynthesis and directed fermentation has recently been reported (Delzer et al., 1984; Bormann et al., 1985). Nikkomycin quantitation in the fermentation process using high-performance liquid chromatography has been described (Fiedler et al., 1981).

During the course of this study, several reports on the uptake of nikkomycins by various organisms were published (McCarthy, 1983; McCarthy et al., 1985; Gooday et al., 1985), the results of which are discussed in detail in Chapter 5. Nikkomycins have been shown to be effective inhibitors of chitin synthesis in the Mexican bean beetle Epilachna varivestis (Schluter, 1982) and to inhibit a chitin synthetase preparation from Agaricus bisporus mycelium (Hanseler et al., 1983).

A third class of peptide-nucleoside drugs, the neopolyoxins, has also been described (Kobinata et al., 1980; Uramoto et al., 1980, 1982). These compounds, produced by Streptomyces cacaoi subsp. asoensis, have planar structures equivalent to the nikkomycins and there is still some doubt as to whether they might be identical compounds.
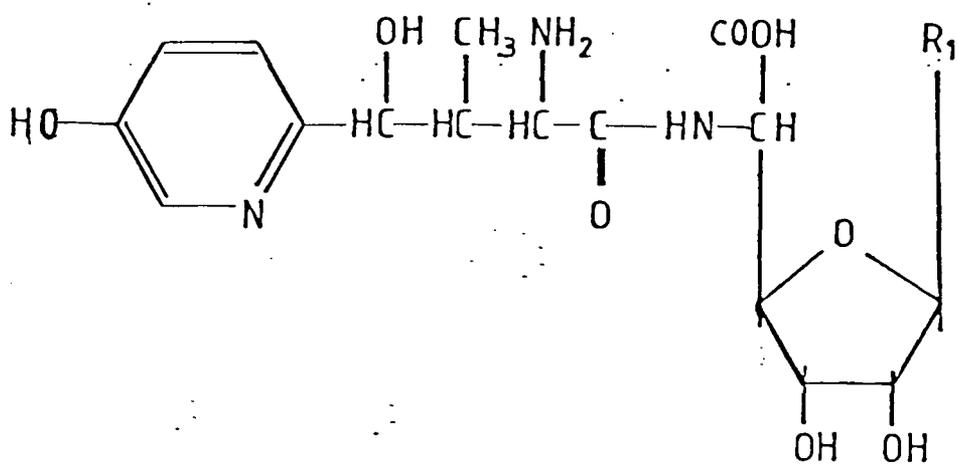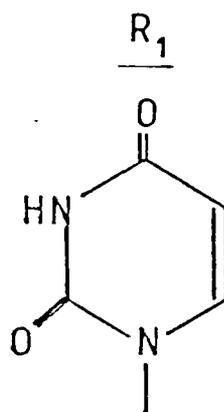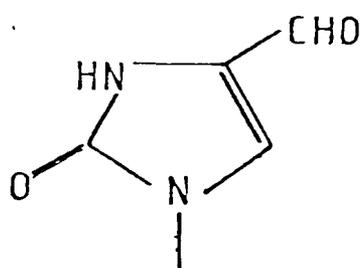
Figure 1.4   Structures of Nikkomycins X and Z

CHAPTER   2

MATERIALS   AND   METHODS

CHAPTER 2   MATERIALS AND METHODS

2.1 Introduction

This chapter is concerned primarily with the assays used to monitor peptide and amino acid uptake, together with drug sensitivity assays, which were used extensively throughout the course of this study.

Other specific methodologies are described in the appropriate sections. For the purpose of brevity, materials and instrumentation used extensively throughout this work are described together in the following sections.

2.2 Materials and Instrumentation

2.2.1 Peptides and Other Materials

Lys-Lys and His-His were gifts from Dr. J. Morley, ICI Pharmaceuticals Division, Alderly Park, Cheshire. Met-Met-Met was from Bachem U.K., Saffron Walden. All other peptides and amino acids were from Sigma (London) Limited, Poole, Dorset BDH Poole, Dorset and Uniscience Limited, Cambridge.

Ala-$[U^{14}C]$Ala and Ala-Ala-$[U^{14}C]$Ala were gifts from Dr. J.W. Lloyd, Roche Products Limited, Welwyn Garden City. Gly-$[U^{14}C]$Phe and $[U^{14}C]$Leu and $[U^{14}C]$Phe were from the Radiochemical Centre, Amersham.

All metabolic inhibitors and protein modification agents were obtained from Sigma (London) Limited, Poole, Dorset. General reagents were, when possible, of analytical grade and supplied by B.D.H. Limited, Poole, Dorset and Hopkins & Williams, Chadwell Heath, Essex.

Fluorescamine and dansyl chloride were from Sigma (London) Limited. Polyamide Sheets (15 x 15 cm) were from B.D.H. Limited.

Polyethylene vials ("Pico" vials) and Soluene 350 Tissue Solubilizer were from Packard Instruments. NE260 micellar scintillant was from Nuclear Enterprises Limited.

"Difco Bacto-Agar" (Difco Laboratories, Michigan, U.S.A.) was used extensively, Yeast Morphology Agar with amino acids (Difco 0393) being used occasionally. Yeast Nitrogen Base lacking amino acids and ammonium sulphate (Difco 0335-15) was used extensively. Tissue culture plates and microtitre tissue culture plates were from Flow Labs. Limited, Irvine Ayrshire. Drug Sensitivity Discs were from Oxoid Limited, Basingstoke, Herts.

2.2.2 Chemical Nomenclature

Abbreviations for amino acids and peptides are in accordance with the recommendations published by the 1972 IUPAC-IUB Commission (Eur. J. Biochem **27** 201-207). The nomenclature of isotopically-labelled compounds is in accordance with that as published by the IUPAC in 1978 (Eur. J. Biochem **86** 9-25). A one-letter notation for amino acids is in accordance with that published by the IUPAC-IUB Commission in 1968 (Eur. J. Biochem **5** 151-153).

The abbreviations used for all other reagents are listed in Appendix I.

2.2.3 Instrumentation

Liquid scintillation counting was performed on a Packard Prias Tri-Carb Model (PL/PLD), the detection window being adjusted to monitor ^{14}C-emission.

Manual fluorescence assays using fluorescamine, were performed on a Baird Atomic Fluoripoint (FD 100) stectrofluorimeter. For the automated fluorescence assay, a Perkin Elmer Model 1000 fluorescence spectrophotometer equipped with a flow cell (part No. 5201-9520) was

used. Analysis of transport data was performed by a 380Z microcomputer (Research Machines Limited, Cambridge) interfaced to the spectrofluorimeter.

Cell densities were measured using a Bauch and Lomb Spectronic 20 or Pye Unicam SP1800 Spectrophotometer.

2.3 Methods for the Assay of Peptide and Amino Acid Transport

2.3.1 Introduction

In the present work, use is made of two, highly sensitive fluorescence assays (fluorescamine, manual and automated assays; and to a lesser extent a dansyl chloride assay) together with radiotracer assays.

Work described in the early literature was generally based on more indirect methods (reviewed in Nisbet, 1980 and Payne, 1980a) which are only briefly discussed here.

2.3.2 Indirect Methods

Early studies on the nature of peptide transport were performed by monitoring the growth response of organisms (and its amino acids auxotrophs) to peptides as sources of required amino acids. Because of their indirect nature these tests allow only limited conclusions to be made concerning the rate and specificity of transport.

Other methods based on the supply of an amino acid in peptide form have been developed. In one method, conditions for the induced synthesis of β-galactosidase are established for an E.coli amino acid auxotroph, just before addition of a peptide as the sole source of the essential amino acid (Bell et al., 1977). From monitoring the rate of enzyme synthesis which is proportional to the amount of the required amino acid supplied, information about the peptide transport systems can be obtained.

In another method an E.coli double amino acid auxotroph was used (Payne & Bell, 1977c), one of the required amino acids being supplied free, in excess, and radioactively-labelled; the other was supplied in peptide form. Protein synthesis can only proceed in the presence of both required amino acids and therefore the rate of incorporation of label into protein is proportional to the supply of the unlabelled peptide amino acid. Both methods have advantages over growth tests but suffer from the fact that transport is not being monitored directly.

A more recent assay uses synthetic peptides with glycine residues α-substituted with thiophenol to determine spectrophotometrically the affinities of peptides for their transport systems in E.coli (Perry & Gilvarg, 1984). These peptides are recognised as substrates both by the peptide transport systems and intracellular peptidases, intracellular cleavage resulting in the release of thiophenol which exits rapidly from the cell, its release being determined spectrophotometrically on reaction with the sulphydryl reagent 5,5'-dithiobis(2-nitrobenzoic acid). Again, this method suffers from the fact that it is not a direct assay of transport per se, though it can yield useful information about the affinities of competing peptides (but not their Vmax values). For detailed kinetic parameters to be obtained, direct assays of transport must be used.

2.3.3 Direct Methods

For the assay of peptide transport in microorganisms, the majority of direct studies have used radioactively-labelled peptides. One main disadvantage to this approach is the lack of commercially available substrates, which has resulted in studies being limited to only a few peptides. Rapid intracellular hydrolysis of accumulated radioactively-labelled peptide and subsequent exodus of radioactively-labelled amino acids and/or secondary metabolites may result in an

underestimation of the true rate of peptide transport. (Payne &
Nisbet, 1980b). Kinetic parameters for peptide transport derived from
assaying label accumulation are thus subject to misinterpretation
(Section 4.3.8).

Direct fluorescence assays developed in this laboratory are used
extensively in the work described here The manual fluorescamine assay
(Section 2.5) is an extremely sensitive assay of small peptides in
solution, allowing exact quantitation of low rates of peptide transport
found in some microorganisms. The automated fluorescamine assay
(Section 2.6) is an improved version of this and when interfaced to a
microcomputer allows detailed transport kinetic parameters to be
evaluated. The dansyl chloride assay (Section 2.4) although more time
consuming, is invaluable in studying peptide transport, because it
allows simultaneous monitoring of peptide and amino acids, both extra-
and intracellularly.

The standard methodology of these three direct assays of peptide
and amino acid transport are discussed in the following sections.

2.4 Dansyl Chloride Assay

2.4.1 Introduction

Dansyl chloride (1-dimethylamino-naphthalene-5-sulphonyl-
chloride) reacts with primary and secondary amines to form fluorescent
sulphonamide derivatives which can be detected down to nanomole amounts
(Hartley, 1970). Dansyl chloride thus reacts with the α-amino groups
of small peptides. Dansylated derivatives may be separated
reproducibly by using thin layer chromatography (reviewed by Seiler,
1970), and quantified by the intensities of the fluorescent spots
relative to standards. This assay is therefore suitable for

monitoring the simultaneous disappearance of different peptides from an incubation medium, allowing competition studies to be performed which are not feasible with the other fluorescence assays. Cell extracts may also be monitored with this assay.

2.4.2 Standard Method for Monitoring Incubation Media and Cell Extracts

Candida albicans strains A and B2630 were grown overnight in liquid Pro-Medium, harvested and resuspended in PCG incubation buffer pH 4.5 and incubated with peptide substrates as described (Section 4.2.2). Samples of incubation media (0.3 ml) were collected periodically and filtered to remove cells, filtrate being collected in Eppendorf centrifuge tubes (1 ml) and stored at -20°C.

Similarly cells were harvested, resuspended and incubated with peptide, and samples (1 ml) of the suspension collected periodically for preparation of cell extracts (Section 4.2.3). Again samples of the boiled, filtered suspension were stored at -20°C before dansylation according to the following methodology described in Payne & Bell (1979) and Nisbet & Payne (1979a).

Briefly, samples containing 0 - 10 nmol peptide (typically 100 ul) were placed in Durham tubes (6 x 30 mm) together with a diaminopimelic acid (DAP) or ornithine standard (10 ul, 0.5 mM), and evaporated to dryness in vacuo. Sodium bicarbonate (20 ul, 200 mM in deionised water) was added to each tube to increase the pH to about 9, followed by an equal volume of dansyl chloride (2.5 mg ml^{-1}, in acetone). Tubes were sealed with Nescofilm, and complete dansylation achieved by incubation at 45°C for 90 min. The mixture was evaporated to dryness in vacuo and the residue redissolved in aqueous pyridine (10 ul, 1:1 vol/vol). Samples (5 ul) were spotted near one corner of polyamide sheets (15 x 15 cm) and chromatographed till the solvent

front had travelled about three quarters of the plate (approx 45 min)

in the following solvent systems.

1)   1st dimension, H_2O:formic acid (98.5 : 1.5, vol/vol)

2)   2nd dimension, acetic acid : toluene (10 : 90, vol/vol)

3)   3rd dimension, methanol : butyl acetate : acetic acid (40 : 60 : 2,

      by volume)

Chromatograms were viewed under long wave UV light, the

fluorescent derivatives being quantified by reference to the

intensities of standards (usually run on the reverse side of the

polyamide sheet), with allowance for the intensity of the internal

standards (diaminopimelic acid or ornithine) on each plate. When

required, plates were photographed under UV light using Ilfodata HS23

film (Ilford) and a Wratten No.3 filter (Kodak). Plates were reused

after washing in acetone:water:880 ammonia (50 : 46 : 4, by volume) for

at least 3 h.

2.4.3 Discussion

A large excess of dansyl chloride must be used in order to obtain

a quantitative reaction with peptides. In the standard method (10 nmol

to be dansylated), a twenty fold molar excess of label is present.

Chromatography in the first dimension often gave poor resolution

due to retardation of insoluble material (phosphate, citrate and

glucose from the incubation media) at the origin. When resolution was

poor, plates were finally rerun in the first dimension to 'round up'

the fluorescent spots.

Dansylated derivatives are easily identifiable from their

positions on a chromatogram relative to the internal standard, as

derivatives ran consistently to the same relative positions.

Differences in the colours of the fluorescent spots also aids identification (Nisbet, 1980). The relative positions of some dansyl peptides and amino acids are shown in Fig. 2.1. The positions of the two main by-products of the dansylation reaction, dansyl hydroxide (formed on reaction with water) and dansyl amide are also shown. Transport rates were estimated from the rate of disappearance of peptide from the medium. The intensity of chromatographed derivatives were estimated by comparison with a set of standard dansyl peptide and amino acids. This value was corrected to the intensity of the internal diaminopimelic or ornithine standard on each sheet, and that of a standard amount of the derivative in question. Quantitation by this method was shown to be accurate to 5% (Payne & Bell, 1979).

In most experiments, conditions were set to give at least 50% uptake of substrate (usually 0.1 mM), by adjusting both the cell density and period of incubation with the peptide. Such a change in the intensity of the fluorescent spots (10 nmol down to 5 nmol, for 50% uptake at initial substrate concentration 0.1 mM) is easily quantifiable.

Further aspects of the dansyl chloride assay are discussed elsewhere (Payne & Bell, 1979: Higgins, 1979; Nisbet, 1980).

2.4.4. Concluding Remarks

The dansyl chloride assay has the advantage of allowing simultaneous monitoring of both peptide and amino acid levels inside and outside the cell. Exodus of constituent amino acids of transported peptides, following their intracellular cleavage by peptidases, has been demonstrated in E.coli (Payne & Bell, 1979) by using this technique. However, it was shown that for Sacc. cerevisiae transport of peptides does not result in exodus of the constituent free amino
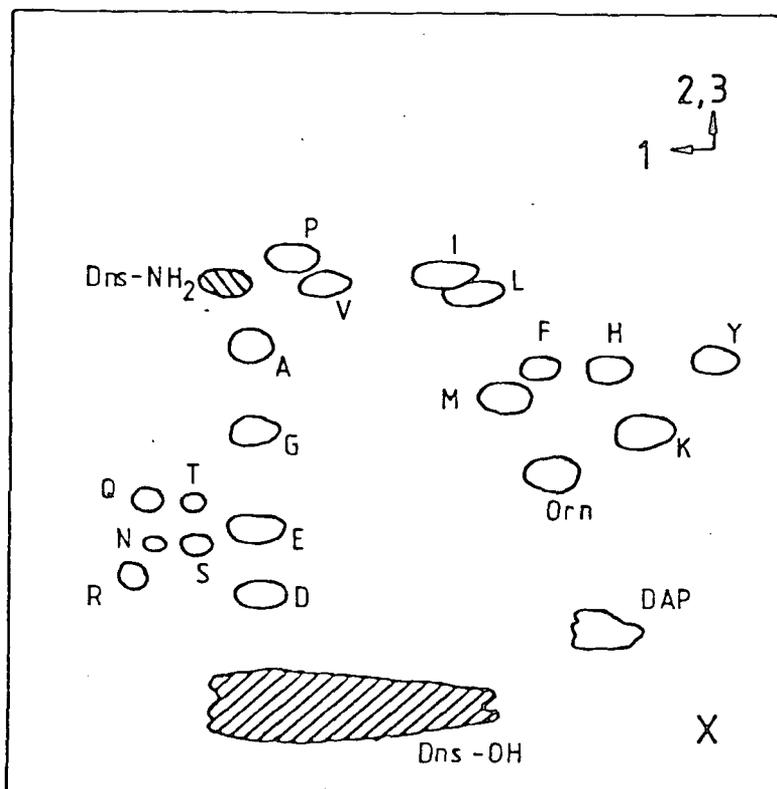
Figure 2.1 Positions of Dansyl-Amino Acids After Chromatography

in Three Solvents

A, Alanine; R, Arginine; N, Asparagine; D, Aspartic acid;

Q, Glutamine; E, Glutamic acid; G, Glycine; H, Histidine;

I, Isoleucine; L, Leucine; K, Lysine; M, Methionine;

F, Phenylalanine; P, Proline; S, Serine; T, Threonine;

Y, Tyrosine; V, Valine.

The by-products of dansylation, dansyl-hydroxide (Dns-OH) and dansyl-ammonia (Dns-NH$_2$) are indicated by cross-hatching. The positions of the internal standards diaminopimelic acid (DAP) and ornithine (Orn) are also indicated.

acids, which instead undergo metabolism (deamination) before efflux (Nisbet, 1980).

The quantitation of low rates of peptide transport using this assay is subject to error, because of the problem of estimating the small loss from the background high intensity of dansylated derivatives. Low rates of peptide transport in yeast are better estimated by using the fluorescamine assays described in the following sections. However the dansyl chloride assay has the advantage that one can observe the intact uptake of peptide by microorganisms and thus comment on active transport mechanisms using this approach. Thus, the dansyl chloride assay was used only briefly to establish general principles of peptide transport in Candida albicans.

2.5 Fluorescamine : A Manual Assay of Peptide Concentration

2.5.1 Introduction

Fluorescamine (4 phenylspiro-furan-2(3H),1'-phthalan 3,3' dione) reacts in milliseconds with the unprotonated form of primary amino groups to form fluorescent derivatives (Udenfriend et al., 1972). The reaction shows a marked pH dependence; the difference between the pKb values of the N-terminal α-amino groups of peptides and amino acids being sufficient to give selective labelling of peptides at pH 6.2 (Perrett et al., 1975). The reactions of fluorescamine are very complex (Stein et al., 1974; Chen et al, 1978) and are not yet fully understood. Neither fluorescamine itself, nor the products it forms on reaction with water or ammonia, are fluorescent. These characteristics have been exploited in developing a solution assay for peptide transport (Nisbet & Payne, 1979a) and a continuous flow assay (Nisbet & Payne, 1981). A review of these fluorescamine assays is found elsewhere (Nisbet, 1980).

At pH 6, the fluorescent yield of the reaction of fluorescamine with primary amines is lower than at pH 9, but the yields of small peptides are much greater than those of free amino acids, allowing the assay of peptides in the presence of amino acids. This is extremely useful for systems where peptide transport is followed by a rapid exodus of constituent amino acids (Nisbet, 1980). Such an exodus was shown not to occur in the yeast Sacc. cerevisiae (Nisbet, 1980).

2.5.2 Standard Method

A micro-manual fluorescamine assay modified somewhat from that described by Nisbet, (1980), was used throughout these studies.

Yeast cells were grown overnight in liquid media, harvested and resuspended in PCG incubation buffer, and incubated with peptide (0.1 - 0.5 mM) or amino acid (0.2 - 0.5 mM) as previously described (Section 4.2.2). Samples of the incubation media were collected periodically and filtered to remove cells. Filtrates were collected in Eppendorf centrifuge tubes (1 ml) and either stored at $-20^{o}C$ or assayed immediately according to the following procedure.

Briefly, duplicate samples (50 ul) of filtrate from substrate uptake incubations (0 - 25 nmol) were added to di-sodium hydrogen orthophosphate-sodium citrate (0.25 ml, 0.14 M phosphate, pH 6.5) in test tubes (100 x 12 mm diam). While the contents were being rapidly mixed using a vortex mixer, fluorescamine (0.1 ml 0.5 mg ml^{-1} in Analar acetone) was added. Samples were left for 2 min at room temperature for the fluorescence to stabilize, and made up to a readable volume by the addition of reaction buffer (phosphate-citrate, 2.5 ml pH 6.5). Fluorescence was read on a Baird Atomic Fluoripoint Spectrofluorimeter (Model FP 100), excitation 390 nm, emission 485 nm., within 15 min of the initial reaction with fluorescamine. For peptide

uptake studies, because of the variation in peptide transport rates for batches of cells grown and harvested at different times (Section 4.3.6), an Ala-Ala standard was always incorporated for each batch of cells, rates being expressed as a percentage of the rate of uptake of Ala-Ala (0.1 - 0.5 mM as appropriate).

During the course of this study the fluorescent yield of a standard Ala-Ala solution (0.1 mM) under set conditions, decreased due to a deterioration of the efficiency of the Baird Atomic Fluoripoint. To compensate for this, in the later stages of this work, several changes were made in methodology, in particular the pH of the buffer was increased. Thus assay conditions were modified as follows : filtrate samples (100 ul) in duplicate, were added to dipotassium hydrogen orthophosphate (0.25 ml, 0.2 M, pH 9.2), followed by fluorescamine (0.1 ml, 1 mg ml^{-1} in Analar acetone) with vortex mixing. Finally 2 ml of buffer (K$_2$HPO$_4$) was added to make the solution to a readable volume.

2.5.3 Discussion

The amount of peptide assayed in these studies was typically in the range 0 - 50 nmol per tube. The fluorescent yield of the fluorescamine reaction is linear over this range, for both peptides and amino acids (Higgins, 1979; Nisbet, 1980).

Under standard conditions employed here, it is acceptable to increase the assay pH to 9, because exodus of interfering substances such as constituent amino acids of transported peptides was shown not to occur in Candida albicans (Section 4.3.3), a situation similar to that found in Sacc. cerevisiae (Nisbet, 1980). In bacteria such as E.coli where exodus may be marked and rapid following peptide uptake, use of a high pH would lead to interference with the assay of peptides.

Usually however, assays do not require the uptake of extensive amounts of the peptide, initial rates of transport may be derived during the uptake of a small proportion of substrate, which minimises interference from amino acid efflux.

The yield of the fluorescamine reaction is dependent on the concentration of fluorescamine, presumably because of competition between peptide and water for the reagent. A concentration of 0.5 mg ml^{-1} in Analar acetone was used routinely (50 ug fluorescamine per assay tube), to compromise between obtaining sufficient yield and the use of what is an expensive reagent.

Further details of the chemistry of the fluorescamine reaction, its standard methodology and controls, are reviewed elsewhere, (Higgins, 1979; Nisbet, 1980).

2.5.4 Concluding Remarks

The standard micro-manual fluorescamine assay described here is an extremely sensitive and rapid assay for the uptake of small peptides and amino acids. This assay allows more exact quantitation of the low rates of peptide transport found in yeast, than does the dansyl chloride assay. Used together these two assays permit a detailed characterisation of peptide transport systems in microorganisms.

2.6 An Automated Assay of Peptide Concentration Using Fluorescamine

2.6.1 Introduction

The fact that fluorescamine reacts with unprotonated primary amines in milliseconds (Chen et al., 1978; DeBarnado et al., 1974) has allowed the development of a continuous assay of peptide (and amino acid) uptake (Payne & Nisbet, 1981), which incorporates the discrete steps of the manual fluorescamine assay. The assay system described by

Payne & Nisbet (1981) has been subsequently modified (J.W. Payne & J.T. Gleaves, unpublished results). The system now incorporates two specially designed mixing chambers and an in line pH meter, which measures the pH of the effluent from the flow cell in the spectrofluorimeter. The system is interfaced to a Research Machines 380Z microcomputer, which in part, allows the system to be controlled via the peristalic pumps. The microcomputer has a high resolution graphic display and collects and analyzes the transport data directly, providing statistically evaluated transport kinetic parameters (J.T. Gleaves & J.W. Payne, unpublished results).

2.6.2 Standard Method

Candida albicans B2630 and 6406 were grown overnight in liquid media, harvested and resuspended in PCG buffer (10 ml) as previously described (Section 4.2.2). Cells (0.8 - 1.2 (mg. dry wt.)ml^{-1}) were incubated for up to 20 min at 28°C in a thermostatted incubation vessel before addition of substrate, cells being maintained in suspension by a small magnetic stirrer bar. A pump speed of 50 ml h^{-1} was used throughout these studies - thus a 12 min assay period is just possible with a 10 ml cell suspension. Cells were incubated in PCG for up to 32 min, a period equivalent to the incubation period in the standard manual fluorescamine assay, conditions in which the glucose supply does not become limiting (Section 4.3.1).

After harvesting, a file is created for logging of transport data, using a DLOG command programme. An identifying string, with details of each particular assay, is added to each datafile. In the standard assay, PCG incubation buffer was pumped through the system for several minutes, to allow a stable baseline of fluorescence to be attained. Four minutes before addition of substrate to the cell

suspension, the pumps were momentarily stopped and the filter assembly transferred into a peptide solution (typically 10 ml, in PCG buffer) at the same concentration (0.02 - 0.2 mM) as that to be used in the assay. The pumps were restarted by a control key on the microcomputer keyboard, and simultaneously datalogging was started. After 4 min, the filter assembly was rapidly transferred into the cell suspension and simultaneously peptide was added to give the same concentration as in the standard solution. After the required incubation period, the filter assembly is rapidly (to avoid bubbles entering the system) replaced into PCG incubation buffer, and pumping is continued to re-establish the baseline. A typical overall assay period was 20 min, 4 min, to establish the fluorescence plateau of substrate concentration, 11 min actual incubation time and 5 min to re-establish the baseline. The datafile was then closed and the system prepared for the next run.

The test solutions are pumped via a needle in the filter assembly and the cells are removed by a glass fibre filter (Whatman GF/C, 25 mm) supported in a modified Swinnex filter holder. Glass fibre filters were shown to have a greater capacity than cellulose acetate membrane filters (Payne & Nisbet, 1981). The filtered solution (PCG incubation buffer, pH 4.5) was mixed with the second reaction buffer (dipotassium hydrogen sulphate, 0.2 M) from Channel B, in the first reaction chamber. This brings the pH at about 9, which will result in a high fluorescence yield when the peptide is reacted with fluorescamine, the reaction being highly pH-dependant (Section 2.5.1). The use of a pH as high as this allows a more sensitive monitoring of peptide uptake, this being permissible with Candida albicans as exodus of peptide-derived amino acids does not occur in this yeast (Section 4.3.3). This feature is particularly useful, in yeasts, as their

typical uptake rates are much lower than those found with bacteria (Payne & Nisbet, 1981)   The resulting solution was reacted with fluorescamine (0.15 mg.ml^{-1} in isopropanol, introduced via Channel C) in the second mixing chamber, and this final solution was passed through a delay coil (5.7 m) to allow fluorescence to stabilise.   The fluorescence was measured in a Perkin Elmer Model 1000 spectrofluorimeter (excitation 390 nm, emission 480 nm), any air-bubbles by-passing the flow cell via a debubbler, and the pH of the effluent was continuously measured.   The flow rate for all three channels was controlled by the peristaltic pumps, a rate of 50 ml h^{-1} (0.83 ml min^{-1}) was used throughout these studies.

A continuous record of both fluorescence and pH of the reaction solution during the incubation was produced by the chart recorder. Runs that produced smooth traces of fluorescence and where no change in pH was observed were subsequently used for data analysis (see following section).

After use, Decon detergent (2% v/v) was pumped through all three channels for 10 min, followed by distilled water for 20 min.   Distilled water was also pumped through the system for 20 min prior to use. Isopropanol was used as a solvent for the fluorescamine because it is not detrimental to the peristaltic pump tubing (unlike acetone) (Payne & Nisbet, 1981).

Modifications to the system described by Payne & Nisbet (1981) include the following :-

1)   The original three channel peristaltic pump was replaced with two 2132 Microperspex Peristaltic Pumps (L.K.B).

2)   The original mixing chambers were replaced with specially machined mixing chambers produced by the Biotechnology

Department, Teesside Polytechnic, Cleveland. These chambers
incorporated a small magnetic spinner, positioned at the junction
of the three arms, which was driven by a mini-magnetic stirrer.

3)   A pH meter (Corning pH meter 145), with a special thin-film
electrode suitable for monitoring flowing solutions, was
incorporated into the system to ensure that any observed changes
in fluorescence were not a consequence of unwanted pH variations.

2.6.3 Data Analysis

The interfacing of the system with a microcomputer enabled direct
analysis of transport data, together with the facility for statistical
evaluation of transport kinetic parameters. Data analysis was carried
out by a series of ALGOL programmes, written by Dr. J.T. Gleaves
(unpublished results).

Transport data from the continuous-flow assay were collected
every 0.08 s and the average of eight readings was recorded every 0.64
s by a 380Z microcomputer (Research Machines Limited, Cambridge)
interfaced to the spectrofluorimeter. The data represents peptide
concentration in the incubation medium (cell suspension in PCG) against
time. The rate of peptide uptake at any moment can be determined from
the rate of change of peptide concentration in the medium, which is
measured from the slope and fluorescence yield of the computer-drawn
curve. Programmes allow the computer to fit Michaelis-Menten hyperbola
to the data using a least squares method; estimates of the best
fitting Vmax and Km parameters are produced together with an analysis
of variance for the data. Contour diagrams linking Vmax and Km values
with defined confidence limits can also be generated.

Transport data are stored on 'floppy discs' (72 Kbytes (disc
side)$^{-1}$), each datafile being approximately 7 Kb. Command programmes

are stored on separate 'Command Discs'. Kinetic analysis of datafiles is briefly as follows :-

1.   ARUNG GRAPH - This programme presents the data on screen with a high-resolution graphics display. The data maybe compacted as required by use of a 'shrink factor'. The respective heights of the baseline and plateau (in arbitrary fluorescence units) are measured and the substrate concentration entered. The programme then allows analysis of the transport data in a choice of two forms - either as a ST plot (substrate against time) or as a VS plot (rate against substrate), the calculated results being stored in an output file. The computer can then be used to measure the gradient of the curve between two moveable arrows, expressing the result either in terms of substrate concentration at the mid-point of the pointers against time, or as the rate of uptake ($nmol.min^{-1}ml^{-1}$) for a given substrate concentration at the mid point of the arrows. Both the increment between the arrows and the point from which analysis of the curve starts can be varied· The computer then sequentially analyses identical sections of the curve, logging all results into the output file.

2.   TXED - this programme facilitates editing of the compiled output file, e.g. allowing if needed the erasure of unwanted data. This programme also allows a series of output files to be compiled into a single 'string' which can be analyzed sequentially, removing the need to load files individually for analysis. TXED is also used for the addition of identifying commands to the output file which are essential for kinetic analysis.

3.   KINETICS - this programme is used for kinetic analysis of the output file, either in a VS or ST mode· The computer will print

the output file and produce a hyperbola of best fit, calculated

by the least squares method. From this plot the programme will

calculate the best estimates of the parameters Km and Vmax. Data

from these computations are logged into a second output file.

4. PRINTOUT - this programme allows the printing of the output

files produced by the Kinetics programme.

2.7 Use of Radioactively-labelled Substrates

2.7.1 Introduction

Radioactively-labelled substrates have been used in the majority

of direct studies of peptide transport reported in the literature.

However, it has been shown that the use of these substrates may lead to

gross underestimates of the true rates of transport (Payne & Nisbet,

1980b; Nisbet, 1980).

In these studies, rates of uptake measured by the conventional

assay of monitoring accumulated counts following addition of

radioactively-labelled substrate to cell suspensions, were compared

with results obtained using the fluorescence methodology (Section

4.3.8). Transport rates obtained by using radioactively-labelled

substrates were derived by the following methodology.

2.7.2 Standard Method

Uptake of Ala-[$U^{14}C$]Ala (3.09×10^{-2} uCi ml^{-1}), Ala-Ala-[$U^{14}C$]Ala

(6.06×10^{-3} uCi ml^{-1}), Gly-[$U^{14}C$]Phe (5×10^{-3} uCi ml^{-1}) and

[$U^{14}C$]Leu (2.5×10^{-2} uCi ml^{-1}) by Candida albicans B2630, were

measured using the following methodology.

Candida albicans strain B2630 was grown overnight in Pro- or Pep-

Medium, harvested and resuspended in incubation buffer (Section 4.2.2).

Organisms (4 ml, 1 - 2 (mg dry wt.)ml^{-1}) were preincubated for 10 min

at 28oC, cells being agitated by a small magnetic stirer bar.

Radioactively-labelled peptide was added (from a 10 mM stock solution in distilled water) to a concentration of 0.1 mM (5 x 10^{-3} - 3.09 x 10^{-2} uCi ml^{-1}), amino acids being routinely added at 0.5 mM (2.5 x 10^{-2} uCi ml^{-1}) After addition of substrate, samples (500 ul) were removed periodically, filtered under vacuum (Whatman GF/C, 25 mm diam), and the filters washed with saline (0.9% w/v, approx. 20 ml, room temp.).

After washing, filters were added to liquid scintillation vials containing Soluene 350 tissue solubilizer (1 ml) capped and incubated overnight at 45°C. NE260 micellar scintillent (5 ml), was added, vials were incubated for at least 6 h at room temperature (to decrease chemiluminescence), before measuring accumulated counts.

Using essentially the same methodology as above, a linear relationship between the amount of Ala-[U^{14}C]Ala and counts detected, over the range 1 - 250 nmol peptide has been previously demonstrated (Nisbet, 1980).

Similarly, a linear relationship between the amount of Gly-[U^{14}C]Phe (5 - 50 nmol) and counts detected, was demonstrated using the standard methodology described here (data not shown).

It has previously been demonstrated that the efficiency of counting the [^{14}C]-labelled peptides both in the presence and absence of added bacteria (S. faecalis and E. coli) was the same, indicating that no detectable attenuation of β -emission is occurring (Nisbet, 1980). The efficiency of [^{14}C] counting for the Soluene-NE260 system has been shown to be about 70% (Nisbet, 1980).

The use of the tissue solubilizer in the scintillant system used in this study, should eliminate the possibility of attenuation of β-emission from "intracellular" accumulated radioactivity (Nisbet, 1980). The β-emission of [^{14}C] should not be significantly attenuated

by materials of the density found in yeast cells. Incorporation in to macromolecules (e.g. protein) should likewise have negligible effect. These conclusions are supported by the report (Zarybnicky & Reich, 1980) that attenuation of β-emission from accumulated label by whole bacterial cells should not exceed 4%.

A comparison of uptake rates measured by accumulation of radioactively-labelled substrates and by the standard manual fluorescamine assay is presented elsewhere (Section 4.3.8).

2.8 Methods for the Assay of Drug Toxicity

2.8.1 A Description of the Available Methods

Antifungal drug sensitivity tests fall into two distinct categories and are performed either in liquid or on solid agar surfaces (Holt, 1975). In the former a standard inoculum of the yeast is challenged by a range of drug concentrations in defined nutrient media; in agar diffusion methods, an even lawn of yeast inoculum or a radial streak encounters a variable zone of drug concentrations diffusing radially from a drug reservoir. In this study, use is made of both categories of sensitivity testing, standard methodology being presented in the following sections

2.8.2 Organisms and Growth Conditions

2.8.2.1 Organisms

Candida albicans strains A, B, B2630, 6406, 6406/8 (Amphotericin B resistant), 500/8/1 (5-fluorocytosine resistant), Candida tropicalis, Candida parapsilosis (Leech) and Candida parapsilosis (Worme & Lambert), and a Saccharomyces cerevisiae were kindly provided by Dr. K.J. Barrett-Bee, I.C.I., Pharmaceuticals Division, Cheshire.

<u>Saccharomyces</u> <u>cerevisiae</u> haploid wild-type strain Σ 1278b (mating type α) and a peptide transport-deficient mutant Σ 1278b <u>gpp</u> were described previously (Nisbet & Payne, 1979b; Nisbet, 1980), the wild-type having originally been provided by Prof. A.A. Eddy, Dept. of Biochemistry, UMIST, Manchester.

2.8.2.2 Growth Conditions

The presence of peptides in complex media precludes the use of such media in the study of peptide drug sensitivity testing and mutant screening. This is because of possible competition for transport with the drug under test and also the possibility of selecting for revertants of peptide transport-deficient mutants on prolonged subculturing on complex, peptide-based media. Therefore, for this study, simple, defined minimal media were used throughout.

Cultures were maintained on 1.5% (w/v) Bacto-Agar plates containing glucose (2%, w/v), Yeast Nitrogen Base lacking amino acids and ammonium sulphate (1.7 mg ml^{-1}, Difco 0335-15), in which proline (4 mg ml^{-1}) was the sole nitrogen source (referred to as Pro-Medium plates). Subculturing was routinely carried out every four weeks, plates being stored at 4°C.

For growth in liquid culture the same medium minus agar was used (Pro-Medium). Cultures were grown overnight at 28°C, with shaking, and subcultured into liquid medium as required.

2.9 Standard Methodology

2.9.1 Introduction

Throughout the course of this study, the limited amounts of peptide drugs available precluded extensive drug sensitivity testing. Care was taken to minimise drug usage in the various tests available.

Drug sensitivity and cross-resistance testing was performed both on solid agar and in liquid minimal media, standard methodologies being given in the following sections.

2.9.2 Agar Dilution Assay

The sensitivities of several strains of yeasts to the basic polyoxin fractions (Section 3.3.2) was examined by an agar dilution assay.

Yeast Morphology Agar with amino acids (Difco 0393) containing polyoxin fractions (10 ml, 2 mg ml^{-1}) was serially diluted with fresh YMA agar to give a range of polyoxin concentrations from 2 - 0.062 mg ml^{-1}. To the wells of a tissue culture plate (Linbro, 24 flat bottom wells plate^{-1}, 1.7 x 1.6 cm) 1 ml of each agar dilution was added. Wells were surface inoculated (1 x 10^3 cells well^{-1}, 100 ul volume), plates were sealed and incubated at 30°C for 45 h. Growth was scored as the appearance of surface colonies or as a lawn of cells, Minimum Inhibitory Concentration (MIC) being read as the lowest concentration in which no growth is visible. MIC values derived here were compared with those obtained in liquid media by using a microtitre plate assay (Section 2.9.3).

2.9.3 Microtitre Plate Assay

Sensitivity of the different strains to the various polyoxins was determined using a microtitre plate assay, based on the procedure of Fisher & Armstrong (1977).

Into the wells of a tissue culture plate (Falcon Micro Test II) was added 50 ul 2x concentrated Pro-Medium; 25 ul inoculum containing 1 x 10^3 cells of a fresh overnight culture resuspended in 10 mM phosphate citrate buffer pH 4.5; 25 ul aqueous polyoxin solutions (10, 2, 0.4 or 0.08 mg ml^{-1}), or 25 ul water as control. The final volume

in each well was 0.1 ml   The microtitre plates were covered with

fitted sterile plastic lids and incubated at 30°C for 45 h. Growth was

scored as the deposition of a clearly visible white sediment of cells,

the minimum inhibitory concentration (MIC) being read as the lowest

polyoxin concentration in which no cellular sediment was visible.

A cell suspension containing no nutrient (0.1 ml, 1 x 10^4 cells

ml^{-1} in distilled water), was placed in the well of a microtitre plate,

and incubated as described (30°C for 45 h). This inoculum did not

produce a cellular sediment after 45 h, i.e. that growth had not

occurred, which was attributed to the absence of supplied nutrient.

Thus, the deposition of a visible cellular sediment (due to the growth

of the initial inoculum) was a legitimate parameter for assaying

growth. This precluded the use of indicator dyes to monitor acid

production by glucose fermentation, as the parameter for the

measurement of growth response to antifungal compounds (Fisher &

Armstrong, 1977).

The microtitre plate assay uses minimal quantities of the limited

amounts of drugs available, providing a rapid, sensitive profile for a

range of yeasts, together with MIC values.

2.9.4 Radial Streak Assays

Routinely, sensitivity to the various drugs was measured using a

radial streak technique (Payne et al., 1984).

For this assay a series of fresh (less than five days old) master

plates was prepared for subculturing onto the test plates, as it was

found that an inoculum taken from a fresh yeast streak, produced a more

even streak on transferring to a test plate. Also it was easier to

pick a small inoculum (using a fine platinum wire) from a fresh master

streak relative to an old one, as the fresh streaks are fairly moist.

A sterile drug sensitivity disc (Oxoid) was placed at the centre of a Pro-Medium plate and up to 20 cultures were streaked from the disc outwards to the edge of plate, a reference wild type always being included. The blank disc was replaced with a drug-impregnated disc, and plates were incubated for 45 h at $28^{o}C$ before measuring the distance from the centre of the disc to the beginning of streak growth, i.e. in effect the radius of the inhibition zones. For several drug screens there was no clear total inhibition zone, but streaks tapered to the centre of the plate Here, the distance from the centre of the disc to the end of the taper, i.e. the start of non-inhibited growth, was measured. This method was used to screen presumptive mutants isolated from drug inhibition zones (Chapter 5) for drug sensitivity relative to a wild-type control strain, and also to test for cross-resistance to the different drugs.

This method has the advantage of economising on drug expenditure, allowing up to 20 strains to be screened per drug-impregnated sensitivity disc. Because of the limited quantities of drugs available in this study, such a consideration is of obvious advantage.

2.9.5 Seeded Soft-Agar Overlays

Drug sensitivity testing was also performed using seeded soft-agar overlays. Sterile soft overlays (agar, 1% w/v., glucose, 2% w/v., proline, 4 mg ml^{-1}., yeast nitrogen base, 1.7 mg ml^{-1}) were maintained in a heating block at $45^{o}C$ till required for use. Overlays were seeded with cells diluted from an overnight culture grown in Pro-Medium (500 ul, 1 x 10^5 colony forming units ml^{-1}) and poured onto a Pro-Medium plate. Plates were allowed to set for 1 h at room temperature before drug-impregnated discs were placed on top of the overlay. Plates were incubated at $28^{o}C$ for 45 h before measuring diameters of inhibition zones

This method was particularly useful for obtaining quantitative data on drug sensitivity patterns especially when separate discs for different classes of peptide drug (e.g. di-, and tripeptide drugs) are included on the same seeded plate. Drug sensitivities of presumptive mutants were always compared with those exhibited by the parental wild-type.

2.9.6 Growth Tests

The growth of Candida albicans strains in liquid medium containing Polyoxin D was followed by monitoring viable counts over a short incubation period (12 h). To conserve the limited stocks of polyoxin D, this assay was performed in a very small volume (100 ul) because a high drug concentration (2.5 mg ml^{-1}) is required (Section 5.5.3).

Cells were grown in liquid Pro-Medium overnight, harvested, resuspended and diluted in fresh medium to approx. 5×10^4 colony forming units ml^{-1}. Polyoxin D (25 mg ml^{-1} in water, 10 ul) was added to the cell suspension (90 ul). For viable count estimations, aliquots (10 ul) were removed periodically, diluted appropriately, duplicate samples (50 ul) were spread on to Pro-Medium plates, and incubated at 28°C for 45 h. After this time, colonies were counted and results expressed as number of colony forming units ml^{-1} against time. Controls without inhibitors were performed in parallel.

The nature of drug toxicity (fungicidal or fungistatic) can be demonstrated by the above growth test. A decrease in the viable count in the presence of the drug indicates the drug is fungicidal, whereas if the viable count remained the same in the initial inoculum, the drug would have a fungistatic mode of action.

2.9.7 Concluding Remarks

The drug sensitivity assays described in this section give valuable information about drug sensitivities of the various yeast strains and their derived drug-resistant mutants. These assays have been used both for rapid screening of presumptive mutants and a more detailed analysis of cross-resistance patterns for selected strains.

CHAPTER   3

ISOLATION OF POLYOXINS AND THEIR CHITIN SYNTHETASE

INHIBITORY PROPERTIES

ISOLATION AND CHARACTERIZATION OF POLYOXINS

3.1 Introduction

A series of polyoxin complexes and their individual components were isolated from a batch of crude agricultural fungicide (Polyoxin Z), by using methods modified somewhat from those described by Isono et al., (1967). The exact composition of Polyoxin Z (a fine, brown, chalk-like powder) was unknown, but was stated to contain 2% polyoxin complex, again the nature of which was unspecified. Polyoxin Z was expected to contain polyoxins A, B and D as its principal components, as these are the major components produced under standard fermentation conditions (Isono et al., 1965). The original isolation procedures (Suzuki et al., 1965; 1966; Isono et al., 1965; 1967; 1968), have been scaled down and modified according to the availability of the various ion-exchange resins and other materials.

Acidic and basic polyoxin fractions were obtained from the Polyoxin Z sample, further resolution of the individual components was achieved by cellulose column chromatography. The basic polyoxin fraction was used in drug sensitivity testing and mutant isolation studies (Chapter 5).

3.2 Materials and Methods

3.2 1 Materials

Dowex 50W-X8 (H^+ form, 100-200 mesh) Amberlite IR-4B (OH^- form) and 'Darco' Activated Carbon (12 x 20 mesh) were from Fluka A.G. Buchs S.G. (Glossop, Derbyshire). Dowex 50W-X16 was from Bio Rad, Watford, Herts. Cellulose thin layer chromatography plates (20 x 20 cm, 1 mm layer) were from E. Merck Darmstadt.

UDP-N-Acetyl-D-[U^{14}C]-Glucosamine was from the Radiochemical

Centre, Amersham. UDP-N-Acetyl-D-Glucosamine, trypsin and soybean

trypsin inhibitor were from Sigma (London) Limited, Poole, Dorset.

Polyoxin Z W.P. (1980) A.I. 2.2% (500 g) was from Nihon Nohyaku, Tokyo,

Japan. Purified polyoxins A, B and D were from Dr. K. Isono, Institute

of Physical and Chemical Research, Wako-shi, Japan.

3.2.2 Column Preparation and Reactivation

Standard glass chromatography columns (2 x 50 cm) were used

throughout this study. All glassware was washed with dilute Teepol,

including two rinses with ethanol and two final rinses with distilled

water. A cotton wool plug was inserted at the neck of each column just

above the tap, and the resin (25 - 100 ml) loaded as a slurry,

activated carbon (25 - 50 ml) being added dry. Columns were tapped

repeatedly to allow the resin to settle. All resins were washed before

use, which tended to free trapped air from the columns, resulting in

tighter packing of the resins.

Columns were loaded with solutions using a long necked funnel,

care being taken not to disturb the packed resin. The rate of flow

through the column was regulated with a tap at the base of the column,

fractions being collected in conical flasks (typically 500 ml). Low

elution rates (0.1 - 0.2 ml min^{-1}) were used throughout this study to

ensure complete adsorption/elution of the polyoxin components. The

following columns were used :-

1) Dowex 50W-X8 (H^{+} form, 100 - 200 mesh) 100 ml resin.

The column (2 x 32 cm) was washed with distilled water (500 ml),

the effluent pH drops to 2 at the start of the wash, rising to pH

5. The column was then washed with HCl (200 ml, 0.2 M) to ensure

the resin was fully in the H^{+} form.

2) <u>Activated Carbon</u> (Darco 12 x 20 mesh) 100 ml.

The column (2 x 32 cm) was washed with distilled water (300 ml) before use, which served to remove fine particulate material. Smaller columns (50 or 25 ml) were washed with an appropriate volume of distilled water.

3) <u>Amberlite IR-4B</u> 50 ml resin.

The resin was provided in the hydroxyl form (OH^-) requiring conversion to the chloride (Cl^-) form. The column (2 x 16 cm) was washed with distilled water (250 ml), effluent pH was initially 8 The column was then washed with NaCl (500 ml, 0.2 M), the pH of the effluent falls and remains at 7

4) <u>Buffered Dowex 50 W-X8 Column</u> 50 ml resin

The column (2 x 16 cm) was washed with distilled water (200 ml), followed by KH_2PO_4-HCl, pH 2, (200 ml 0.5 M) and finally KH_2PO_4-HCl, pH 2 (200 ml, 0.1 M) before use.

5) <u>Dowex 50W-X16</u> (H^+ form, 200 - 400 mesh) 25 ml.

The column was washed with distilled water (250 ml) and then with HCl (100 ml, 0.2 M) to ensure that the resin was fully in the H^+ form.

The Dowex columns were reactivated by extensive washing with distilled water, and then washing with the appropriate acid or buffer. Amberlite columns were washed with distilled water (200 ml) before washing with NaCl (200 ml, 2 M). The activated carbon was not reused in these studies.

3.2.3 <u>Thin Layer Chromatography</u>

Analysis of fractions from ion exchange (Sections 3.3.2, 3.3.3, 3.3.4) and cellulose column chromatography columns (Section 3.3.5) was by thin layer chromatography (TLC) on cellulose plates (20 x 20 cm).

Literature Rf values for paper chromatography of polyoxins (Isono et al., 1967) differed from values obtained by cellulose thin layer chromatography (Table 3.1), therefore it was not possible to use quoted literature values for the presumptive identification of polyoxin components.

Samples (5 - 15 ul) of polyoxin fractions were spotted onto plates using a Hamilton syringe, diameter of spots being kept to a minimum (3 mm) by rapid evaporation of solvent from the plate using a hot air blower. To facilitate spotting the plates, a very fine pencil line was drawn to mark the point of origin for each sample. Chromatograms were run in standard sealed chromatography tanks, the air in the tanks being saturated with solvent by placing a filter paper soaked in solvent around the inside edges of the tank. Chromatograms were developed for approx. 1 h, or until the solvent front had ran at least 10 cm from the origin, dried and developed with ninhydrin (Section 3.2.4).

For analysis of polyoxin-containing fractions from ion-exchange and activated carbon columns, chromatograms were developed with a propanol/pyridine/acetic acid/water (15 : 10 : 3 : 12 by volume) system. For fractions obtained from cellulose column chromatography the butanol/acetic acid/water (4 : 1 : 2 by volume) solvent system of Isono et al., (1967) was used.

Examples of cellulose thin layer chromatography plates are presented in the following sections (Figs 3.1, 3.3, 3.4).

3.2.4 Detection of Polyoxins in Solution and on Thin Layer

    Chromatography Plates

Polyoxins were routinely analysed by thin layer chromatography (Section 3.2.3) Chromatograms were dried in a fume hood to remove solvent (the solvent front being marked), before development with
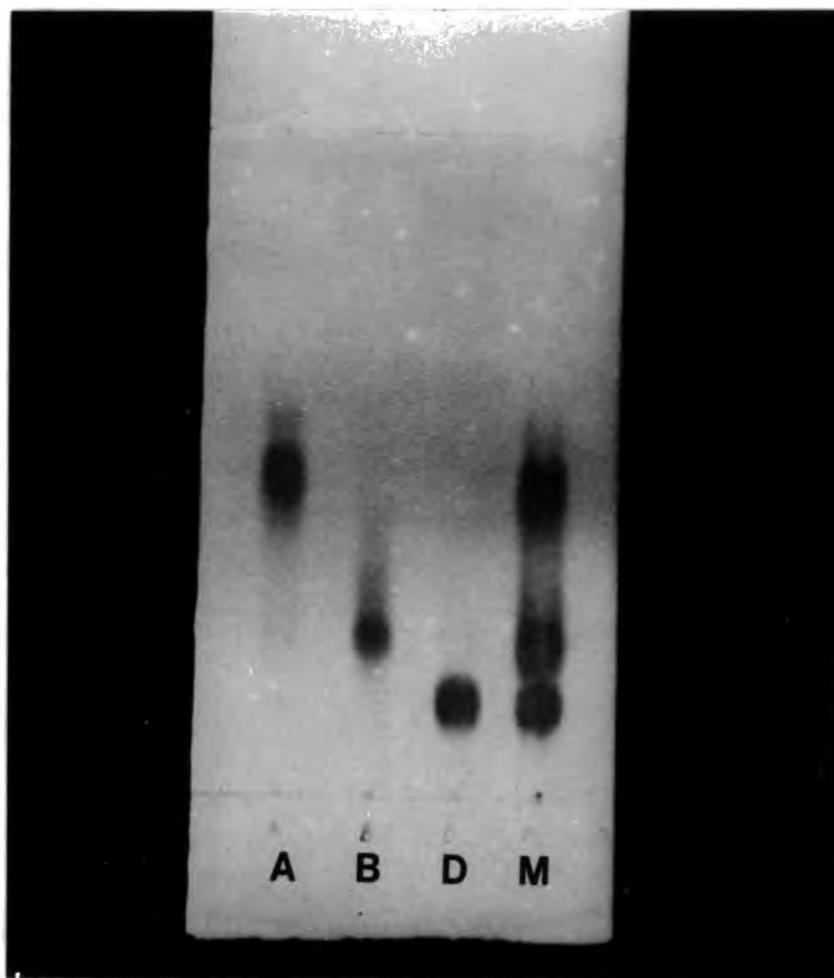
Figure 3.1   Thin Layer Chromatography of Polyoxin Standards

Thin layer chromatography of polyoxin standards A, (A);   B, (B);   D,
(D) and a mix of polyoxins A, B and D, (M), after chromatography using
n-propanol/pyridine/glacial acetic acid/water (15 : 10 : 3 : 12)
(Section 3.2.3).

Rf Values

Polyoxin	1) PPAH	2) PHENOL	3) BAH
A	0.48	0.62 (0.53)	0.31 (0.19)
B	0.24	0 18 (0 18)	0.12 (0.07)
D	0.15	0.15 (0.06)	0.12 (0.07)

TABLE 3.1   Thin Layer Chromatography of Polyoxins

Thin layer chromatography of polyoxin standards A, B and D (25 - 33 ug) was achieved as described (Section 3.2.3), using different solvent systems.   Chromatograms were developed with ninhydrin (Section 3.2.4) and Rf values measured.

Solvent System

1) PPAH - n-Propanol : Pyridine : Glacial Acetic Acid : Water

   (15 : 10 : 3 : 12 by volume)

2) Phenol - (75% w/v in water)

3) BAH - Butanol : Acetic Acid : Water (4 : 1 : 2 by volume)

Values quoted are the average of at least two determinations.

Figures in parentheses are the Rf values of polyoxins A, B, D after paper chromatography of polyoxins, taken from Isono et al. (1977)

ninhydrin (0.2% w/v in Analar acetone, prepared fresh)   Plates were
sprayed with ninhydrin, and developed by gently warming the plates over
a hot plate.   Polyoxins produced purple spots on reaction with
ninhydrin.   Chromatograms were photographed immediately as the
intensity decreased after a few hours.

Ninhydrin development enabled detection of Polyoxins to 2 ug
$spot^{-1}$ (5 ul, 0.4 mg ml^{-1})   Polyoxins A, B and D (25 - 33 ug $spot^{-1}$)
were often included on chromatograms, giving an internal control for
spot intensity on ninhydrin development.

For screening fractions from cellulose column chromatography, the
absorbance at 262 nm was measured with a Pye Unicam SP8-100 UV-VIS
Spectrophotometer (1 cm path length).   UV absorbing fractions were
subsequently analysed by TLC (Section 3.2.3).

3 2.5   Physicochemical Analysis of Purified Polyoxins

Physicochemical analysis of purified polyoxins was performed with
the assistance of the Natural Products Department, I.C.I.
Pharmaceuticals.

UV spectra of isolated polyoxins (in HCl, 0.05 M) were measured
with a Pye Unicam SP8-100 UV-VIS Spectrophotometer.   NMR spectra
(samples in D_2O) were recorded using a 400 MHz spectrometer.

3.3   Large Scale Preparation of Polyoxin

3.3.1   Extraction of Polyoxins from Polyoxin Z

Polyoxin Z (150 g) was dissolved in distilled water (750 ml), the
solution being stirred continually (using a magnetic stirrer bar) for
5h, after which time the solution was filtered (Whatman No. 1   11 cm).
The insoluble base was resuspended in distilled water (500 ml), stirred

for 5 h and refiltered; and again resuspended (distilled water 500 ml), and stirred overnight before refiltering. The filtered solutions were combined and concentrated in vacuo to approx. 600 ml. The insoluble base was concentrated in vacuo to dryness and kept for future reference.

Because of the high solubility of polyoxins in water (Isono et al., 1965; 1967), this treatment was expected to completely solubilize the polyoxin complex present in the Polyoxin Z, (150 g of Polyoxin Z would be expected to yield 3 g of crude polyoxin complex). The polyoxin extract (600 ml) was acidified to pH 2 with HCl (10 M), before adsorption onto Dowex 50W-X8(H$^+$), see following section.

The isolation procedure employed in this study is outlined in Fig. 3.2.

3.3.2 Preparation of Crude Polyoxin Complex

A Dowex 50W-X8 (H$^+$ form, 100 - 200 mesh) column was prepared as previously described (Section 3.2.2). The acidified polyoxin complex (600 ml) was applied to the Dowex column (2 x 32 cm), the column was then washed with distilled water (200 ml) to remove salts and non-adsorbed material. Polyoxins were eluted with ammonium hydroxide (0.6 M, 1100 ml).

From preliminary experiments the polyoxins were known to elute at between 300 - 1000 ml of NH$_4$OH (Fig 3.3), so for this large scale isolation, two fractions (250 ml and 850 ml) were collected, the second fraction being expected to contain the polyoxin complex.

Fraction 2 (850 ml) of the Dowex eluent was applied to an activated carbon column (75 ml), the column was then washed with distilled water (400 ml). Polyoxins were eluted with 60% acetone (1000 ml), concentrated in vacuo to dryness, and redissolved in distilled water (20 ml).

Polyoxin Z
|
Acidified Filtrate
|
Dowex 50W-X8 (H^+)

| NH_4OH (0.6M)

Eluate
|
Activated Carbon

| Acetone (40% v/v)

Eluate
|
Amberlite IR-4B (Cl^-)

Effluent

|
Dowex 50W-X8 (H^+)

| Phosphate Buffer

Activated Carbon

| Acetone (60% v/v)

Basic Polyoxin Fraction

(Polyoxins A,B,C,G,H,I,J,K,L,M)

Adsorbent

| NaCl(0.6% w/v)

Eluate
|
Activated Carbon

| Methanol-pyridine-H_2O
(5 : 1 : 4 v/v)

Dowex 50W-X16
|
Effluent
|
Activated Carbon

| Methanol-pyridine-H_2O
(5 : 1 : 4 v/v)

Acidic Polyoxin Fraction

(Polyoxins D,E,F)

Figure 3.2   Isolation Procedure for Polyoxins
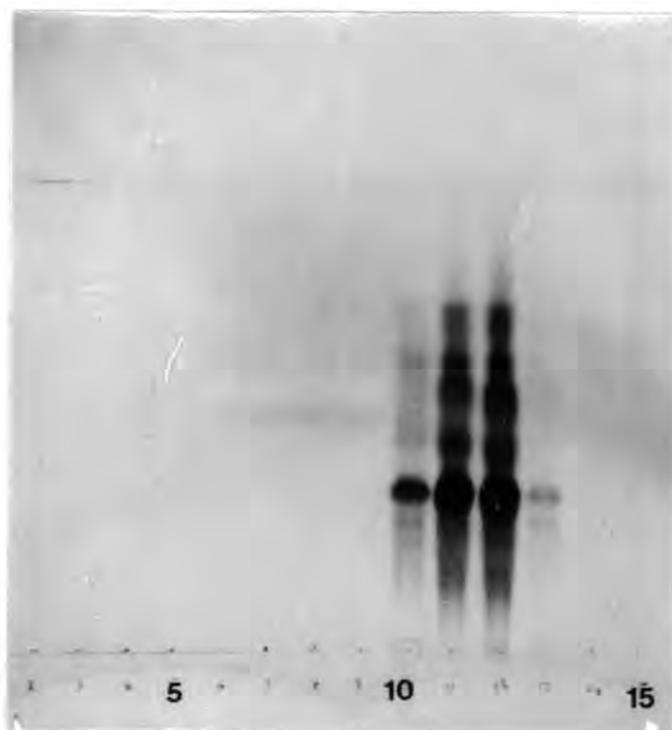
Figure 3.3 Elution of Polyoxin Complex from Dowex 50W-X8: Analysis

of Fractions by Cellulose Thin Layer Chromatography

Samples (10 ul) from fractions eluted from a Dowex 50W-X8 column (100

ml) were spotted onto a cellulose TLC plate (Section 3.2.3) and

chromatographed using n-propanol/pyridine/acetic acid/water (15 : 10 :

3 : 12) as the solvent system. Plates were developed with ninhydrin

(Section 3.2.4).

Fraction numbers are included at the base of the plate.

Ninhydrin-positive material was found on fractions 10, 11, 12, 13;

fractions 9 - 14 were combined and concentrated in vacuo to dryness.

3 3 3 <u>Isolation of Basic Polyoxins</u>

An Amberlite IR-4B (Cl⁻ form), column (2 x 32 cm) was prepared as previously described (Section 3.2.2). The polyoxin sample (20 ml) from the Dowex column was applied to the Amberlite column and washed with distilled water (350 ml). The effluent and washings were combined and concentrated <u>in vacuo</u> to dryness to give 660 mg of mixed basic polyoxins.

The crude basic polyoxins were dissolved in K_2HPO_4-HCl buffer, pH 2 (10 ml, 0.1 M) and applied to a Dowex 50W-X8 column (2 x 16 cm) prepared as described previously (Section 3.2.2), the column was then washed with KH_2PO_4-HCl, pH 2 buffer (400 ml, 0.1 M). Polyoxins were eluted with KH_2PO_4, pH 5 (500 ml, 0.1 M) and applied to an activated carbon column (50 g). The column was washed with distilled water (100 ml) and eluted with acetone (500 ml, 60% w/v). The eluate was concentrated <u>in vacuo</u> to dryness to give 290 mg of crude basic polyoxins.

Typical yields of basic polyoxins obtained by this procedure were 2 - 6 mg of crude basic polyoxin per g of Polyoxin Z.

3.3.4 <u>Isolation of Acidic Polyoxins</u>

The Amberlite IR-4B column of the preceding section was eluted with NaCl (500 ml, 0.6% w/v). The eluate (500 ml) was applied to an activated carbon column (20 g), and the column was washed with distilled water (200 ml). The polyoxins were eluted with methanol pyridine-water (5 : 1 : 4 by volume, 500 ml) and the total eluent concentrated <u>in vacuo</u> to dryness.

A Dowex 50W-X16 column (25 ml) was prepared as previously described (Section 3.2.2). The acidic polyoxin complex was redissolved in HCl (10 ml, 0.2 M) applied to the column, which was then washed through with HCl (400 ml, 0.2 M). The effluent was applied to an activated carbon column (10 g), and the column washed with distilled water (150 ml). Polyoxins were eluted with methanol-pyridine-water (5 : 1 : 4 by volume, 500 ml) and concentrated in vacuo to dryness to yield the acidic polyoxin complex (215 mg).

The yield of acidic polyoxins obtained by this procedure was 1.4 mg per g of Polyoxin Z.

3.3.5 Cellulose Column Chromatography

Resolution of the individual components of the acidic and basic polyoxin fractions was achieved by using cellulose column chromatography.

Microgranular Cellulose Powder (Whatman CC31) was used throughout this study, giving greater resolution (with a corresponding slower elution time) than cellulose fibre (Whatman CF11).

A thin layer of sand (13 mm) was laid over a cotton-wool plug, cellulose powder (15 - 100 g, depending on column size) was added and packed tightly by tapping the column on a hard surface. A top layer of sand (3 mm) was laid over the cellulose, this facilitated even loading of the column (essential for maximum resolution) and prevented the top of the column from being disturbed.

Samples of polyoxin (10 - 100 mg) were dissolved in butanol-glacial acetic acid-water (4 : 1 : 2 by volume, 1 - 2ml) loaded onto the column and eluted with the same solvent system. For larger quantities of polyoxin complex (100 - 500 mg) longer columns of cellulose (100 g) were used. Fractions (2 - 7 ml) were analysed for

polyoxin content by thin layer chromatography (Section 3.2.3) or by absorbance at 262 nm (Section 3.2.4). Fractions containing homogeneous polyoxin components, i.e. those giving a single spot on TLC analysis, were combined, concentrated in vacuo to dryness and rechromatographed to prove homogeneity. Overlapping fractions were rechromatographed using smaller cellulose columns (typically 20 g).

From the initial chromatography of the acidic and basic polyoxin complexes (Section 3.3.3 and 3.3.4), a series of fractions (60 x 7 ml) were obtained. Fractions containing polyoxins of very similar Rf value on TLC analysis, were combined and rechromatographed on smaller cellulose columns. Examples of TLC analyses of fractions from cellulose column chromatography of polyoxin complexes are shown in Fig. 3.4.

Each polyoxin was finally recrystallised from aqueous ethanol. Each of the polyoxins showed a single spot on thin layer chromatography analysis, exhibiting minimal tailing, Rf values were recorded. Yields of purified polyoxin components were rather low (2 - 20 mg).

Unresolved fractions were combined, concentrated in vacuo to dryness and rechromatographed.

3.3.6 Isolated Polyoxins

In addition to the acidic and basic polyoxin complexes obtained by ion-exchange procedures, a series of purified polyoxin components were isolated by cellulose column chromatography (Table 3.2).

The lack of sufficient standards for TLC and other physicochemical data precluded positive identification of most of the isolated components. Separation of the polyoxin complexes in to acidic (polyoxins D, E and F) and basic (polyoxins A, B, C, G, H and I) components at the Amberlite IR-4B stage of the isolation procedure (Section 3.3.2), aided component identification.

Figure 3.4 TLC Analysis of Fractions Obtained by Cellulose Column

Chromatography

Fractions from cellulose column chromatography were analyzed by TLC (Section 3.2.3). Samples (10 ul) from fractions (2.5 ml) were spotted on cellulose plates and chromatographed with butanol-acetic acid-water (4 : 1 : 2 by volume) as the solvent system. Plates were developed with ninhydrin.

A   Analysis of fractions from a cellulose column (15 g) loaded with recombined fractions (30 mg) from earlier column.

B   Analysis of fractions from a cellulose column (100 g) loaded with basic polyoxin fraction (98 mg).
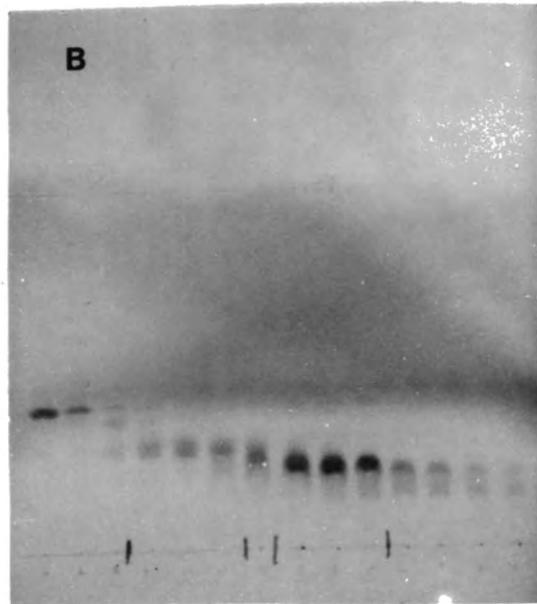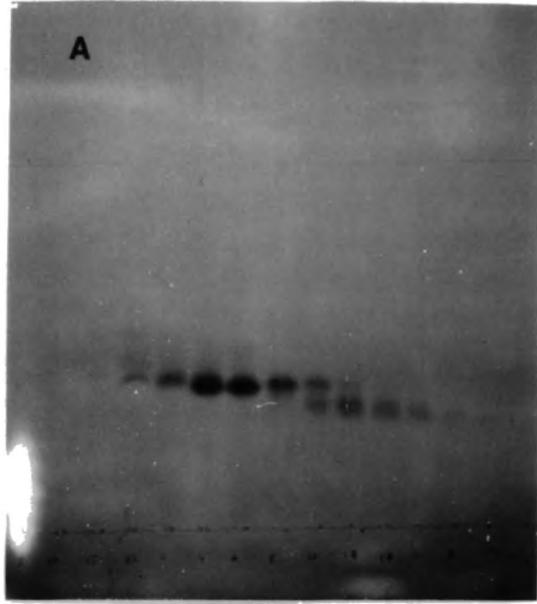
Positive identification of purified polyoxins by NMR spectrometry was unfortunately limited because of restricted access to the 400 MHz spectrometer. Physicochemical properties of polyoxin components were very similar (Isono et al., 1967), which coupled with the small quantities of isolated polyoxins, prevented positive identification (UV spectra and E_{max} values were very similar).

3.4 Concluding Discussion

The procedures described in the preceeding sections resulted in the isolation of some 7 mg of partially purified polyoxin complex (combined acidic and basic fractions) per g of crude agricultural fungicide (Polyoxin Z). The original Polyoxin Z was stated to contain 2% polyoxin complex, the nature of which was unspecified. On the assumption that this complex comprised solely of polyoxin components, a 35% yield was achieved by the described isolation procedures, which is reasonable when one considers the number of stages in this protocol.

Resolution of the basic polyoxin complex into individual components proved difficult, in part due to the number of components present in this fraction, (a maximum of 10 polyoxins will be present, if all the possible components are present) compounded by the fact that polyoxins have very similar structures and correspondingly very similar Rf values and physicochemical properties. Yields of individual components were low (2 - 27 mg), because very small amounts of basic polyoxin complex were available for resolution (upto 500 mg); whereas studies in the literature reported resolution of much larger quantities of basic polyoxin complex typically 5 g. (Isono et al., 1965; 1967). Resolution of such low quantities of polyoxin would probably be better achieved by reversed-phase high-performance liquid chromatography as recently described by Shenbagamurthi et al, (1982).

	Rf	Mass (mg)
Polyoxin A	0.17	27
Polyoxin B	0.09	4
Unknown 1	0.31	6
Unknown 2	0.14	2
Unknown 3	0.08	27
Unknown 4	0.04	27

TABLE 3.2 Table of Isolated Polyoxins

Polyoxins were isolated by cellulose column chromatography (Section 3.2.3), from the crude basic polyoxin complex.

Polyoxins A and B were formally identified by NMR and TLC, the lack of suitable standards precluded positive identification of other components.

Rf values derived by thin layer chromatography (Section 3.2.3) on cellulose plates with butanol : glacial acetic acid : water (4 : 1 : 2, by volume) as the solvent system.

Unfortunately, there was insufficient time to attempt to resolve the acidic polyoxin complex because one of the resins used in the isolation procedure (Dowex 50W-X16) proved difficult to procure and did not become available until towards the completion of this phase of the studies (carried out at I.C.I. Pharmaceuticals Division, Cheshire, UK). Future resolution of this complex should prove profitable as it only contains three components (polyoxins D, E and F)

Identification of resolved components proved difficult because of the lack of material for extensive chemical analysis of individual components (as reviewed by Isono et al. 1969). The lack of sufficient standards and similarity of physicochemical data precluded positive identification of most of the isolated polyoxin components. Identification by NMR spectrometry was limited because of restricted access to the spectrometer; it had been hoped that differences in the spectra due to differences in structure would be sufficient to identify positively individual components. The purified polyoxins A, B and D provided by Dr. K. Isono (Institute of Physical and Chemical Research, Wako-shi, Japan), were not of sufficient purity for NMR analysis - it has been hoped to obtain standard spectra from these samples for comparison (and identification) with spectra from polyoxin components isolated in these studies. Attempts to obtain NMR spectra for individual polyoxins from Dr. K. Isono were unsuccessful.

Thus the positive identification of individual polyoxin components was prevented by a combination of factors. However, the basic polyoxin fraction was shown to have equivalent toxicity to purified polyoxins towards intact cells (Section 5.3) and to be inhibitory to a chitin synthetase preparation (Section 3.7.3), permitting its use for mutant studies (Chapter 5).

ASSAY OF CHITIN SYNTHETASE AND INHIBITORY PROPERTIES OF ISOLATED POLYOXINS

3.5 Introduction

A particulate chitin synthetase preparation from Saccharomyces

cerevisiae can be isolated in an inactive zymogen form (Cabib, 1972),

and activated in vitro by incubation with trypsin. A particulate

chitin synthetase preparation from Candida albicans was used in these

studies to examine the inhibitory properties of isolated polyoxin

components. The following sections describe the preparation of a

chitin synthetase preparation which is stable with high specific and

total activities, and the estimation of kinetic parameters in the

presence and absence of inhibitors. The methods for preparation and

assay of chitin synthetase are based on a somewhat modified ICI

protocol, which in turn is based on the earlier work of Gooday and de

Roussett Hall (1975); de Roussett Hall and Gooday (1975); Adams and

Gooday (1980); and Brillinger (1980).

3 6 Methods

3.6.1 Growth and Harvesting of Organisms

Candida albicans strains A and B2630 were grown overnight in

Sab~~ur~~rauds Glucose medium (6 x 1 1) at 37°C, liquid cultures being

innoculated from agar slopes. Exponential phase cells were harvested

by centrifugation (2000 rpm, 15 min) and washed twice with distilled

water. Cells were resuspended in Trizma-HCl (50 mM) pH 7.5, $MgCl_2$ (10

mM), EDTA (1 mM) and sucrose (250 mM), before disruption

Cells were disintegrated using a Braun Disintegrator (for 4 x 1

min period at full speed) with glass beads (440 - 540 um diameter), the

suspension being continuously cooled at 4°C by vapourising liquid

~~nitrogen~~ CO_2. A particulate chitin synthetase preparation was obtained by

fractionation of the cell homogenate as described in Section 3.6.2.

3.6.2 Preparation of Chitin Synthetase

The cell homogenate obtained by Braun disintegration was fractionated as shown (Figure 3.5). The 108,000xg pellet (containing the chitin synthetase activity) was resuspended in Trizma-HCl pH 7.5 (50 mM, 15 - 20 ml), using a fine glass rod to disperse the pellet, and immediately frozen at -20° The activity was known to be stable for 3 - 6 months (Dr. K. Barrett-Bee, pers. commun.). When the cell homogenate volume is kept to a minimum (as above) the 108,000xg supernatant fraction can be used as a source of chitinase activity, this fraction also being stored at -20°C.

The protein concentration of the resuspend^{ed} 108,000xg pellet was estimated by the method of Lowry et al. (1951) using Lab Trol (DADE) as a standard. Protein concentrations were typically 10 - 12 mg protein ml.$^{-1}$.

3.6.3 Activation of Chitin Synthetase

The chitin synthetase prepared from Candida albicans is present as a zymogen which essentially has no activity. However, activation in vitro can be achieved by limited proteolysis which presumably takes place in vivo via an endogenous activator. The assay in vitro of chitin synthetase is achieved by activation by trypsin (see below), proteolysis being terminated by the addition of trypsin inhibitor.

Preincubate : Chitin synthetase zymogen + Trypsin + Assay buffer (Trizma, 63 mM., MgCl$_2$, 12.5 mM., EDTA, 1.75 mM), pH 8.5. 1 - 5 min.

Add : Trypsin inhibitor to stop activation.

Optimal concentrations for this activation step :

10 vol. Enz prep (10 - 12 mg ml^{-1}) + 1 vol. Trypsin (2 mg ml^{-1}) + 3 vol. Assay buffer, pH 8.5

preincubate at 37°C for 1 - 5 min

Add 1 vol. Soybean Trypsin Inhibitor (3 mg ml^{-1}).

Braun disintegrator

4 x 1 min

Cell homogenate

spin 5,000 xg   10 min

5,000 xg   pellet
⟶   discard

5,000 xg supernatant

spin 108,000 xg   60 min

108,000 xg pellet + Supernatant

resuspend in Trizma-HCl (15 - 20 ml, 50 mM) pH 7.5
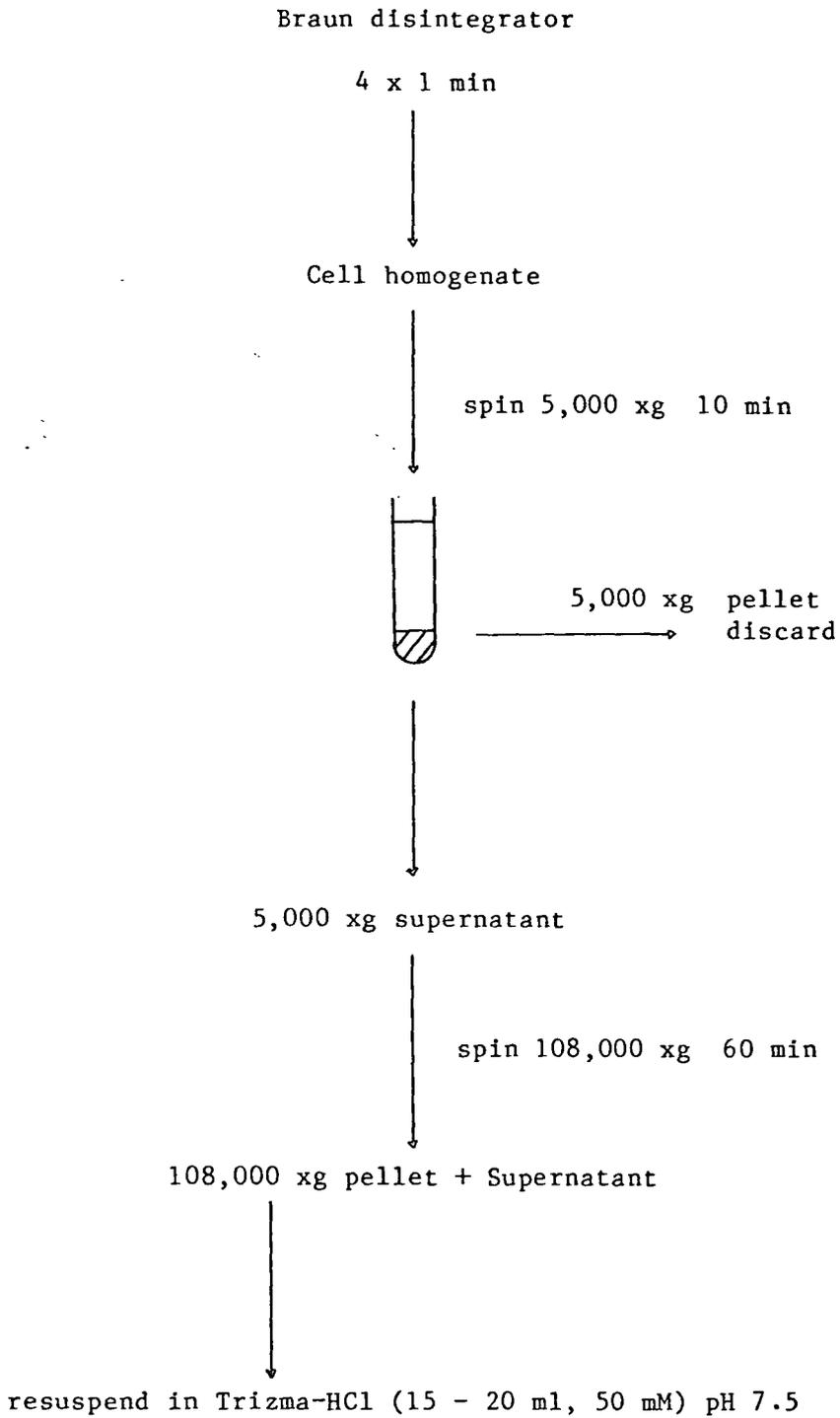
Figure 3.5   Chitin Synthetase Preparation - Fractionation of Cell

Homogenate

Optimal activation will vary with the protein concentration of each preparation, but the above preparation was known to be adequate for the 108,000xg fraction typically obtained from 6 x 1l. of culture medium (Dr. K. Barrett-Bee, pers. commun.). Maximum activation may be achieved by varying the period of incubation with trypsin and/or the trypsin concentration. The activated enzyme was generally used immediately, or alternatively, stored on ice till required.

Because of the presence of endogenous activator the zymogen-enzyme mixture as isolated has some activity (Fig 3.6), this intrinsic activity was estimated by dilution of the zymogen-enzyme preparation to the same extent as activated enzyme (i.e. 2 : 3 dilution); before assaying for chitin synthetase activity (Section 3.6.4).

3.6.4 Assay of Chitin Synthetase

Chitin synthetase activity was assayed by a somewhat modified method of Gooday and de Rousset-Hall (1975), using the following reagents –

10 ul enzyme preparation–zymogen

–activated

10 ul 1 : 3 v/v UDP-N-Acetyl-D-[U^{14}C]-Glucosamine : UDP-N-Acetyl-D-Glucosamine (10 – 50 mM), final substrate concentration 1.5 – 7 5 mM. Specific activity of stock UDP-NAG solutions (1.25 uCi ml.$^{-1}$).

25 ul Trizma-HCl (63 mM), MgCl$_2$ (12.5 mM), EDTA (1.75 mM). pH 8.5

5 ul Test compound dissolved in distilled water

Assays (50 ul final vol.) were performed in plastic Eppendorf centrifuge tubes (1.5 ml). Reactions were initiated by addition of enzyme (both enzyme and reagents were pre-equilibrated at 37°C) and continued at 37°C for 20 – 90 min. Reactions were terminated by
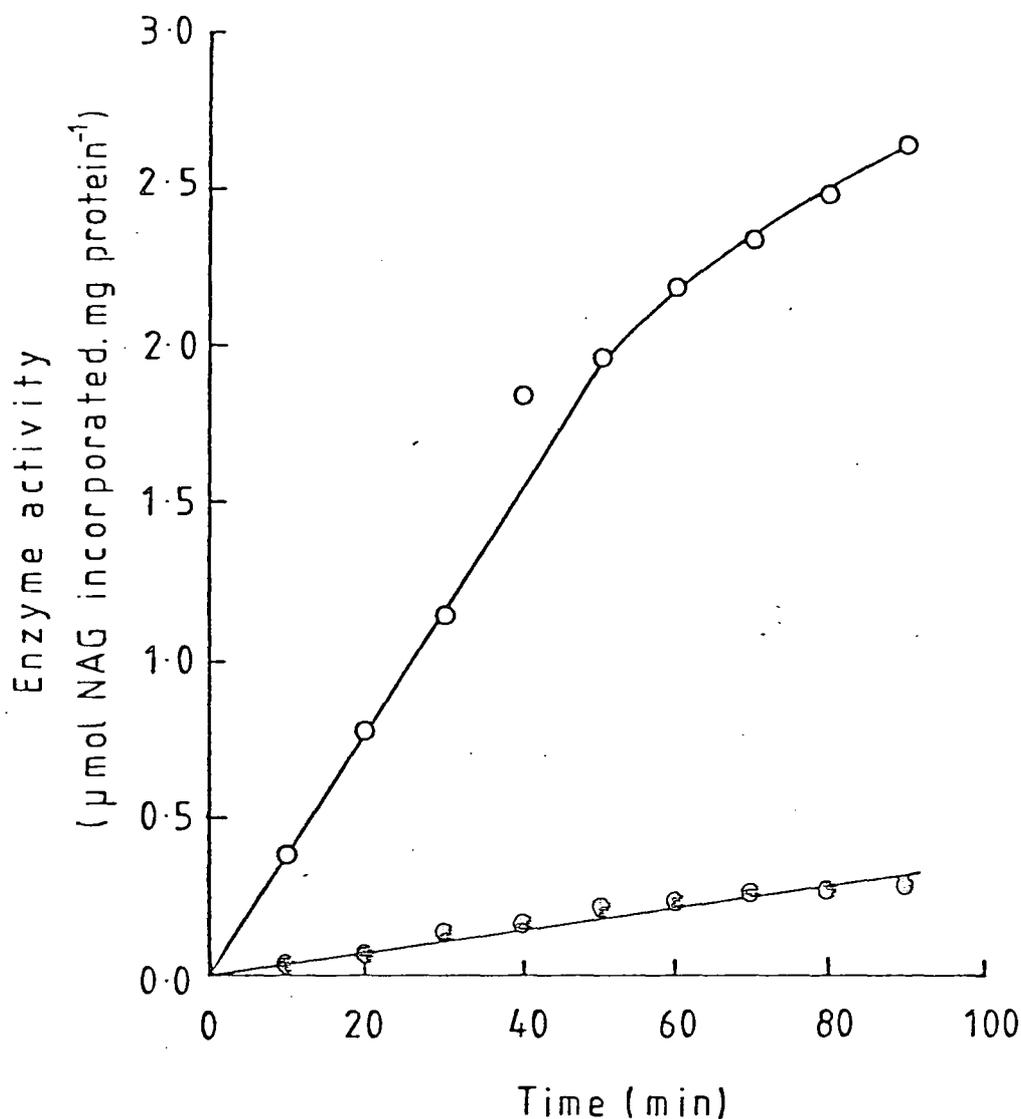
Figure 3.6   Activation of Chitin Synthetase – Comparison of

Activities of Zymogen and Activated Enzyme

Chitin synthetase zymogen (0.4 ml) was activated as described (Section

3.6.4) or diluted appropriately (0.4 ml zymogen + 0.12 ml assay buffer,

pH 8.5 + 80 ul distilled water), the zymogen was incubated with trypsin

for 5 min, before addition of soybean trypsin inhibitor.   The

activities of the zymogen ($\circledcirc$) and activated enzyme ($\circ$) were measured

(Section 3.6.4), enzyme activity being quoted as umol NAG incorporated

mg protein^{-1}.   Initial substrate concentration was 7.5 mM   (1.25 uCi

ml^{-1}).

addition of trichloracetic acid (TCA) (0.9 ml, 10% w/v). Insoluble

products were precipitated by incubation at 4°C for 1 h.

The radioactively-labelled substrate, which becomes incorporated

into macromolecular chitin, was separated from non-incorporated label

by filtration on Whatman GF/F filters (25 mm diam). Filters were

presoaked in TCA (5% w/v)/sodium pyrophosphate (20 mM), to minimise the

amount of radioactively-labelled substrate non-specifically adsorbed

during the filtration procedure. Contents of the reaction tubes were

washed onto filters, and then the filters were washed with distilled

water (15 - 20 ml). Filters were transferred to liquid scintillation

vials and PCS liquid scintillation cocktail (4 ml) added. Liquid

scintillation counting was performed on a Packard-Prias Tri-Carb (Model

PL/PLD).

The specific activity of the stock UDP-NAG solutions (12.5 - 37.5

mM) was 1.25 uCi ml.$^{-1}$. Thus, the total counts added to each sample

vial can be directly equated with the molarity of the substrate and

therefore used to calculate the velocity of the chitin synthetase

activity. Results are quoted as nmol NAG incorporated min.$^{-1}$ mg

protein^{-1}. Control values for the original enzyme-zymogen samples

allowed calculation of the extent of activation achieved by trypsin

digestion.

All experimental sampling points were performed in triplicate,

and the average value for incorporation of radioactively-labelled

substrate into newly synthesised chitin calculated for each time point.

A series of control experiments was performed : there was no

incorporation of radioactively-labelled substrate in the absence of

enzyme or in the presence of boiled enzyme; nor was there non-specific

adsorption of radioactively-labelled substrate on the presoaked

filters, and there was minimal radioactivity remaining in tubes after the described washing procedure.

3.7 Results

3.7.1 Activation of Chitin Synthetase

The enzyme-zymogen preparation (10 - 12 mg protein ml.$^{-1}$) was activated as described (Section 3.6.3) and assayed for chitin synthetase activity (Section 3.6.4) relative to the untreated control.

Typically, activation of the enzyme-zymogen mixture resulted in an eleven fold increase in the initial rate of substrate incorporation (Fig. 3.6) relative to the untreated control. Initial rates of incorporation were 40 nmol min^{-1} mg protein^{-1} for the activated enzyme and 3.6 nmol min.$^{-1}$ mg protein^{-1} for the untreated preparation. Incorporation of substrate was linear for approximately 50 min. for the activated enzyme (initial substrate concentration, 7.5 mM), under the assay conditions described (Section 3.6.4).

3.7.2 Kinetic Parameters for Chitin Synthetase

The affinity of the substrate UDPNAG for an activated enzyme preparation from Candida albicans strain A was examined

The enzyme-zymogen preparation was activated as described (Section 3.6.3) and incubated with radioactively-labelled substrate (1.5 - 7.5 mM) for 20 min. The following kinetic parameters were determined using a Lineweaver-Burke plot Km was 4.9 +/- 0.8 mM, Vmax was 42 +/- 8 nmol.min^{-1} mg protein^{-1}, (quoted values being the average of four determinations, calculated for the same enzyme preparation). A typical Lineweaver-Burke plot is shown (Fig. 3.7).

A control experiment (Fig. 3.8) demonstrated that uptake was linear for upto 20 min at the lowest substrate concentration (1.5 mM),
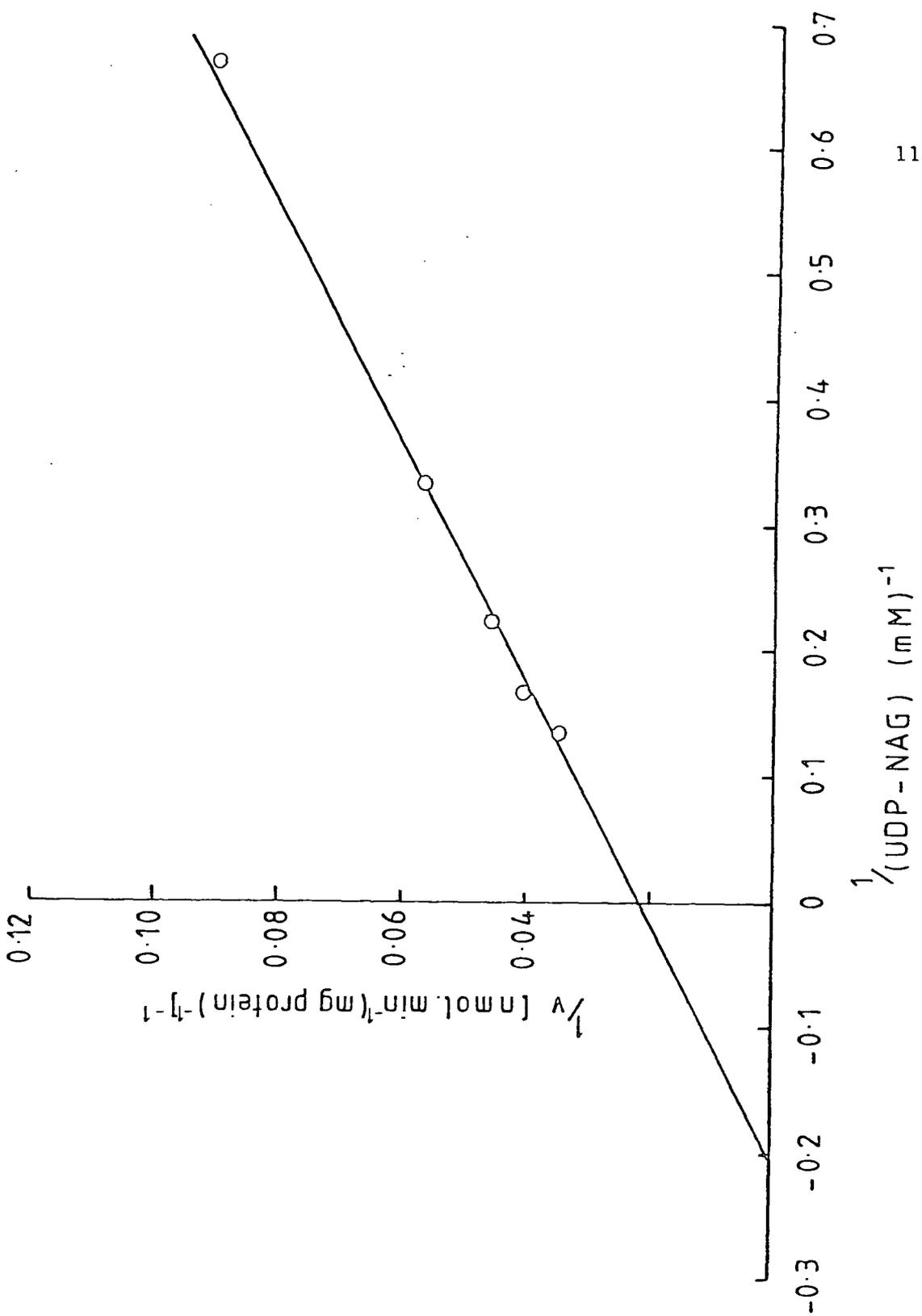
Figure 3.7   Lineweaver–Burke Plot of Chitin Synthetase Activity

Chitin synthetase was activated as described (Section 3.6.3) and enzyme activity assayed (Section 3.6.4), initial rates of NAG incorporation being measured for initial substrate concentrations in the range 1.5 – 7.5 mM.
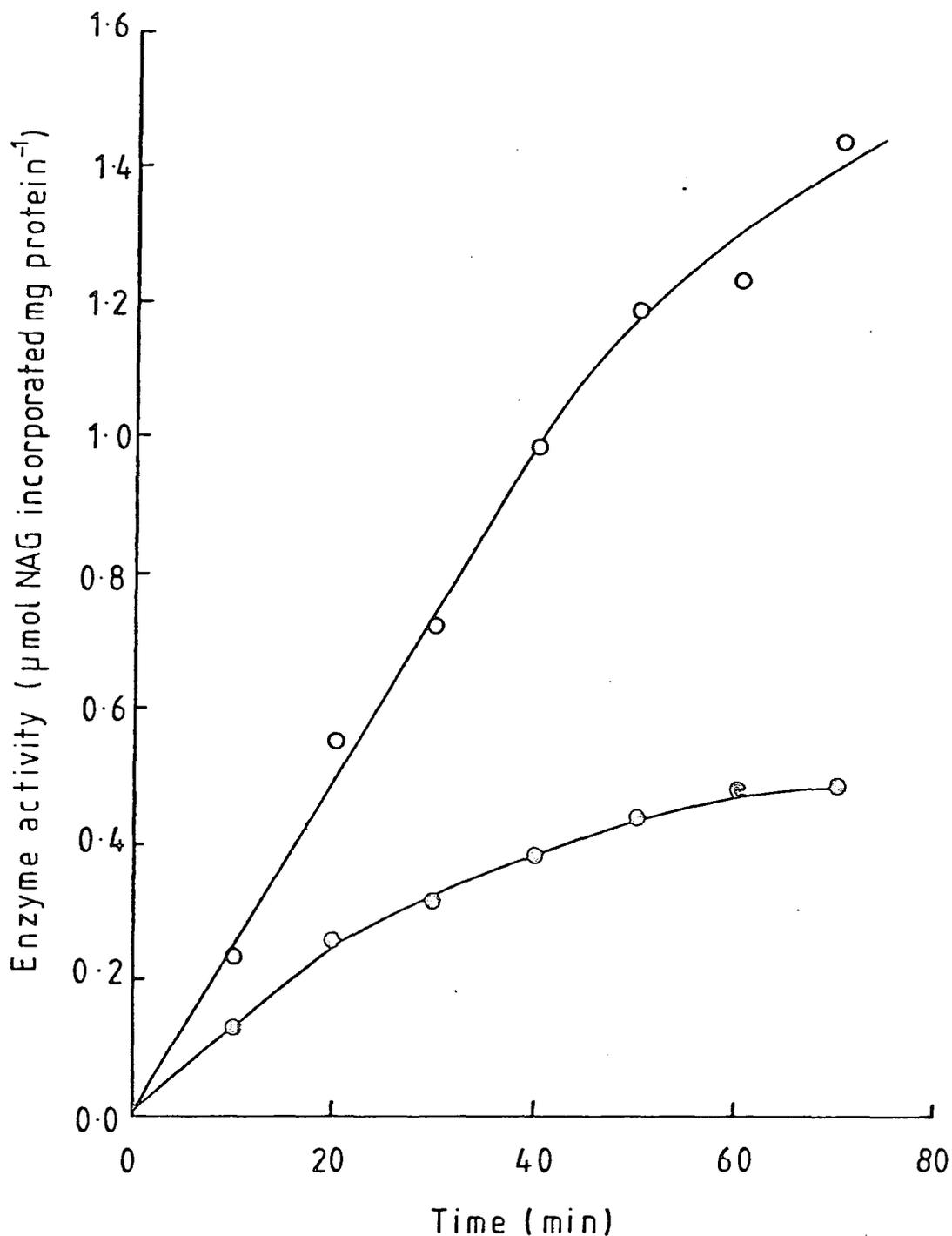
Figure 3.8 Incorporation of NAG into Chitin at Different Initial

UDP-NAG Concentrations

Chitin synthetase was activated as described (Section 3.6.3) and enzyme

activity assayed at 1.5 (O) and 7.5 (O) mM initial UDP-NAG

concentrations (Section 3.6.4).

i.e. that incorporation was proceeding at its maximal rate over the 20 min incubation period used in the preceeding experiment, a condition essential for the accurate estimation of kinetic parameters.

3.7.3 Inhibition of Chitin Synthetase Activity by Polyoxins

Purified polyoxin components isolated from the crude Polyoxin Z mixture (Section 3.3) were tested for inhibitory activity against a chitin synthetase preparation from Candida albicans strain A.

For unidentified polyoxin components, an average molecular weight of 550 - 610 was assumed in order to prepare stock solutions (approx. 1 mM). The inhibitory effects of various polyoxin concentrations (1 - 50 uM) at different initial substrate concentrations (1.5 - 7.5 mM) on chitin synthetase activity were examined by the standard method (Section 3.6.4). Kinetic parameters for inhibition (Ki values) were determined by using a Dixon plot (e.g. Fig. 3.9), and the mode of inhibition derived from a Lineweaver-Burke plot (e.g. Fig. 3.10). Ki values for the various isolated polyoxins are given (Table 3.3).

The results indicated that the polyoxins are competitive inhibitors of chitin synthetase from Candida albicans strain A.

3.8 Concluding Discussion

The particulate chitin synthetase preparation described in the preceeding sections exhibited values for kinetic parameters typical of other literature reports for similar preparations from Candida albicans. Similarly, Ki values for inhibition of this preparation by isolated polyoxins were in agreement with previously published figures (see Gooday, 1979). This justified the use of such a preparation for measuring the inhibition of chitin synthetase activity by polyoxins
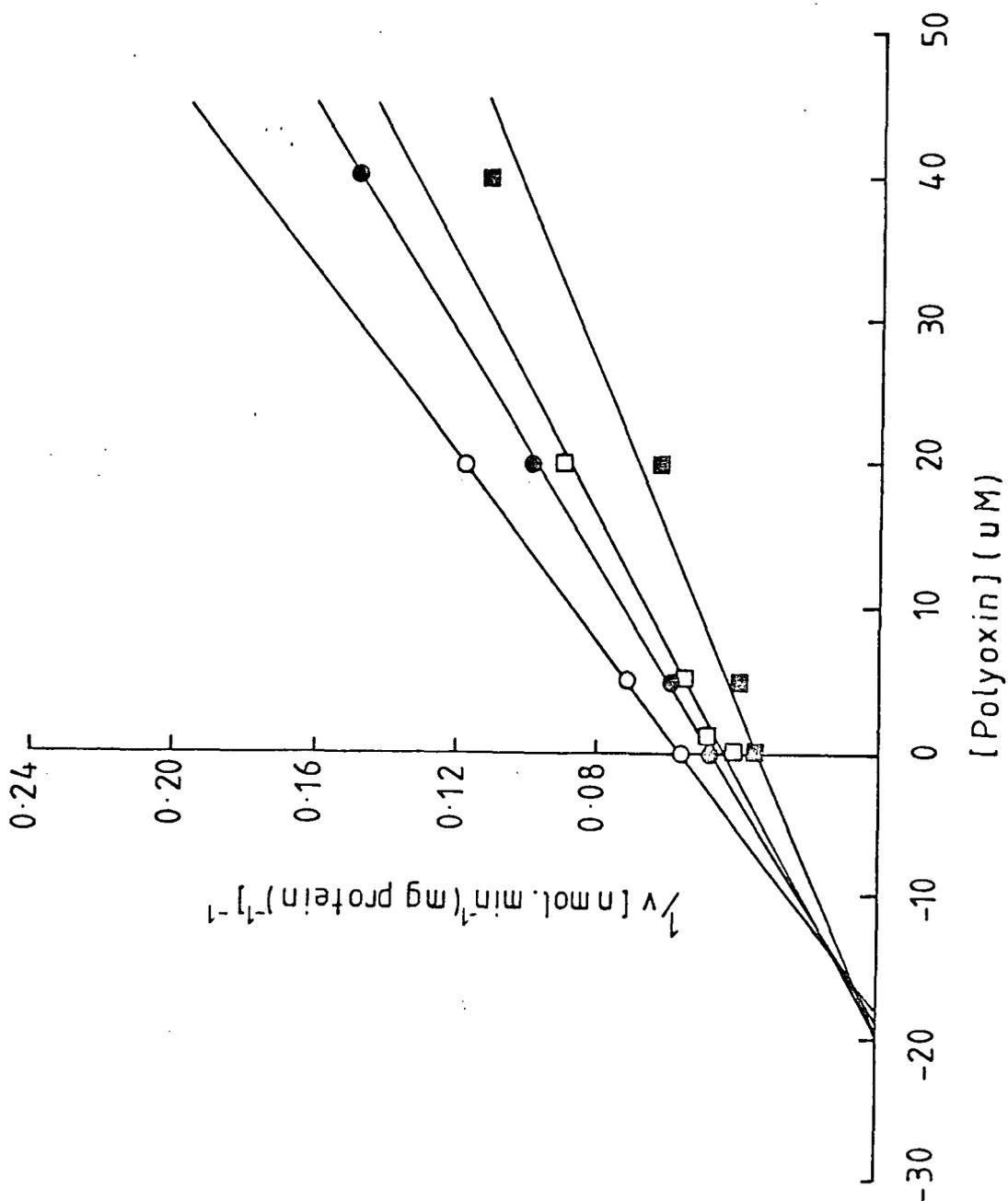
Figure 3.9 Dixon Plot for Inhibition of Chitin Synthetase by

Unknown Polyoxin I

Dixon plot of the velocity against concentration of unknown Polyoxin I

(Section 3.3.6).

Initial substrate concentrations (UDP-NAG) are (○), 3 mM; (○), 4.5

mM; (□), 6 mM and (▧), 7.5 mM.
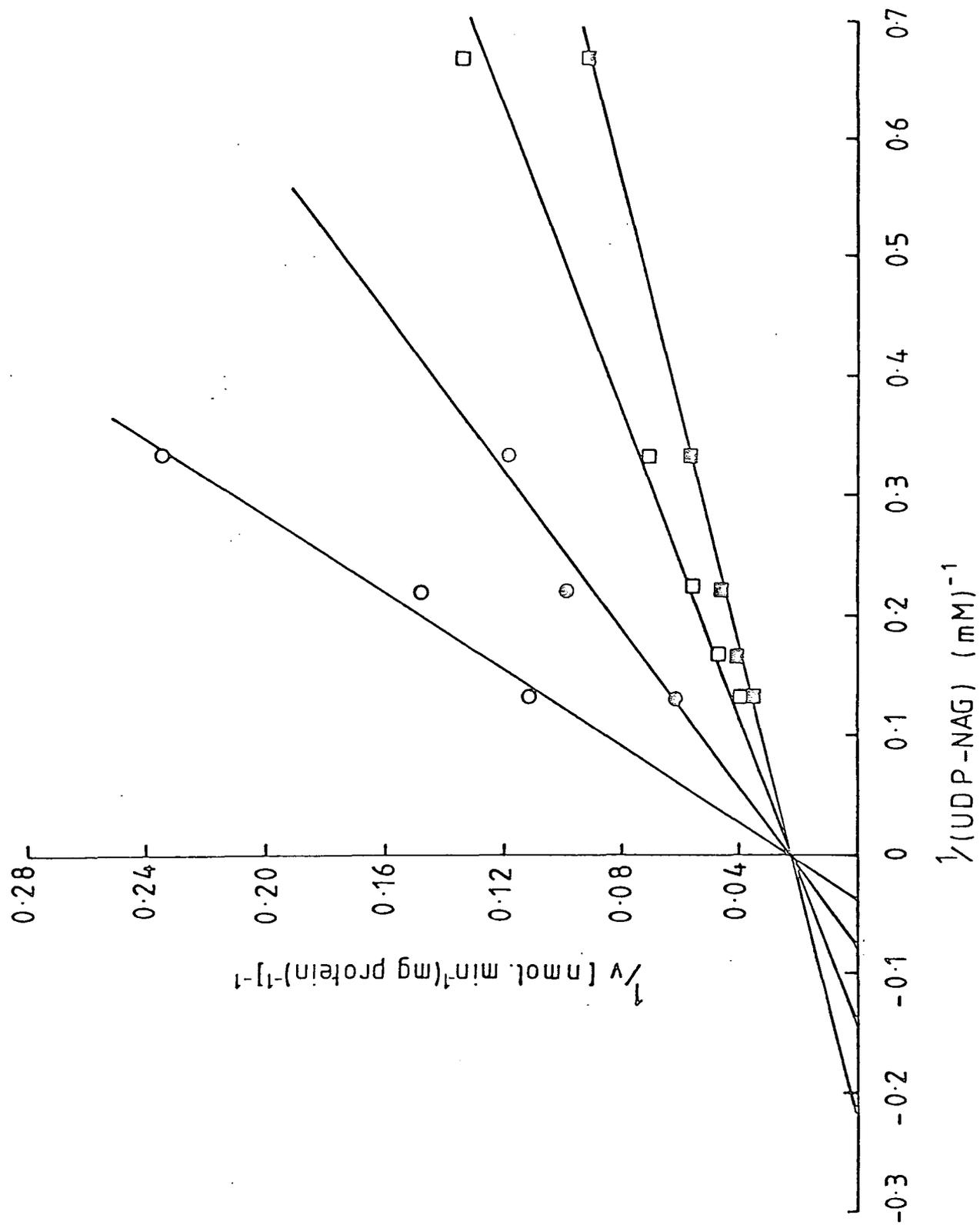
Figure 3.10   Lineweaver-Burke Plot for Inhibition of Chitin

Synthetase by Unknown Polyoxin I

Lineweaver-Burke plot of the velocity against substrate concentration in the presence of Unknown Polyoxin I (Section 3.3.6). Polyoxin concentrations are (⊟), 0 uM; (□), 5 uM; (◦), 20 uM; and (○), 40 uM.

	K_i (uM)
Polyoxin A	9
Polyoxin B	24
Unknown Polyoxin 1	15
Unknown Polyoxin 3	9
Basic Polyoxin Fraction	21

TABLE 3.3 K_i Values for Inhibition of Chitin Synthetase from

Candida albicans strain A

The inhibition of a chitin synthetase preparation from Candida albicans strain A by isolated polyoxins (Section 3.3.6) was measured as described (Sections 3.7.1, 3.7.3).

(Section 3.7.3) and for comparison of chitin synthetase activities of wild-type strains with polyoxin- and nikkomycin-resistant mutants (Section 5.5.7).

Further purification of chitin synthetase and/or solubilisation to obtain higher specific activities was not considered necessary, the measured activity providing sufficient sensitivity for these studies. The isolation procedure described here has the advantage of being rapid and provides a preparation with high total activity, which can be stored for a period of upto six months, enabling several assays to be performed with the same enzyme preparation. The specific activity of this preparation is equivalent to that of a preparation from Coprinus cinereus (Adams & Gooday, 1980), which was also used to screen analogues of the substrate UDP-NAG for inhibition of the enzyme (Adams & Gooday, 1983).

CHAPTER 4

PEPTIDE TRANSPORT AND ITS REGULATION IN CANDIDA ALBICANS

CHAPTER 4   PEPTIDE TRANSPORT AND ITS REGULATION IN CANDIDA ALBICANS

4.1   Introduction

Peptide transport in Candida albicans has received some preliminary investigation (Lichliter et al., 1976;   Logan et al., 1979; Davies, 1980) but many aspects are still poorly understood, in part arising from misinterpretation of results obtained from using radioactively-labelled substrates, which can give erroneous estimates of peptide transport kinetic parameters (Payne & Nisbet, 1980b; Nisbet, 1980).

In this study, the combination of fluorescence and radioactively-labelled substrate assays has led to a deeper understanding of the transport systems in this yeast, in terms of mechanism and specificity of solute uptake, together with a detailed evaluation of kinetic parameters.   Such information has applications in the rational design of antifungal agents, which use peptide transport systems for their facilitated uptake.

4.2   Methods

4.2.1   Media and Growth Conditions

Candida albicans and Sacc. cerevisiae (Section 2.8.2.1) were routinely grown in liquid Pro-Medium (Section 2.8.2.2), cultures being maintained on Pro-Medium plates throughout.   Alternatively, organisms were grown in a Peptone-based liquid medium containing glucose (2%, w/v), Yeast Nitrogen Base (without amino acids and ammonium sulphate; Difco 0335-15) in which Bacto-Peptone (2%, w/v;   B.D.H., Poole, Dorset) was the sole nitrogen source (referred to as Pep-Medium).

Cultures were transferred from Pro-Medium plates to Pro-Medium, grown overnight at 28°C, with shaking.   Cells were kept in liquid Pro-

Medium at $4^{o}C$ for a maximum of 4 weeks and subcultured from this stock as required. Cells for transport assays were routinely grown in 50 ml batches

Growth in liquid media was monitored by measuring the absorbance of a portion of the overnight culture (4 - 5 ml) in a Bausch and Lomb Spectronic 20 spectrophotometer (660 nm, 1 cm diameter tube). It was shown that there was a linear relationship between A_{660} and cell density upto an A_{660} of 0.8. Determinations of dry weight and viable count showed that an A_{660} of 1.16 was equivalent to $1(\text{mg dry wt})\text{ml}^{-1}$ and 8×10^{7} colony forming units ml^{-1}. Alternatively, a Pye Unicam SP 1800 spectrophotometer was used for routine O.D. measurements, a cell density of $1(\text{mg dry wt.})\text{ml}^{-1}$ corresponding to an A_{660} of 1.7.

Growth curves in both Pro- and Pep-Media were obtained. These yielded a generation time of 80 min on Pep-Medium, and 160 min on Pro-Medium; the faster growth on Pep-Medium being attributed to the better quality of Pep-Medium as a sole nitrogen source.

4.2.2 Monitoring Peptide in Medium

Cultures were grown routinely at $28^{o}C$ in liquid Pro- or Pep-Media to a cell density of 0.2 - 0.7 (mg dry wt.) ml^{-1}. Organisms were harvested by membrane filtration (47 mm diameter, 0.45 um pore size, Oxoid), washed on the filter with disodium hydrogen orthophosphate-citric acid, pH 4.5 (2 x 20 ml, 20 mM with respect to phosphate, referred to as phosphate-citrate buffer) at room temperature, and resuspended in the same buffer so that a ten-fold dilution of the suspension gives a cell density of 0.2 - 0.7 (mg dry wt.)ml^{-1}.

Portions of this suspension were diluted two-fold with a glucose solution (1.6%, w/v) to give a final buffer concentration of 10 mM

phosphate and a glucose concentration of 0.8% w/v (referred to as phosphate-citrate-glucose or PCG buffer). Final volumes for uptake assays were 1 - 4 ml.

Uptake assays were carried out at 28°C, cells being maintained in suspension by a small magnetic stirrer bar. After a 10 min pre-incubation, peptide was added (from a 10 mM stock solution in sterile, distilled water) to a concentration of 0.1 - 0.5 mM (typically 0.1 mM for cells grown in Pro-Medium, 0.2 or 0.5 mM for cells grown in Pep-Medium). Samples (0.3 ml) were taken at appropriate time intervals (routinely every 5 min for 25 min) using a 1 ml syringe or automatic pipette, and immediately freed from cells by passing them through a membrane filter (13 mm diameter, 0.45 um pore size, Oxoid) using a Swinnex (Millipore) filter holder. To economise on the use of these expensive filters, filters (13 mm diameter) were cut from sheets of filter paper (Whatman GF/C) using a specially prepared punch. Filtrates were collected in Eppendorf centrifuge tubes, and stored at -20°C.

Monitoring the peptide concentration of the medium by using the manual fluorescamine assay, was performed on 50 - 100 ul aliquots of the filtrates, according to the standard method (Section 2.5.2). Examination of the samples of incubation medium by dansylation was performed on 100 ul of the filtrates, treated by the standard method (Section 2.4.2).

4.2.3 Examination of Cell Extracts

Harvesting and incubation with peptide was performed as in Section 4.2.2. After incubation with peptide, a 1 ml sample of the yeast suspension was harvested on a membrane filter under water vacuum, immediately washed with distilled water (40 ml, room temperature) and

extracted in water (1 ml) in a stoppered tube for 15 min in a boiling water bath. The boiled suspension was cooled, passed through a membrane filter to remove insoluble cellular debris, and the filtrate stored at $-20^{\circ}C$. Portions (100 ul) of the filtrate were dansylated by the standard procedure (Section 2.4.2).

4.3 Characterization of Peptide Transport in Candida albicans

4.3.1 Effect of glucose concentration on Peptide Uptake

The rate of peptide (Leu-Leu) uptake in Sacc. cerevisiae was shown to be dependant on the glucose concentration in the incubation medium (Nisbet, 1980). There was little uptake in the absence of glucose, uptake rates increasing upto a glucose concentration of 0.4% (w/v). Nisbet subsequently used a glucose concentration of 0.8% (w/v), a compromise between the need for excess glucose in the incubation and the interference in the running of chromatograms of dansyl derivatives if too much glucose is present.

The effect of glucose concentration on the rate of peptide uptake in Candida albicans B2630 was examined by manual fluorescamine assay of samples of medium in which cells were incubated with different glucose concentrations (0 - 0.8% w/v). Cells were harvested and resuspended in phosphate-citrate buffer, pH 4.5, as previously described (Section 4.2.2), and preincubated for 10 min before addition of peptide. Peptide uptake (over a 25 min period) was assayed by the standard manual fluorescamine procedure (Section 2.5.2). Thus, cells were starved or incubated in the presence of different concentrations of glucose for a 35 min assay period.

Uptake of Leu-Leu and Ala-Ala by cells grown in either Pro- or Pep-Medium, showed no dependence on the glucose concentration of the incubation medium (Table 4.1).

Rate of Uptake (nmol min^{-1}(mg dry wt)$^{-1}$)

Glucose Conc. (% w/v)	A, Ala-Ala	A, Leu-Leu	B,Ala-Ala
0	1.52	0.73	13.6
0.2	1.67	0.82	13.2
0.4	1.87	0.56	14.3
0 8	1.66	0.62	13.3

TABLE 4.1   The Effect of Glucose Concentration on Peptide Uptake

in Candida albicans B2630

The effect of glucose concentration on the uptake of peptides by Candida albicans B2630 grown either in Pro- (A, 0.1 mm) or Pep-Medium (B, 0.5 mM). Uptake was measured by the standard manual fluorescamine procedure (Section 2.5.2).

A glucose concentration of 0.8% (w/v) was used in subsequent experiments, to ensure that glucose did not become limiting in those experiments in which high cell densities or long incubation periods were used.

The demonstration that peptide transport in Candida albicans is not dependent on external glucose concentration presumably reflects the presence of adequate endogenous energy reserves to drive substrate uptake during these relatively short incubation periods.

4.3.2 Effect of Nitrogen Source Used for Growth on Peptide Uptake

Candida albicans strains B2630, A and 6406 were grown overnight in simple defined media (glucose, 2% w/v., Yeast Nitrogen Base without amino acids or ammonium sulphate, 1.7 mg ml^{-1}), with the following sole nitrogen sources : ammonium sulphate (0.5% w/v), proline (4 mg ml^{-1}) or Bacto-Peptone (2%, w/v). Cells were harvested and resuspended in incubation buffer as described (Section 4.2.2), and rates of Ala-Ala and Leu-Leu transport were measured using the manual fluorescamine assay (Table 4.2).

A nitrogen regulation effect was demonstrated, together with a stimulatory effect on peptide transport by growth on peptone.

4.3.3 Uptake of Peptide is Not Accompanied by Amino Acid Exodus

In preliminary studies, the uptake of several dipeptides by Candida albicans strains B2630 and A, was followed by using the dansyl chloride assay (Section 2.4.2). Cells were grown in Pro-Medium overnight, harvested, resuspended and incubated with peptide as previously described (Section 4.2.2). Uptake of peptide (0.1 mM) was monitored by dansylation of samples of medium during 30 min

| | | Growth Medium | | |
Strain	Substrate	NH_4-	Pro-	Pep-
B2630	Ala-Ala	N.D.	1.25	3.8
B2630	Leu-Leu	N.D.	0.7	2
A	Ala-Ala	-	3.3	9.7*
6406	Ala-Ala	-	3.05	19*

TABLE 4.2   Effect of Nitrogen Source on Peptide Transport

in Candida albicans

Candida albicans strains B2630, A and 6406 were grown overnight in

NH_4-, Pro- or Pep-Medium (ammonium sulphate, 0.5% w/v., L-proline, 4

mg ml^{-1}., Bacto-Peptone, 2% w/v). Cells were harvested, washed and

resuspended in incubation buffer (Section 4.2.2). Rates of uptake of

peptide (0.1 mM) were determined by fluorescamine analysis of samples

of medium, during 30 min incubations.

(N.D. not detectable *, initial substrate concentration 0.2 mM).

Uptake rates are given as nmol min.$^{-1}$(mg dry wt)$^{-1}$

-, not determined

incubations, transport rates being presented elsewhere (Section 4.3.9). In no case was there any evidence of exodus of constituent amino acids of the peptide taken up.

Cell extracts taken from cells preincubated with peptide were shown to have increased intracellular pools of the constituent amino acids. Cells were grown in Pro-Medium overnight, harvested and resuspended in incubation buffer as described (Section 4.2.2). Cells were incubated with peptide (Ala-Ala or Leu-Leu, 0.1 mM) for 30 min, before samples (1 ml) were taken for preparation of cell extracts (Section 4.2.3), and examination using the dansyl chloride assay. Accumulation of constituent amino acids was demonstrated, relative to controls preincubated without peptide, though the increase in intracellular amino acid concentration was not quantified.

Thus, incubation with peptide leads to an increase in the intracellular amino acid pools, and not to an exodus of constituent amino acids. Regrettably, no photographic record was made of these results, because this work was performed as part of an initial study and was not repeated at a later date.

4.3.4   Effect of pH on Peptide Transport Activity

Peptide transport in yeast shows a marked pH dependence, a pH optima for Candida albicans of 3.5 (Logan et al., 1979) and 4.5 (Davies, 1980) having been reported.

Overnight growth of Candida albicans B2630 in Pro-Medium (pH 4.5) to late exponential phase (0.6 (mg dry wt) ml^{-1}) resulted in a decrease in pH to 3.5. Similarly, overnight growth in Pep-Medium (pH 3.7) resulted in a pH decrease to 3.4. Rates of Ala-Ala uptake by strain B2630 grown overnight in Pep-Medium, were determined in a series of PCG

buffers at differing pH (Table 4.3), maximal uptake being at pH 4.5.
Preincubation of cells at pH 7 for 40 min had no effect on the rate of
Ala-Ala uptake, relative to a control batch preincubated for the same
period at pH 4.5 (Section 6.2.1).

Thus, all peptide transport studies were performed at pH 4.5
throughout the course of this study.

4.3.5 Effect of Cell Density at Harvesting on Peptide Transport Activity

The rate of Ala-Ala uptake by strain B2630 was not dependant on
the density at which the organisms were routinely harvested, over the
range 0.3 - 0.8 (mg dry wt)ml^{-1}, both for cells grown in Pro- or Pep-
Medium (Fig 4.1).

4.3.6 Variation in Transport Rates in Different Cell Batches

Some variability in peptide transport rates was detected for
batches of cells grown and harvested on different occasions (Fig 4.2).
Uptake rates do not correlate with the stage of exponential growth at
which cells were harvested (Section 4.3.5).

Similar variations in measured rate were noted in Sacc.
cerevisiae (Becker & Naider, 1977; Nisbet, 1980). Because of this
variability, the rate of uptake of a peptide was always determined
relative to the rate of Ala-Ala uptake in cells grown and harvested in
the same batch. As peptide transport is subject to nitrogen regulation
(Section 4.3.2), the observed variations in rate according to the batch
of cells harvested, may possibly be ascribed to variations in intra-
and extra-cellular levels of nitrogenous metabolites

pH	Rate of Ala-Ala Uptake(nmol.min.$^{-1}$(mg dry wt.)$^{-1}$)
3.6	4.6
4.5	10.8
5.2	6

TABLE 4.3   Effect of pH on Ala-Ala Uptake

Candida albicans B2630 was grown in Pep-Medium overnight, harvested and resuspended in phosphate-citrate-glucose assay buffer (Section 4.2.2), at differing pH. Uptake of Ala-Ala (0.5 mM) was measured by the standard manual fluorescamine assay (Section 2.5.2).
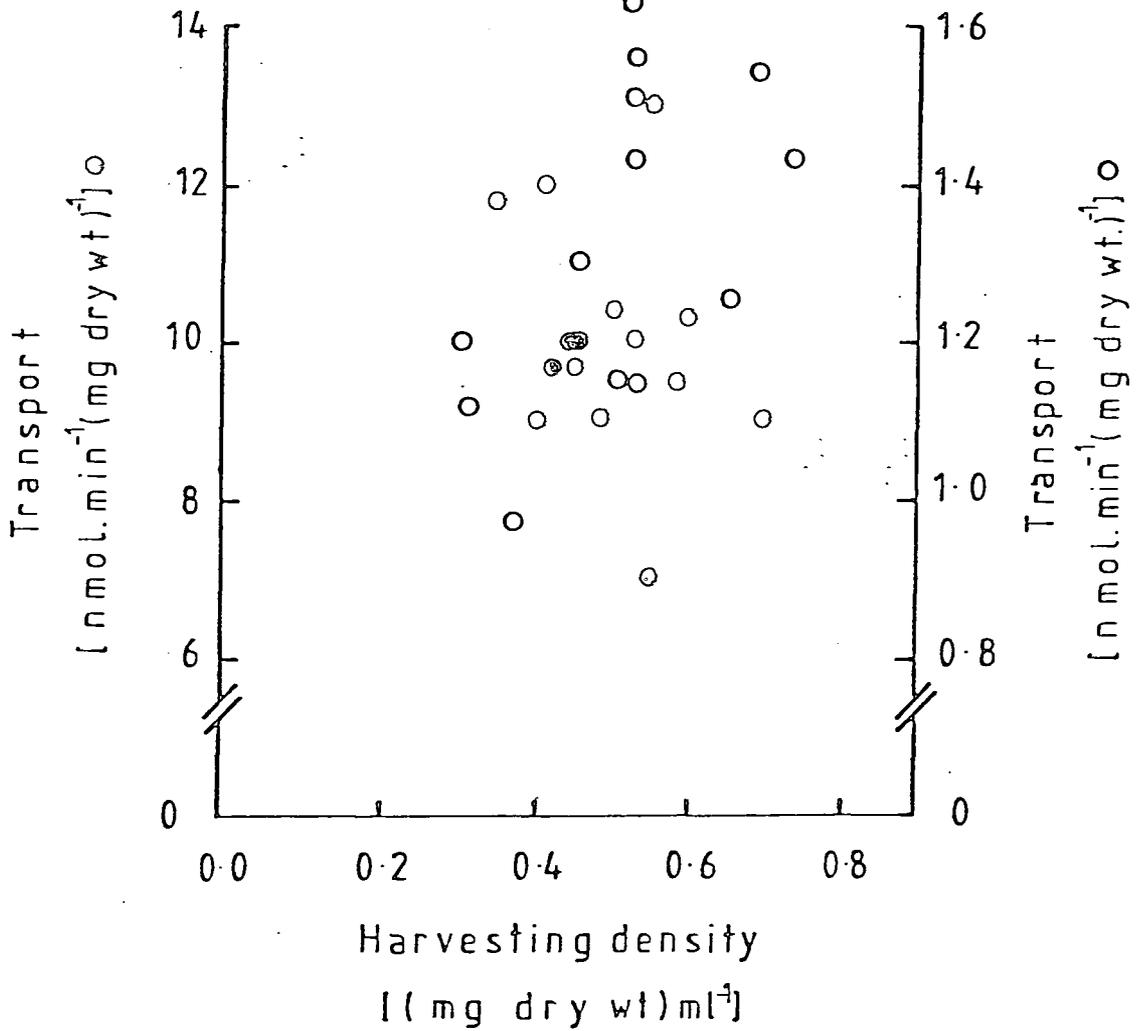
Figure 4.1 Effect of Cell Density at Harvesting on Ala-Ala Uptake

Uptake of Ala-Ala by Candida albicans strain B2630 grown in Pro- (○) and Pep-Medium (○), initial substrate concentration being 0.1 and 0.5 mM respectively.
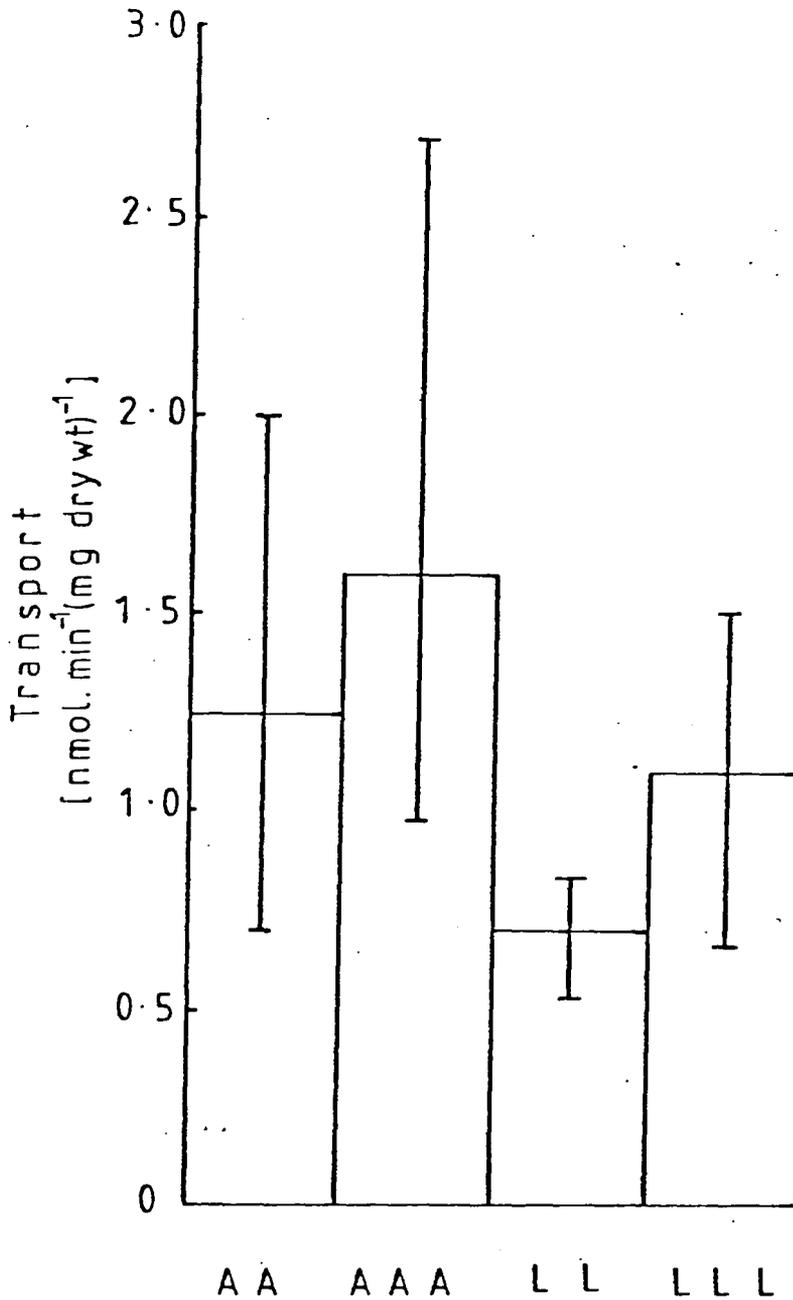
Figure 4.2 Variation in Transport Rates in Different Cell Batches

Candida albicans strain B2630 was grown in Pro-Medium overnight,
harvested and resuspended in incubation buffer (Section 4.2.2). Uptake
of peptide (0.1 mM) was followed by fluorescamine analyses of samples
of medium for up to a 30 min incubation.

The plot shows the mean and range for at least 6 determinations.

AA,   Ala-Ala; AAA, Ala-Ala-Ala; LL, Leu-Leu; LLL, Leu-Leu-Leu

4.3.7 Accumulation of Glycyl-Sarcosine by Strain A : Evidence for

Intact Uptake

The intact accumulation of peptides has been demonstrated in

Sacc. cerevisiae (Nisbet & Payne, 1979a; Nisbet, 1980), by dansylation

of cell extracts following incubation of yeast cells with sarcosine

peptides. The peptide Glycyl-Sarcosine (Gly-Sar) was used in this

study to demonstrate intact uptake in strain A, the first demonstration

of intact accumulation in Candida albicans,

Strain A was grown in Pro-Medium overnight, harvested and

resuspended in incubation buffer (1.5 - 2 (mg dry wt) ml^{-1}). Cells

were incubated with Gly-Sar (5 mM) at 28°C for 1 h, periodic samples (1

ml) being taken for preparation of cell extracts (Section 4.2.3), and

examination by the dansyl chloride assay (Section 2.4.2). Accumulation

of Gly-Sar was demonstrated (Fig 4.3), no free sarcosine was detected

in extracts, implying that the rate of intracellular hydrolysis of Gly-

Sar was very low.

In order to calculate the intracellular concentration of

accumulated Gly-Sar, an intracellular volume of 1.5 ul (mg dry wt)$^{-1}$

was taken. This volume calculated for Sacc. cerevisiae by Nisbet

(1980) was based on literature values for packed cell volume (Arnold,

1973) and protoplast volume (Arnold & Lacy, 1977), was extended for

calculations with Candida albicans. Although this figure is an

approximation, it is unlikely to invalidate conclusions with regard to

peptide accumulation. An intracellular concentration of 18 mM was

calculated (relative to an external concentration of 5 mM), for cells

incubated with Gly-Sar for 1 h, thus, active accumulation was clearly

demonstrated.

Figure 4.3 Chromatograms of Dansylated Derivatives from

Cell Extracts of Candida albicans strain A

Candida albicans strain A was grown in Pro-Medium overnight, harvested

and resuspended in PCG buffer (1.5 (mg. dry wt.)ml^{-1}). Cells were

incubated with Gly-Sar (5 mM) for upto 1 h at 28°C, cell extracts were

prepared (Section 4.2.3) and dansylated as described (Section 2.4.2).

A   Extracts prepared after a 30 min incubation.

B   Extracts prepared after a 60 min incubation.

C   Key to derivatives.   A, alanine;   D, aspartic acid;

E, glutamic acid;   G, glycine;   K, lysine;

P, proline;   S, serine;   T, threonine;   Orn, ornithine;

Dns-NH$_2$, dansyl ammonia;   Dns-OH, dansyl hydroxide;

X, origin

A B C

C panel labels:
2,3
1
K
Orn
P
V
Dns-NH₂
A
G
Gly-Sar
E
T
S
D
Dns-OH
X
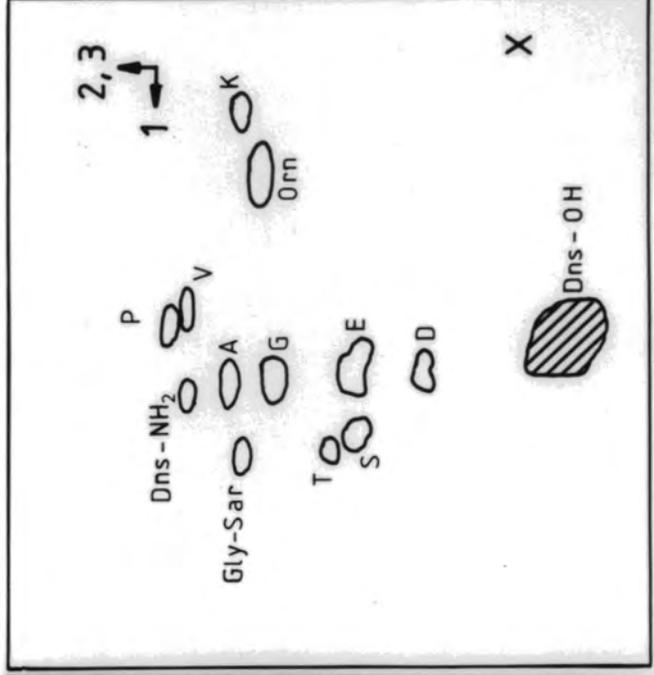
More extensive studies on the mode of uptake of sarcosine peptides were carried out for <u>Sacc.</u> <u>cerevisiae</u> (Nisbet & Payne, 1979a; Nisbet, 1980). Natural peptides were shown to compete with Gly-Sar and Gly-Sar-Sar uptake. Kinetic studies of Gly-Sar uptake gave an apparent K_m of the order 10^{-2} M, though a secondary mode of Gly-Sar uptake was demonstrated, making kinetic analysis difficult.

Detailed kinetic analysis of Gly-Sar uptake was not performed in this study, rather the principle of intact, active accumulation of peptides by <u>Candida</u> <u>albicans</u> was established, by demonstration of Gly-Sar accumulation.

4.3.8 The Uptake of Radioactively-Labelled Substrates by <u>Candida albicans</u> : Simultaneous Monitoring Using Fluorescamine and Radioactivity Assays

The uptake of Ala-[U^{14}C]Ala by <u>Candida</u> <u>albicans</u> B2630 was monitored simultaneously using samples from the same batch of cells by both fluorescamine (Section 2.5.2) and by accumulation of radioactively-labelled peptide (Section 2.7.2). Assay of peptide uptake by monitoring accumulation of radioactively-labelled peptide gave an underestimate of the true rate of transport measured by the fluorescamine assay (Fig 4.4).

The rates of transport of Ala-Ala, Ala-Ala-Ala and Leu measured by using radioactively-labelled substrates were consistently lower than the rates measured using the fluorescamine assay (Table 4.4).

The apparently lower rates measured by accumulation of radioactively-labelled substrate is attributed to the net flux arising from uptake and concomitant exodus of deaminated amino acid derivatives (Woodward & Cirillo, 1977), and release of [^{14}C]CO$_2$ (formed by
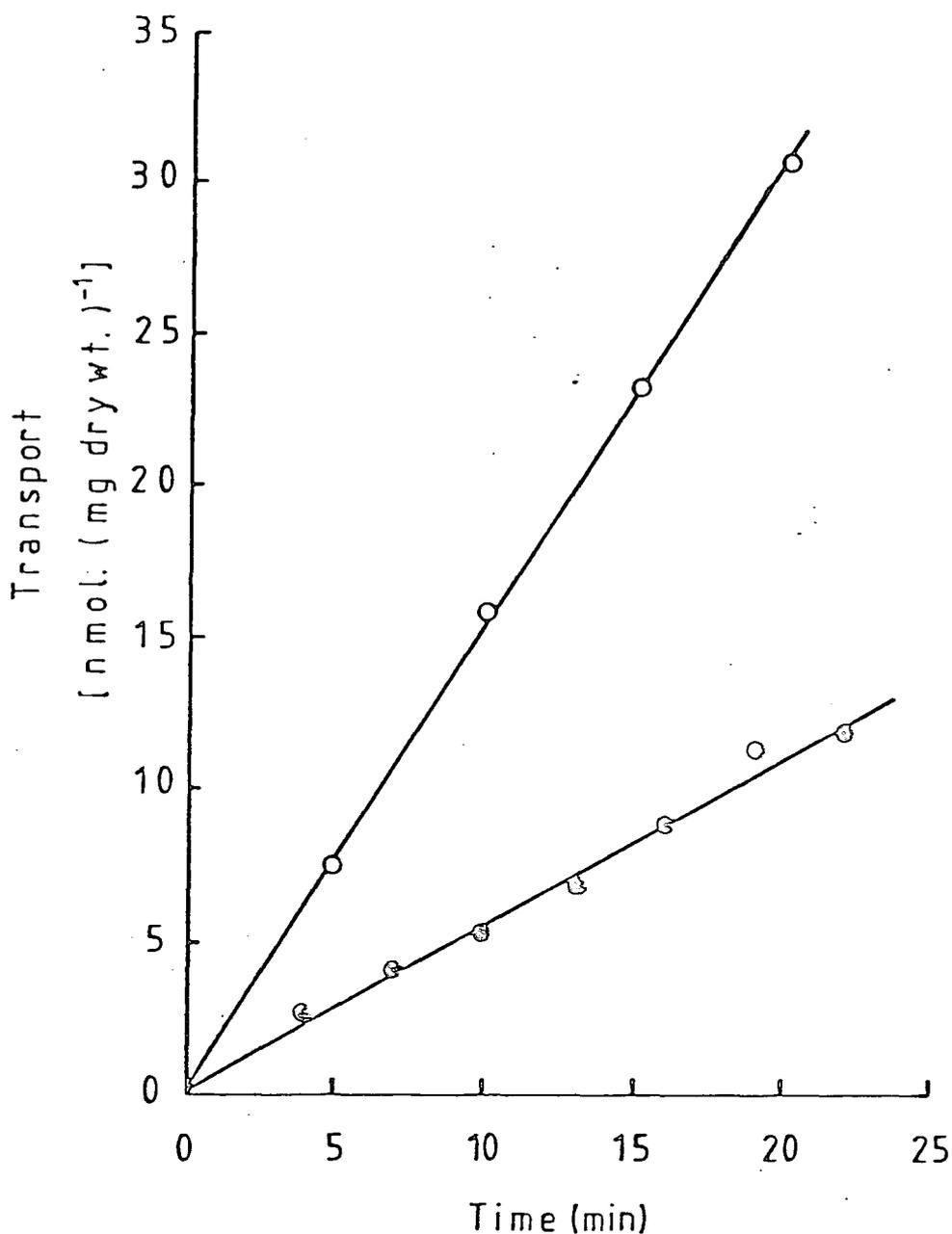
Figure 4.4   Comparison of Radioactivity and Fluorescamine Methods for

the Assay of Peptide Uptake in Candida albicans

Strain B2630 was grown in Pro-Medium overnight, harvested and

resuspended in phosphate-citrate (pH 4.5, 10 mM phosphate) with glucose

(0.8% w/v) to a density of 1.4 (mg. dry wt.)ml^{-1}.   Uptake of Ala-

[U^{14}C]Ala (0.1 mM, 3.09 x 10^{-2} uCi ml^{-1}) was assayed by using

radiotracer (⊙) or fluorescamine (○) methods.

Uptake rates (nmol min^{-1} (mg dry wt)$^{-1}$)

	Fluorescamine Assay	Radiotracer Assay
1) Ala-Ala	1.25	0.41
2) Ala-Ala-Ala	1.6	0.65
3) Leu	8.2	2.8
4) Leu (Pep-grown)	7.3	1.3

TABLE 4.4   Rates of Transport Measured by Using Radioactively-Labelled

Substrate and Fluorescamine Assays

Candida albicans B2630 was grown in Pro- (1 - 3) or in Pep-Medium (4),

harvested and resuspended in PCG buffer (Section 4.2.2).

Uptake of Ala-[U^{14}C]Ala (0.1 mM, 3.09 x 10^{-2} uCi ml^{-1}), Ala-Ala-

[U^{14}]Ala (0.1 mM, 6.06 x 10^{-3} uCi ml^{-1}) and [U^{14}C]Leu (0.5 mM, 2.5 x

10^{-2} uCi ml^{-1}) was measured by monitoring the accumulation of

radioactively-labelled substrate (Section 2.7.2).   Uptake of the

corresponding unlabelled substrates was measured by the standard manual

fluorescamine assay (Section 2.5.2), under identical conditions and

substrate concentrations.

decarboxylation reactions following transamination of the intracellularly released [U^{14}C]-labelled amino acids), in an analagous manner to that shown for Sacc. cerevisiae and certain other micro-organisms (Payne & Nisbet, 1980b). Selective exodus of peptide-derived amino acids has been shown not to occur in Candida albicans (Section 4.3.3), thus, the release of [^{14}C]-labelled amino acids (as seen for example with E.coli), cannot explain the differences in rate obtained when using the fluorescamine and radioactively-labelled substrate assays.

The rates of [^{14}C]Leu transport exhibited by cells grown in Pro- and Pep-Medium were shown to be significantly different (Fig 4.6), whereas, when assayed using fluorescamine, no such significant difference was observed (see Section 4.4.4). This difference was attributed to differences in the extent of metabolism of accumulated label, and associated differences in the extent of exodus of peptide-derived secondary metabolites.

4.3.9 The Specificity of Uptake by Candida albicans

The uptake of a range of peptides by Candida albicans B2630 was measured using the standard manual fluorescamine assay (Table 4.5). Because the rate of peptide uptake showed some variation when measured for different batches of yeast (Section 4.3.6), the rate of uptake of a peptide was always determined relative to the rate of Ala-Ala uptake in cells grown and harvested in the same batch.

In a preliminary study, the rates of uptake of a selected range of peptides by Candida albicans strains B2630 and A, were measured by dansyl chloride assay of samples of medium (Section 2.4.2). Uptake rates determined by this assay (Table 4.6) were in agreement with those

Peptide	Rate	Peptide	Rate	Peptide	Rate
L-Ala-L-Ala	100	Ala-Gly	29-42	Gly-Ala	33-50
L-Ala-D-Ala	ND	Ala-Leu	131-303	Leu-Ala	37-40
D-Ala-L-Ala	ND	Ala-Met	144-145	Met-Ala	28-60
L-Ala-L-Ala-L-Ala	145-174	Ala-Phe	105-169	Phe-Ala	47-90
L-Ala-L-Ala-D-Ala	ND	Ala-Tyr	69-90	Tyr-Ala	96-120
L-Ala-D-Ala-L-Ala	ND	Ala-Asp	ND	His-Ala	130-176
D-Ala-D-Ala-D-Ala	ND	Ala-Ser	54-66	Leu-Leu-Leu	52-125
L-Ala-L-Ala-L-Ala-L-Ala	155-207	Gly-Phe	21-50	Gly-Ala-Ala	22-48
L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	132-204	Phe-Gly	53-100	Met-Met-Met	165-18?
Gly-Gly	62-92	Arg-Arg	ND	Val-Val-Val	ND
Gly-Gly-Gly	ND	Lys-Lys	ND	Glu-Ala-Ala	ND
Gly-Gly-Gly-Gly	ND	Leu-Leu	45-67		
		His-His	165-183		

TABLE 4.5   Uptake of Peptides by Strain B2630

Rates of uptake of peptide (0.1 mM) were determined by manual fluorescamine analysis of samples of medium during 25 min incubations. Cell density 1 – 2.5 (mg dry wt.)ml^{-1}. Rates are expressed relative to that of an Ala-Ala control (0.7 – 2 nmol min^{-1}(mg dry wt)$^{-1}$) determined for each experiment. Values given are the range of at least two determinations.

(N.D., not detectable).

Rate of Uptake (nmol min^{-1}(mg dry wt)$^{-1}$)

Peptide	Strain B2630	Strain A
Ala–Ala	1	3
Ala–Ala–Ala	1.5	4
Leu–Leu	0.8	–
Leu–Leu–Leu	1	–
Met–Met	1.5	–
Gly–Phe	–	0.3

TABLE 4.6   Uptake of Peptides by Strains B2630 and A

Uptake of peptide (0.1 mM) was monitored by dansylation of samples of medium during 30 min incubations.   Results are the average of at least two determinations.

–;   not determined

values determined by the manual fluorescamine assay (Table 4.5), though they were not determined relative to an Ala-Ala control.

The following characteristics of peptide uptake in strain B2630 can be deduced from the results presented in Table 4.5 :-

1.   Strain B2630 takes up a range of di-pentapeptides

2.   The transport system(s) have a pronounced specificity for L-stereoisomers, uptake of peptides containing D-stereoisomers being undetectable.

3.   Peptides containing glycine are generally poor transport substrates, transport of tri- and tetraglycine being undetectable.

The results from this study are discussed in conjunction with those of earlier studies in Section 4.7, particularly with reference to the development of rationally designed peptide-antifungal drugs.

4.3.10 Kinetic Parameters for Uptake in Strain B2630

Uptake of peptides at different initial substrate concentrations was assayed by using the standard fluorescamine procedure. Because of the possibility of variability in rates of peptide uptake between different batches of yeast (Section 4.3.6), initial rates of uptake were always determined for cells suspensions taken from the same batch of organisms, harvested at the same time.

Double reciprocal plots of the data yielded values for the kinetic parameters Km and Vmax for several peptides (Fig 4.5, Table 4.7). Km values were of the same order of magnitude as those described in Sacc. cerevisiae (Naider & Becker, 1977; Nisbet, 1980).
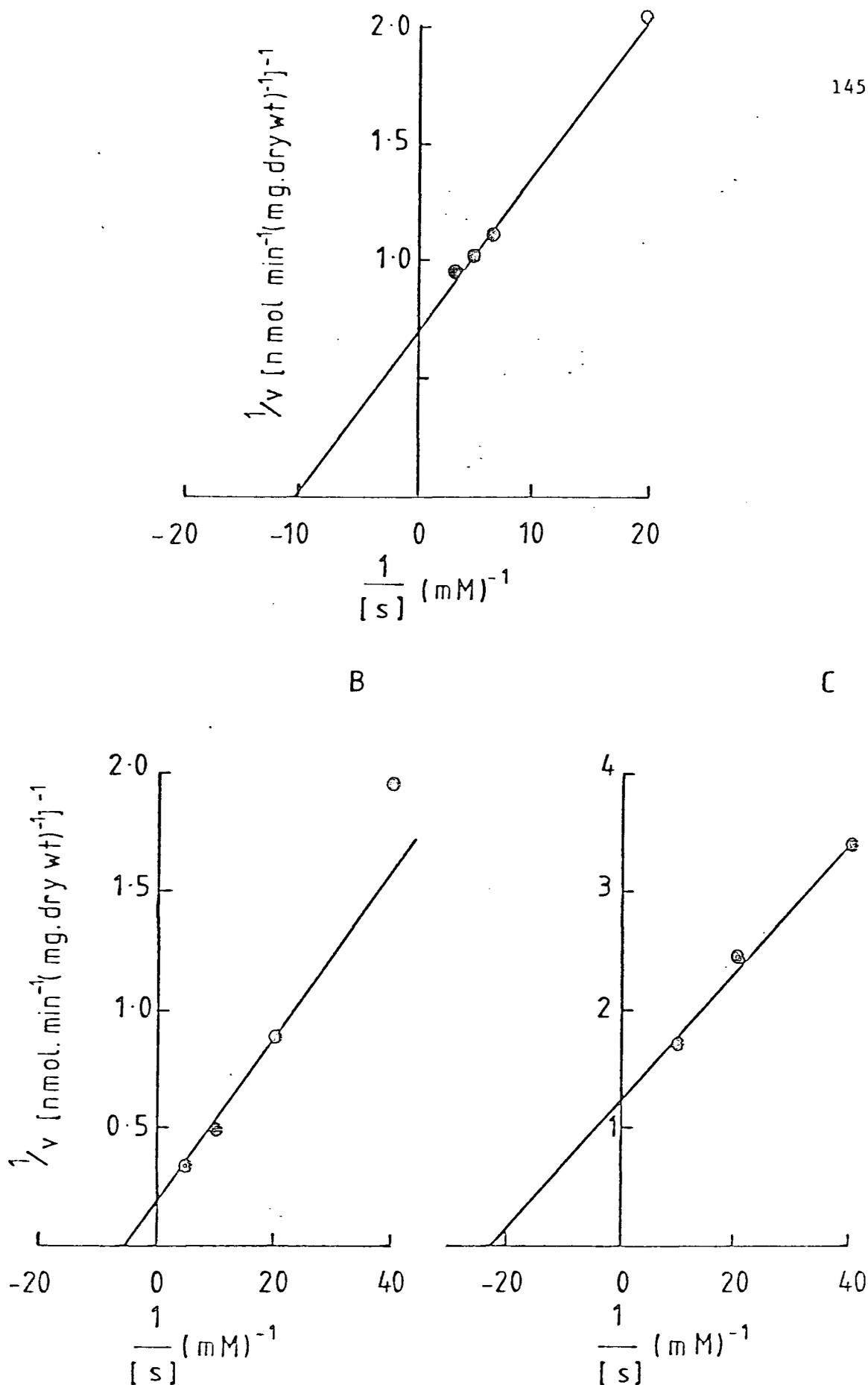
Figure 4.5 Affinity of Peptide for Uptake by Strain B2630

Uptake rates of A) Ala-Ala, B) Ala-Ala-Ala and C) Leu-Leu were

determined by the standard manual fluorescamine procedure.

Peptide	Km (uM)	Vmax(nmol·min^{-1}(mg·dry wt)$^{-1}$)
Ala-Ala	100	1.5
Ala-Ala-Ala	200	5
Leu-Leu	40	0.8

TABLE 4.7 Affinity of Peptides for Uptake in Strain B2630

Initial rates of peptide uptake by Candida albicans B2630 (grown in Pro-Medium), were determined by the standard manual fluorescamine procedure (Section 2.5.2).

A more detailed kinetic analysis of peptide transport in strain B2630 is presented elsewhere (Section 4.5), statistically evaluated transport kinetic parameters being evaluated using a microcomputer interfaced with the automated fluorescence assay system.

4.3.11 The Stimulatory Effect of Peptone on the Rate of Peptide Transport in Candida albicans B2630

To check the generality of the stimulatory effect of peptone on transport seen with Ala-Ala, transport was investigated for several other representative peptides in Candida albicans B2630. Cells were grown overnight in Pro- or Pep-Medium, harvested and resuspended in incubation buffer (Section 4.2.2) and assayed for peptide uptake by the standard manual fluorescamine procedure (Section 2.5.2).

The ratios of transport rates relative to their internal Ala-Ala standard were shown to be approximately constant for both nitrogen sources (Table 4.8)

4.4 Regulation of Peptide Transport in Candida Albicans

4.4.1 Introduction

Certain of the aspects described in the preliminary studies above, were subsequently investigated in greater detail. The studies are discussed in the following sections.

4.4.2 Effect of Nitrogen Source on Uptake of Ala-Ala

The effect of nitrogen source on the transport of peptides was further investigated (Table 4.9). Candida albicans strain B2630 was grown overnight in simple defined media supplemented with different nitrogen sources (ammonium sulphate, Casein Acid Hydrolysate, proline and Bacto-Peptone). Cells were harvested and resuspended as described (Section 4.2.2) and transport of Ala-Ala (0.1 - 0.5 mM) assayed by the standard fluorescamine procedure (Section 2.5.2).

Growth Medium

Peptide	Pro-	Pep-
Ala-Ala	100	100
Ala-Ala-Ala	105-166	89-126
Leu-Leu-Leu	52-125	58-87
Gly-Phe	21-60	39-75

TABLE 4.8   Uptake of Di- and Tripeptides by Candida Albicans

B2630 Grown in Pro- or Pep-Medium

Candida albicans B2630 was grown overnight in Pro- or Pep-Medium, harvested and resuspended in incubation buffer (Section 4.2.2).   Uptake of di- and tripeptides was assayed by the standard manual fluorescamine assay (Section 2.5.2).

Rates are expressed relative to that of an Ala-Ala control (at same initial substrate concentration, 0.1 mM). Average uptake rates of Ala-Ala are 1.25 nmol. min.$^{-1}$(mg dry wt.)$^{-1}$ for cells grown overnight in Pro-Medium   and   3.83 nmol.min.$^{-1}$(mg dry wt.)$^{-1}$ for cells grown overnight in Pep-Medium.

Growth Medium	Uptake Rate $[\text{nmol.min.}^{-1}(\text{mg.dry.wt})^{-1}]$
1. NH_4-	ND (100)
2. Pro-	1.25 (100)
3. Pro- + NH_4-	ND (100)
4. Bacto-Peptone	7.5 (200)
5. Bacto-Peptone	10.6 (500)
6. Bacto-Peptone + NH_4-	10.3 (500)
7. Bacto-Peptone + Pro-	9.5 (500)
8. Trypicase Peptone (Pancreatic Digest of Casein)	10.8 (500)
9. Casein Acid Hydrolysate	1.2 (100)

TABLE 4.9   Effect of Nitrogen Source on Uptake of Ala-Ala

Candida albicans strain B2630 was grown overnight in simple defined media (glucose, 2% w/v., Yeast Nitrogen Base without ammonium sulphate or amino acids, 1.7 mg ml^{-1}), supplemented with the following nitrogen sources ammonium sulphate (0.5%, w/v); L-proline (4 mg ml^{-1}); Casein Acid Hydrolysate (2%, w/v); Bacto-Peptone (2%, w/v) or Trypticase-Peptone (2%, w/v). Cells were harvested and resuspended in incubation buffer (Section 4.2.2), transport rates being derived by the manual fluorescamine assay. Values for initial substrate concentration (uM) are given in parentheses. Results are the average of at least two determinations.

The presence of peptone was shown to override ammonium repression of peptide transport. Three levels of peptide transport were demonstrated, a minimal level due to ammonium repression, an enhanced level exhibited by cells grown on a poor nitrogen source (i.e. amino acids), and a maximal level due to the stimulatory effect of peptides in the growth media.

The molecular form of supplemented nitrogen source, (either free amino acids or combined in peptide form) was shown to be important. Two digests were examined (total acid hydrolysate and enzymic hydrolysate) that were derived from the same protein (casein), the total amino acid content of which must be very similar. The demonstration that Ala-Ala transport is stimulated by growth on two different peptone-based media, shows that the phenomenon is not dependant on a particular commercial brand of peptone.

4.4.3 Kinetics of Peptide Transport Regulation

The timing of the changes in peptide transport rates was examined in cells switched from Pro- to Pep-Medium (Step Up), and from Pep- to Pro-Medium (Step Down).

Cells were grown overnight in one media, harvested by membrane filtration (25 mm diameter, 0.45 um pore size, Oxoid), washed on the filter with phosphate-citrate buffer, pH 4.5 (20 mM with respect to phosphate, 2 x 20 ml) at room temperature. Cells were resuspended in the second media (1 - 2 ml) to a high cell density (5 - 20 (mg dry wt.)ml^{-1}) by vortexing. On resuspension, a portion (approx. 1 ml) of the cell suspension was used to inoculate a flask containing fresh medium (50 ml), pre-equilibrated at 28°C in a water bath. The flask was swirled to evenly disperse the inoculum, and growth allowed to

commence by incubation at 28°C in a shaking water bath. This treatment

gave a final cell density of 0.2 - 0.4 (mg dry wt.)ml^{-1}, providing

sufficient cells on reharvesting for transport assays. From

calculation of the cell density at the time of harvesting, and assuming

that the harvesting and resuspension stages are 90% efficient (some

cells are invariably lost during these procedures), an appropriate

volume of cells can be taken from the overnight batch for resuspension

to the required cell density in the second medium. Cells were

reharvested, washed and resuspended in incubation media by the standard

methodology (Section 4.2.2) and assayed for transport of Ala-Ala (0.5

mM) by using the standard manual fluorescamine assay (Section 2.5.2).

A control experiment in which cells were grown in Pep-Medium

overnight, harvested and resuspended in fresh Pep-Medium (for 30 min),

and reassayed for Ala-Ala uptake, showed that the above treatment per

se did not affect peptide transport (Table 4.10; experiment 6).

Transfer of cells from Pro- to Pep-Medium (Step Up) showed that

Ala-Ala uptake was increased within 100 min, to the level exhibited by

cells grown overnight in Pep-Medium (Table 4.10; experiment 5).

Similarly, transfer of cells from Pep- to Pro-Medium (Step Down)

showed that Ala-Ala uptake was decreased within 100 min, to the level

exhibited by cells grown overnight in Pro-Medium (Table 4.10,

experiment 7).

Experiments in which cells were transferred from Pro- to Pep-

Medium and assayed at 100, 200 and 300 min showed no increase in Ala-

Ala transport after the initial assay at 100 min (Table 4.11).

A more stringent analysis of the kinetics of Step Up (Table 4.12)

and Step Down (Table 4.13) showed both processes to occur within 30 min

of transfer from one growth medium to another.

Overnight Growth Medium	Transfer to	Rate Ala-Ala Transport $(nmol.min^{-1}(mg\ dry\ wt.)^{-1})$
1) NH_4-	Pep- (100)	12.4
2) Casein Acid Hydrolysate	Pep- (100)	11.6
3) Pro-	Pep- + NH_4- (200)	12
4) Pro-	Trypticase Peptone (200)	10.8
5) Pro-	Pep- (100)	11
6) Pep-	Pep- (30)	10.5
7) Pep-	Pro- (100)	1.25

TABLE 4.10   Transfer of Cells from an Overnight Culture to

Fresh Media : Effect on Transport of Ala-Ala

Candida albicans B2630 was grown overnight in a defined growth medium, harvested and resuspended in a different medium as described in Section 4.4.3. Cells were incubated at 28°C, figures in parentheses give period (min) of incubation. Cells were reharvested and resuspended in incubation buffer (Section 4.2.2) and assayed for Ala-Ala transport by the standard manual fluorescamine assay (Section 2.5.2). Initial Ala-Ala concentration was 0.5 mM, except for (7), 0.1 mM. Media composition is as described in Table 4.9.

Period of Incubation in Pep-Medium (min)	Transport Rate $(nmol.min.^{-1}(mg\ dry\ wt.)^{-1})$
100	9.9
200	10.3
300	10.5

TABLE 4.11   Kinetics of Step Up of Peptide Transport

Candida albicans B2630 was grown overnight in Pro-Medium, harvested and resuspended in Pep-Medium (50 ml, 0.1(mg dry wt.) ml^{-1}). Samples were taken periodically, reharvested and resuspended in incubation buffer (Section 4.2.2), and assayed for Ala-Ala (0.5 mM) uptake by the manual fluorescamine assay (Section 2.5.2).

Control rates for cells grown in Pro-Medium overnight (equivalent to 0 min incubation in Pep-Medium), is 1.25 nmol.min.$^{-1}$(mg dry wt.)$^{-1}$ (Section 4.3.6).

Period of Incubation in Pep-Medium (min)	Transport Rate $nmol.min.^{-1}(mg\ dry\ wt.)^{-1})$
20	8.1
60	9.2
100	7.7

TABLE 4.12   Kinetics of Step Up of Peptide Transport

Candida albicans B2630 was grown overnight in Pro-Medium, harvested and resuspended in Pep-Medium (50 ml, 0.33 (mg dry wt.)ml^{-1}). Samples were taken periodically, reharvested and resuspended in incubation buffer (Section 4.2.2), and assayed for Ala-Ala (0.5 mM) uptake, by the manual fluorescamine assay (Section 2.5.2). Control rate for cells grown overnight in Pro-Medium (equivalent to 0 min incubation in Pep-Medium), is 1.25 nmol.min.$^{-1}$(mg dry wt.)$^{-1}$ (Section 4.3.6).

Period of Incubation in Pro-Medium (min)	Transport Rate $(nmol.min.^{-1}(mg\ dry\ wt.)^{-1})$
0	9.1 (0.5)
30	1.37 (0.1)
60	1.35 (0.1)

TABLE 4.13   Kinetics of Step Down of Peptide Transport

Candida albicans B2630 was grown overnight in Pep-Medium, harvested and resuspended in Pro-Medium (50 ml, 0.3 (mg dry wt.)ml^{-1}). Samples were taken periodically, reharvested and suspended in incubation buffer (Section 4.2.2), and assayed for Ala-Ala uptake by the manual fluorescamine assay (Section 2.5.2).

Initial substrate concentration (mM) is given in parentheses.

Step Up and Step Down of transport was also demonstrated for the peptide Gly-Phe (Table 4.14), cells being transferred from one medium to another as previously described.

Step Up was shown to occur in cells transferred from overnight growth in Pro-Medium to Pep-Medium, containing 0.2% w/v., Bacto-Peptone; typically Pep-Medium contains 2% w/v., Bacto-Peptone. Cells grown overnight in Pro-Medium were also transferred to a minimal medium (glucose, 2% w/v., Yeast Nitrogen Base without ammonium sulphate and amino acids, 1.7 mg ml^{-1}) in which Ala-Ala (0.2% w/v) was the sole nitrogen source. Cells were incubated at 28°C for 100 min before reharvesting and assaying for Ala-Ala and Ala-Ala-Ala transport (Table 4.15). Step Up of peptide transport was shown not to occur when cells were transferred to medium containing Ala-Ala as the sole nitrogen source.

Thus the kinetics of changes in peptide transport uptake was shown to be rapid, occurring within 30 min of transfer from one media to another. The effect of inhibition of protein synthesis on the peptone induced increase in peptide transport was examined (Section 4.4.5).

4.4.4 Specificity of Peptone-Induced Increase in Transport Rates

To examine whether the peptone effect was specific for peptide transport, the transport rates of representative amino acids was examined in Candida albicans B2630 grown overnight in Pro- and Pep-Medium.

Cells were grown overnight in Pro- or Pep-Medium, harvested and resuspended in incubation buffer (Section 4.2.2), and assayed for

Transport Rate (nmol.min.$^{-1}$(mg dry wt.)$^{-1}$)

Overnight Growth Medium	Transfer to	Ala–Ala	Gly–Phe
1) Pro–		1.25	0.6 (21 – 60)
2) Pep–		10.6	6.2 (39 – 75)
3) Pro–	Pep–	9.5	4.6 (49)
4) Pep–	Pro–	1.1	0.5 (45)

TABLE 4.14   Step Up and Step Down of Gly–Phe Transport

Candida albicans was grown overnight in a defined growth medium, harvested and resuspended in a different medium as described in Section 4.4.3. Cells were incubated at 28°C for 200 min before reharvesting and assaying for uptake of Gly–Phe (0.5 mM for cells resuspended in Pep–Medium, (3);   0.1 mM for cells resuspended in Pro–Medium, (4)). Control rates for Ala–Ala (1) and Gly–Phe (2) for cells grown in Pro– and Pep–Medium are given.   Figures in parentheses are the rates expressed relative to that of an Ala–Ala control;   values given in (1) and (2) being the range of at least two determinations

<div align="center">

Transport Rate

$(nmol.min.^{-1}(mg\ dry\ wt.)^{-1})$

</div>

Transfer to	Ala-Ala	Ala-Ala-Ala
1) Bacto-Peptone	3.6	6.1
2) Ala-Ala	1.1	1.4

TABLE 4.15 Effect of Ala-Ala as a Sole Nitrogen Source

on Step Up of Peptide Transport

Candida albicans B2630 was grown overnight in Pro-Medium, harvested and transferred to minimal media (glucose, 2% w/v., Yeast Nitrogen Base without ammonium sulphate and amino acids, 1.7 mg ml^{-1}) in which Bacto-Peptone (0.2% w/v) or Ala-Ala (0.2% w/v) were the sole nitrogen sources. Cells were incubated at 28°C for 100 min before reharvesting and assaying for peptide transport by the manual fluorescamine assay (Section 2.5.2). Initial substrate concentration was 0.1 mM.

transport of amino acids (0.5 mM) by using both fluorescamine (Section 2.5.2) and radiotracer assays (Section 2.7.2). Results are presented in Fig. 4.6.

The rates of amino acid transport as determined by the standard manual fluorescamine assay, were shown to be independant of nitrogen source. The rates of [^{14}C]-Leu transport exhibited by cells grown in Pro- and Pep-Medium were shown to be significantly different. This difference was attributed to differences in the extent of intracellular metabolism of accumulated label (Section 4.3.8). When assayed using fluorescamine no such significant difference in the rate of Leu transport was observed by cells grown in Pro- and Pep-Medium.

4.4.5 Effect of Protein Synthesis Inhibition on Peptone Induced Increase in Peptide Transport

4.4.5.1 Introduction

The marked increase in rates of peptide transport was shown to occur within one generation (80 min) on transfer of cells from Pro- to Pep-Medium. To gain further insight into this process, the effect of the protein synthesis inhibitor cycloheximide on the Step-Up mechanism was examined, together with its effects on growth, and on the incorporation of radioactively-labelled amino acids into protein.

4.4.5.2 Effect of Cycloheximide on Transport Following Transfer from Proline- to Peptone-Medium

Candida albicans strain B2630 was grown in Pro-Medium overnight, harvested by membrane filtration and washed with phosphate-citrate buffer, pH 4.5 (Section 4.2.2). Cells were resuspended in phosphate-citrate buffer (1 - 2 ml) to a high cell density (5 - 20 (mg dry wt.)ml^{-1}). A sample (approx. 1 ml) of this suspension was used to
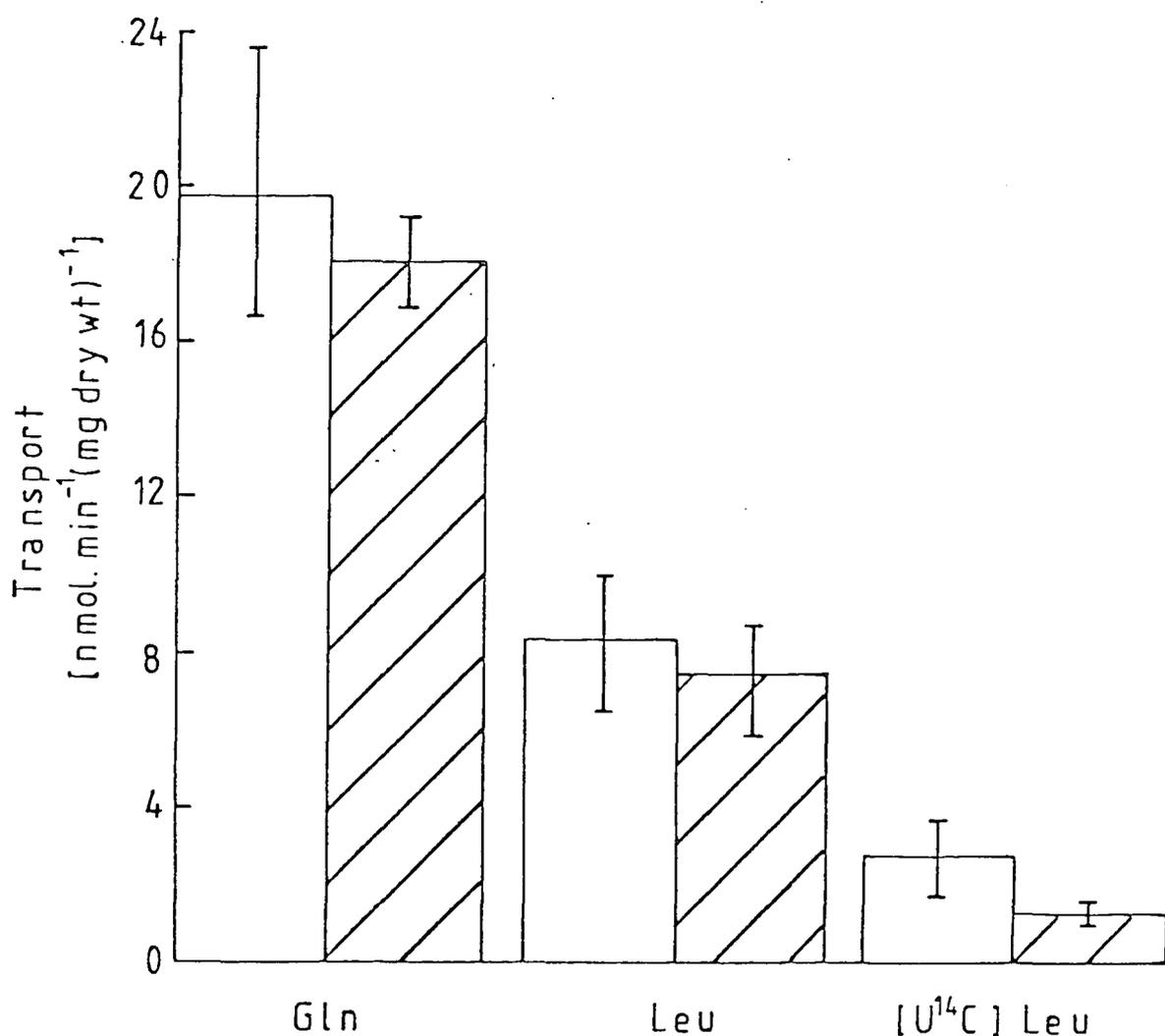
Figure 4.6 Effect of Sole Nitrogen Source on Transport Rates of Amino
          Acids

Candida albicans B2630 was grown in Pro- (□), or Pep-Medium
(▨) overnight, harvested, resuspended in incubation buffer, and
incubated with amino acid. Transport of Gln and Leu (both 0.5 mM) was
measured by the standard manual fluorescamine assay (Section 2.5.2).
Uptake of [U^{14}C]Leu (0.5 mM, 2.5 x 10^{-2} uCi ml^{-1}) was measured as
described (Section 2.7.2).
Results are the mean and range of at least two determinations.

inoculate a flask containing Pep-Medium (50 ml), equilibrated at 28°C, containing cycloheximide (5 mM). A control without cycloheximide was performed at the same time. Cells were incubated at 28°C for 100 min, before reharvesting and assaying for transport by the standard fluorescamine procedure (Section 2.5.2). Measured rates of transport for Ala-Ala and Gly-Phe (both 0.5 mM) for cells incubated in the presence of cycloheximide (5 mM) and for the untreated control, showed no significant difference (Table 4.16).

To confirm that this result was independent of the transport assay employed, the effect of cycloheximide was examined using Gly-[U^{14}C]Phe. Cells were grown in Pro-Medium overnight, harvested and resuspended in Pep-Medium with or without cycloheximide (5 mM) as previously described, incubated at 28°C for 100 min, before reharvesting and assaying for uptake of Gly-[U^{14}C]Phe by the standard method (Section 2.7.2). Measured rates of transport of Gly-[U^{14}C]Phe for cells incubated in the presence (4.0 nmol.min^{-1}(mg dry wt.)$^{-1}$) or absence (3.8 nmol.min^{-1}(mg dry wt)$^{-1}$) of cycloheximide, showed no significant difference (Fig. 4.7).

However when carrying out these latter experiments on Gly-[U^{14}C]Phe uptake anomalous results (Fig.4.8) were frequently encountered in which, apparently, very high initial rates of uptake occurred (c.a. 40 nmol.min.$^{-1}$(mg dry wt.)$^{-1}$). This effect was independant of the presence of cycloheximide and could appear with one half of a batch of cells otherwise producing typical uptake plots. No ready explanation for these effects can be offered but it is possible that either adsorption/absorbtion of the aromatic Phe-peptide onto filters or cells may occur. The nature of these "anomalous" results

Uptake Rate (nmol.min.$^{-1}$(mg dry wt.)$^{-1}$)

Peptide	Control	Cycloheximide Treated
Ala-Ala	11.4	11.7
Ala-Ala	12	11.8
Gly-Phe	5.9	5.4
Gly-Phe	5.0	4.7

TABLE 4.16 Effect of Cycloheximide on Peptide Uptake

Following Transfer from Pro- to Pep-Medium

Candida albicans B2630 was grown overnight in Pro-Medium, harvested, washed and resuspended in Pep-Medium with or without cycloheximide (5 mM), incubated at 28°C for 100 - 130 min, before measuring uptake of peptide (0.5 mM) by the standard manual fluorescamine assay.
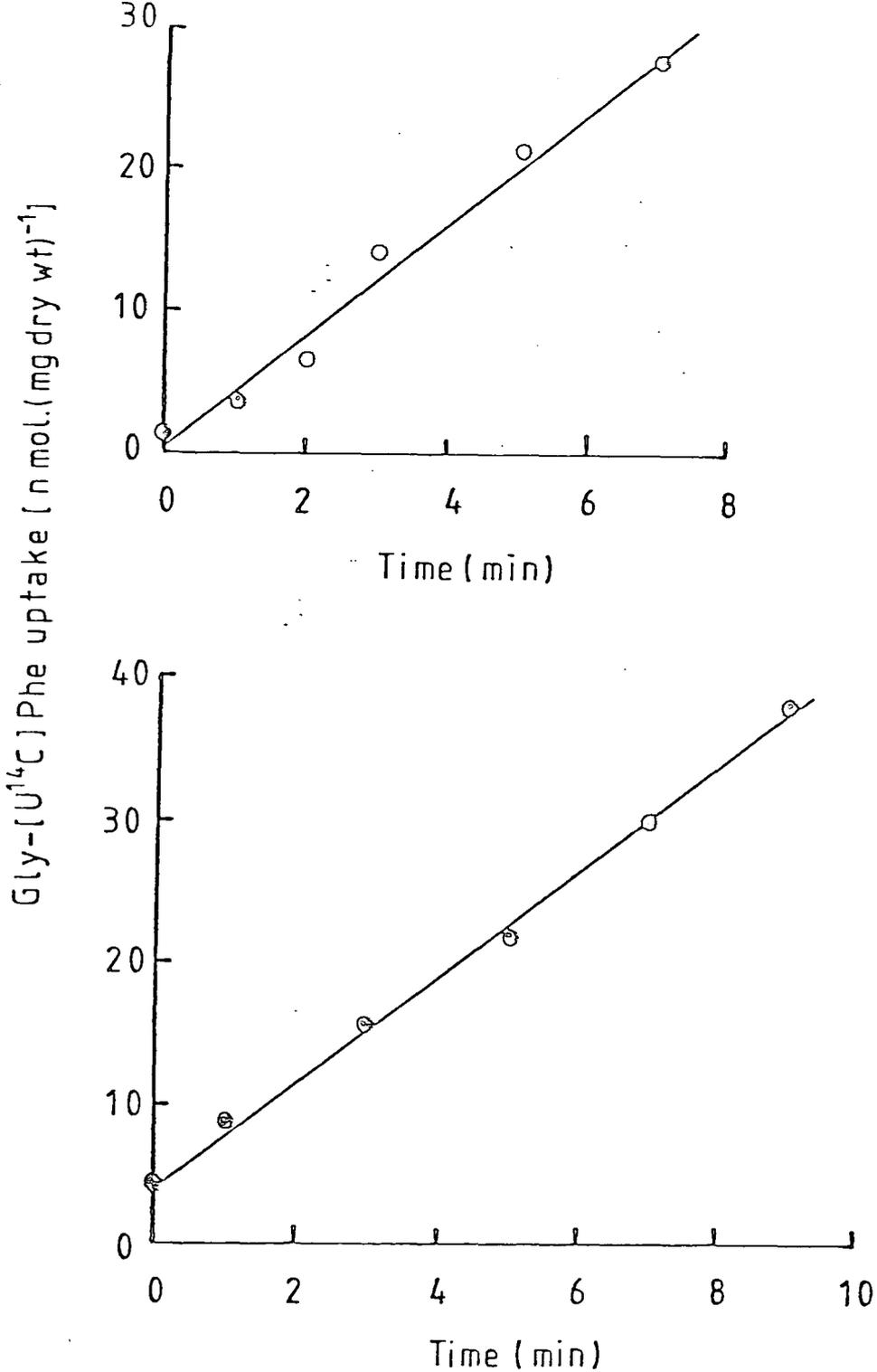
Figure 4.7 Effect of Cycloheximide on the Uptake of Gly-[U^{14}C]Phe

Following Transfer from Pro- to Pep-Medium

Overnight, Pro-Medium grown <u>Candida</u> <u>albicans</u> strain B2630 was harvested, washed and resuspended in Pep-Medium with (A, 1.25 (mg.dry wt.)ml^{-1}) or without (B, 1.7 (mg.dry wt.)ml^{-1}) cycloheximide (5 mM), incubated at 28°C for 110 min, before measuring uptake of Gly-[U^{14}C]Phe (0.5 mM, 2.5 x 10^{-2} uCi ml^{-1}).
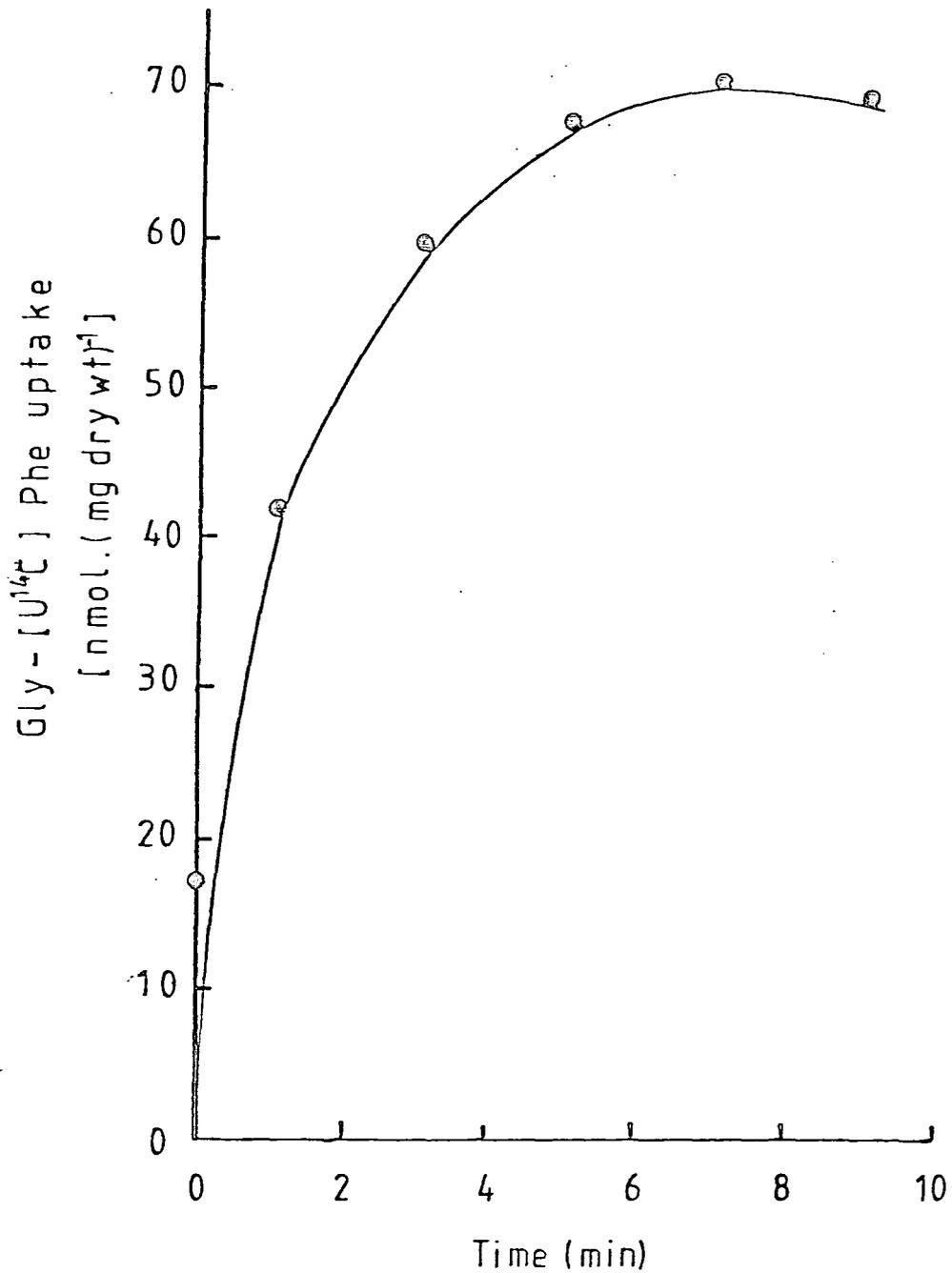
Figure 4.8   Anomalous Uptake of Gly-[U^{14}C]Phe

Cells were incubated in Pep-Medium as described (Fig. 4.8) before assaying for uptake of Gly-[U^{14}C]Phe (0.5 mM, 2.5 x 10^{-2} uCi ml^{-1}, 1.3 (mg. dry wt.) ml^{-1}).

was not investigated further, the "common-sense" results being taken as supportive evidence for the results from the fluorescence assay. It must be stressed, however, that similar "anomalous" results were not obtained when measuring uptake of Ala-[U^{14}C]Ala, Ala-Ala-[U^{14}C]Ala or [U^{14}C]Leu.

Transport of peptides by cells grown for one generation in Pep-Medium (after which time the Step Up of transport is known to have occurred) was thus shown to be unaffected by the presence of cycloheximide in the medium. However, before it can be concluded that synthesis of protein components is not required for the Step Up mechanism, it is necessary to demonstrate that cycloheximide is acting as an effective inhibitor of protein synthesis in this system, these studies are shown in the following sections.

4.4.5.3 Effect of Cycloheximide on Growth of Candida albicans in Peptone-Medium

The effect of cycloheximide on growth of cells after transfer from Pro- to Peptone-Media was examined.

Candida albicans B2630 was grown overnight in Pro-Medium, harvested and resuspended in Pep-Medium with or without cycloheximide (5 mM, 50 ml) as previously described (Section 4.4.5.2). Growth was monitored by measuring absorbance of a portion of the culture (4 - 5 ml) in a Bausch & Lomb Spectronic 20 spectrophotometer (660 nm, 1 cm diam. tube), using Pep-Medium as the reference solution for calibration. Growth curves for the cycloheximide-treated cells and for the untreated control are shown (Fig. 4.9).

Clearly, cycloheximide inhibits growth. This effect presumably arises from inhibition of protein synthesis by cycloheximide, this specific aspect is addressed in the next section.
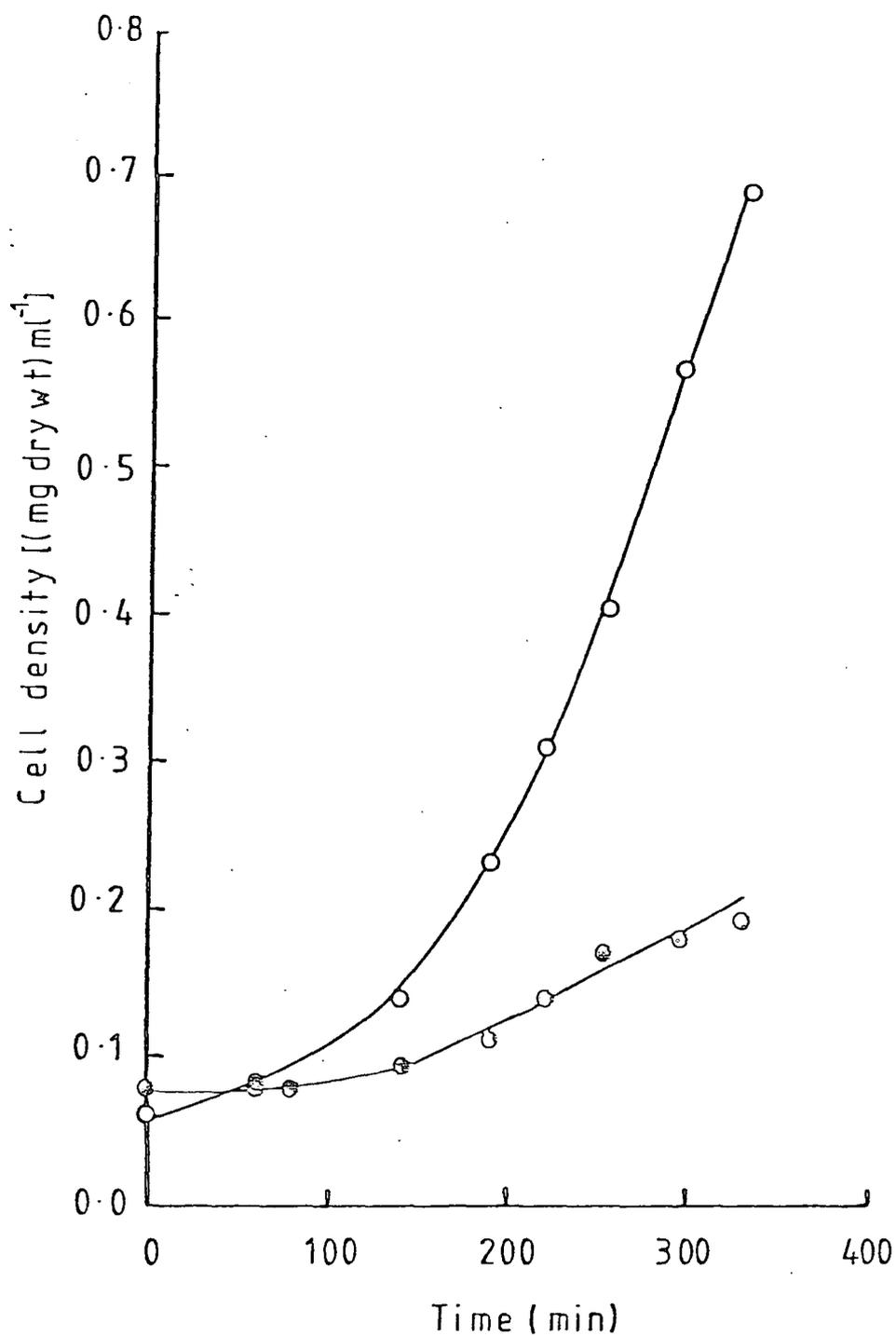
Figure 4.9   Effect of Cycloheximide on the Growth of Candida Albicans
            B2630

Experimental procedure is described in the text (Section 4.4.5.3).

(○) Untreated Control   (⊖) Cycloheximide Treated

4.4.5.4 Inhibition of Protein Synthesis by Cycloheximide

To investigate whether protein synthesis in Candida albicans is inhibited by treatment with cycloheximide, the incorporation of radioactively-labelled amino acids into tri-chloroacetic acid (TCA) insoluble material was measured in cycloheximide-treated cells relative to an untreated control. Phenylalanine and leucine were chosen for this study because their relatively low rates of intracellular metabolism make them a particularly suitable choice for this type of incorporation study.

Cells were grown overnight in Pro-Medium, harvested and washed as described (Section 4.2.2), and resuspended into fresh Pep-Medium (approx. 10 ml, at a cell density of 1.5 - 2 (mg dry wt.) ml^{-1}). Radioactively-labelled amino acids ([U^{14}C]Phe or [U^{14}C]Leu, 0.3 ml, 10 mM, 2.5 uCi ml^{-1}), was immediately added to duplicate samples of this suspension (both 5.1 ml), the suspensions being rapidly mixed by a small magnetic stirrer bar. 30s after addition of the radioactively-labelled amino acid, a sample (0.5 ml) was removed for determination of incorporation of radioactivity into TCA-precipitable material. After a further 30 s, cycloheximide (0.6 ml, 50 mM) or water (0.6 ml) as a control was added, and thereafter samples (0.5 ml) were taken periodically for up to 100 min. Final cell densities were 1.3 - 1.6 (mg dry wt.)ml^{-1}, and the final cycloheximide concentration was 5.45 mM. The samples (0.5 ml), periodically removed, were immediately added to plastic Eppendorf vials containing TCA (1 ml, 10% w/v) and incubated for at least 10 min at room temperature. The samples were filtered under vacuum using glass fibre discs (Whatman GF/C, 25 mm diam.), incubation vials were washed out and the washings passed through the

filter with TCA (40 ml, 2% w/v). Filters were added to polythene liquid scintillation vials, NE260 scintillant (5 ml) was added and radioactivity incorporated into the TCA precipitable material was counted.

Results were plotted as nmol [$U^{14}C$]-labelled amino acid incorporated into TCA-precipitable material (mg dry wt.)$^{-1}$ against period of incubation (min) in Pep-Medium with-, or without cycloheximide (5.45 mM); for both Phe (Fig. 4.10) and Leu (Fig. 4.11). Treatment with cycloheximide was shown to inhibit the incorporation of both Phe and Leu into TCA-precipitable material.

Thus, it may be concluded that the observed Step Up in peptide transport activity seen when cells are transferred to peptone medium does not require de novo synthesis of cycloheximide-inhibitable proteins.

4.5   Characterization of Kinetic Parameters for Uptake

4.5.1   Introduction

To characterise further the peptide transport systems of Candida albicans it was necessary to evaluate the kinetic parameters for uptake, for a series of representative peptides. Kinetic analysis was performed using the automated fluorescamine assay (Section 2.6), modifications of the original system (Payne & Nisbet, 1981) are described in Section 2.6.2. The original system has been interfaced to a 380Z microcomputer (Research Machines Limited) which provides statistically evaluated transport kinetic parameters. An evaluation of the continuous system, with a view to optimisation of data analysis, is presented in the following sections.
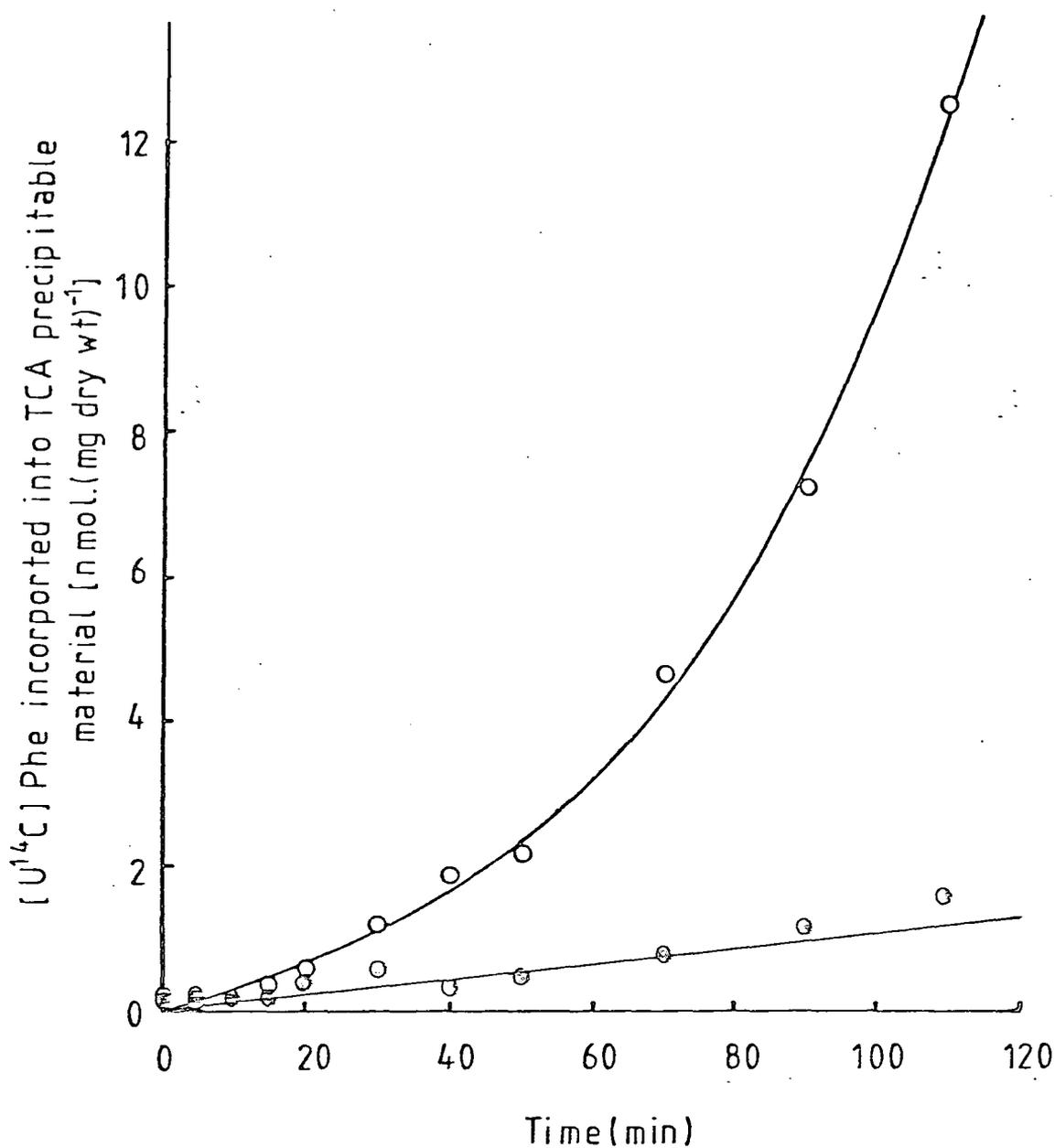
Figure 4.10   Effect of Cycloheximide on the Incorporation of

[U^{14}C]Phe

Experimental procedure is described in the text (Section 4.4.5.4).

Cells (1.6 mg ml^{-1}) were incubated with [U^{14}C]Phe (0.5 mM, 0.125 uCi

ml^{-1}) with (⊚) or without (○) cycloheximide (5.45 mM) for 110 min,

samples (0.5 ml) being taken periodically.   Radioactivity incorporated

into TCA precipitable material was measured as described.
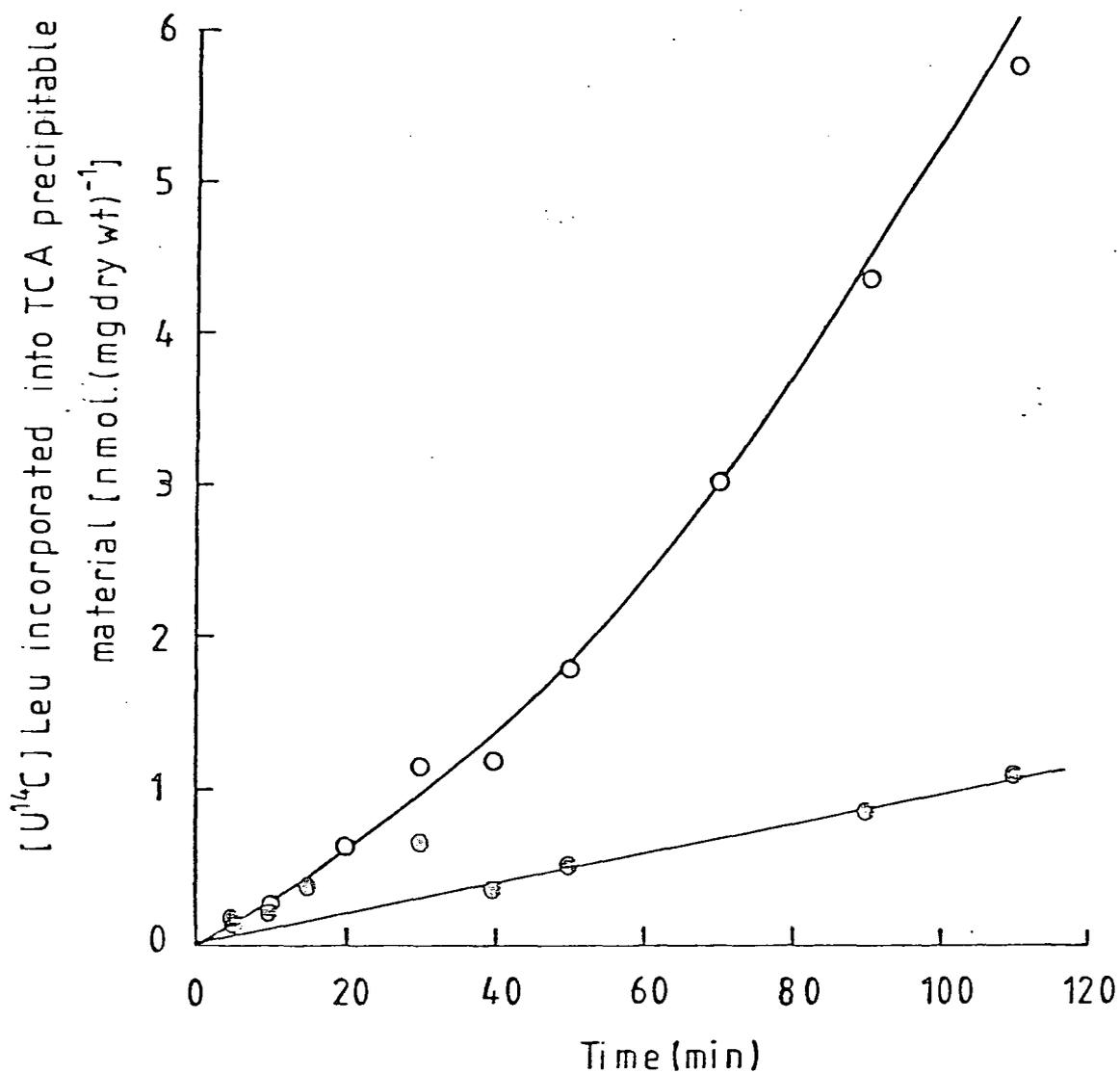
Figure 4.11   Effect of Cycloheximide on the Incorporation of $[U^{14}C]$Leu

Experimental procedure is described in the text (Section 4.4.5.4). Cells(1.3 mg ml^{-1}) were incubated with $[U^{14}C]$Leu (0.5 mM, 0.125 uCi ml^{-1}) with (⊙) or without (○) cycloheximide (5.45 mM) for 110 min, samples (0.5 ml) being taken periodically.   Radioactivity incorporated into TCA precipitable material was measured as described.

Following the optimisation of this technique, the system could be used to characterise the kinetic parameters for a range of peptides for both wild-type and transport-deficient mutant strains. Such studies should characterise individual peptide transport systems and provide data for optimising the sequence of carrier peptides for synthetic peptide drugs. Of particular interest to these studies was to determine whether the Step Up of peptide transport activity (which was shown to occur when cells were transferred from proline- to peptone-based media, Section 4.4); involves any change in the kinetic parameters for uptake. Estimation of the values of Vmax and Km for cells grown in both Pro- and Pep-Medium, is described in the following sections.

4.5.2 Optimisation of Data Analysis

The interfacing of the microcomputer to the automated system permits detailed kinetic analysis with statistical assessment of the data. Following completion of the writing of the software programmes, initial developmental work was performed to optimise data analysis in order to establish a protocol for future use. The following section describes the establishment of a recommended protocol for data analysis, together with a comparison of the effect of analysis in both the VS (velocity against substrate) and ST (substrate against time) modes. The work described here is based on the analysis of a particular output file, DAS7C; for which Candida albicans was grown overnight in Pro-Medium, harvested in the prescribed manner (Section 4.2.2), resuspended in Pep-Medium for 145 min, reharvested, and the uptake of Ala-Ala-Ala (0.1 mM) assayed according to the standard method (Section 2.6.2).

1) <u>Initial Scan of Datafile</u> A visual evaluation is made of the 'quality' of the decay curve (i.e. peptide concentration versus time) with the pH trace from the DLOG programme, (i.e. the data output recorded during the experimental run). Runs that gave a stable fluorescence baseline and plateau, together with a smooth transition from base to plateau and a 'smooth' decay curve were used for further data analysis. Conversely, runs that gave poor output were erased from the disc.

Runs producing quality output were taken for data analysis with the ARUNG GRAPH programme. A shrink factor of 1 (i.e. no data compression) was used throughout these studies for compilation of VS and ST data. The GRAPH programme then requires the entering of a 'filter factor', which determines how wide variation is allowed to either side of the 'typical' data before a point is declared to be an outlier. Any raw data outside this range will not be used subsequently by the programme. A 'filter factor' of 1000, i.e. no filtering was used throughout these studies. The entire data file is scanned to decide on the optimal sections to measure the base and plateau, and the point on the decay curve at which to start compiling VS and ST data. A condensed version of the datalogging file was produced using a shrink factor of 5 (Fig. 4.12). (The graphics display on the monitor screen shows 12 grid squares of data. At shrink factor 1 each grid square represents 25 data points, each data point represents the average of eight fluorescence readings recorded every 0.08 s, i.e. each data point represents 0.64 s, of data; therefore each grid square is 16 s.
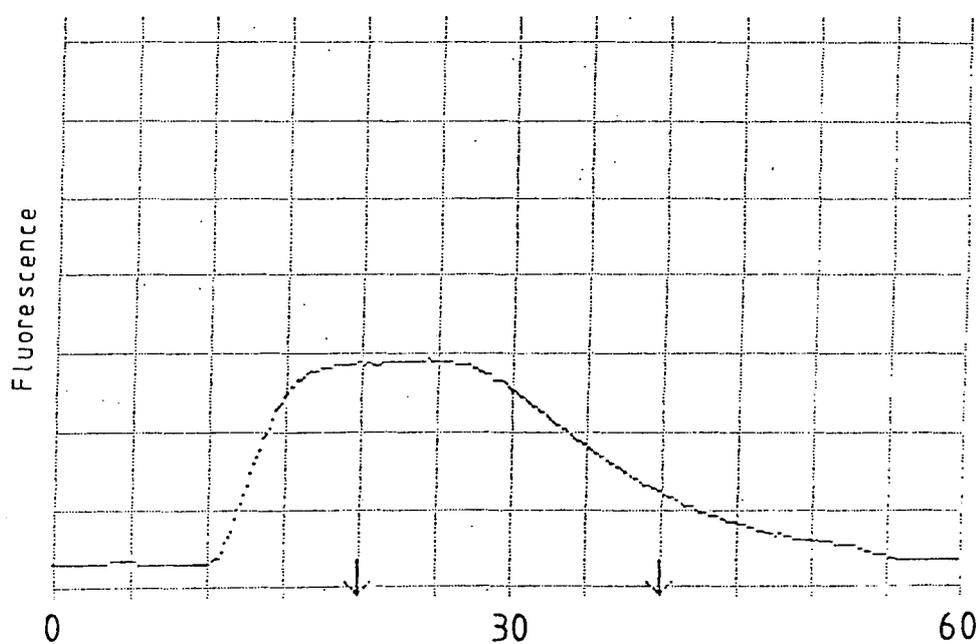
Figure 4.12   Condensed Datalogging File for DAS7C

Plot of fluorescence against time for DAS7C (Ala-Ala-Ala, 0.1 mM, 0.95 (mg.dry wt.)ml^{-1}). Data were condensed with a shrink factor of 5. Figure shows fluorescence (arbitrary units) against time (expressed as number of grid squares) from grid squares 0 - 60.

2) <u>Measurement of Base and Plateau.</u> The respective heights of the baseline and plateau are measured by a Control M function, which sets two moveable pointers (arrows) 4 grid squares apart (Fig. 4.13), the heights being expressed in fluorescence units min^{-1}. If a second analysis of the same data file is carried out, the heights are expressed in concentration units. Typically, baselines were measured from grid squares 4 - 8, plateaus from grid squares 22 - 26. If the trace between 4 grid squares is uneven, the distance between the moveable pointers can be reduced (or enlarged) by a Control D function. After measuring the base and plateau, the settings are checked with a Control Y function, and the concentration (uM) entered, enabling the computer to convert fluorescence readings into concentration units.

3) <u>Sequential Analysis of Decay Curve.</u> The decay curve is then analysed, through using a Control R function, either in a VS or ST mode, the starting point being away from the plateau (typically 6 grid squares from the plateau). For ST analysis, the moveable pointers are left a 1 grid square distance; for VS analysis, the pointers are positioned at 3 grid squares distance. The computer then sequentially analyses identical sections of the curve, with a repeated estimations mode, logging all results into an output file. The output file is closed with a Control Q function, which states the number of data points compiled. With the pointers set at 1 grid square, upto 40 data points can be compiled with the Control R function. The decay curve for file DAS7C is shown in Fig. 4.14.

A

Fluorescence

41,140

0.0   4.0   8.0   12.0
DAS7C 100uM ala3   H=0.58   Assay=0.95   S=
2   Y=4   Pep=145min

slope between arrows = 1.2405 FLU/min

B

Fluorescence

43.08

17.0   21.0   25.0   29.0
DAS7C 100uM ala3   H=0.58   Assay=0.95   S=
2   Y=4   Pep=145min

slope between arrows = 0.9097 FLU/min
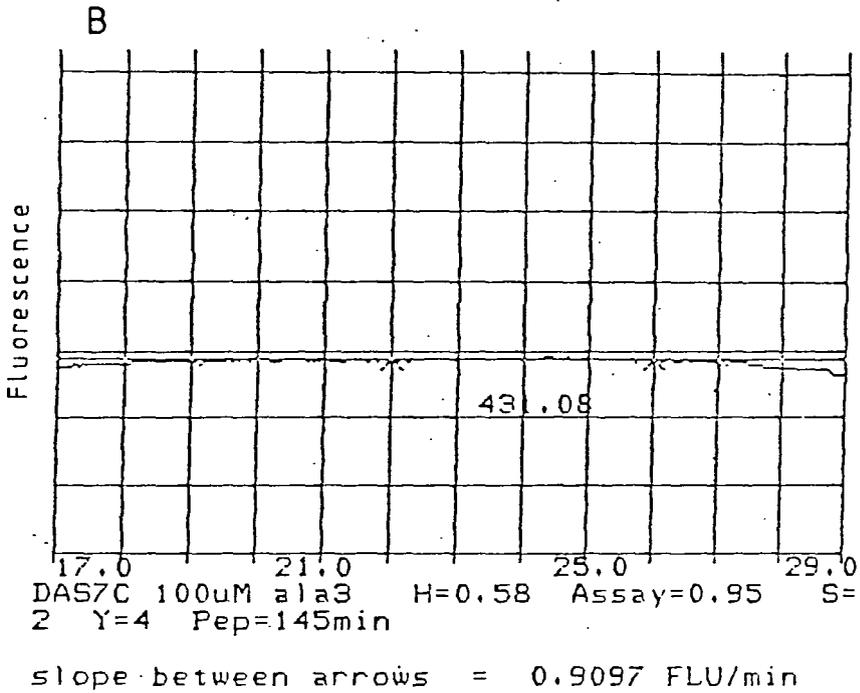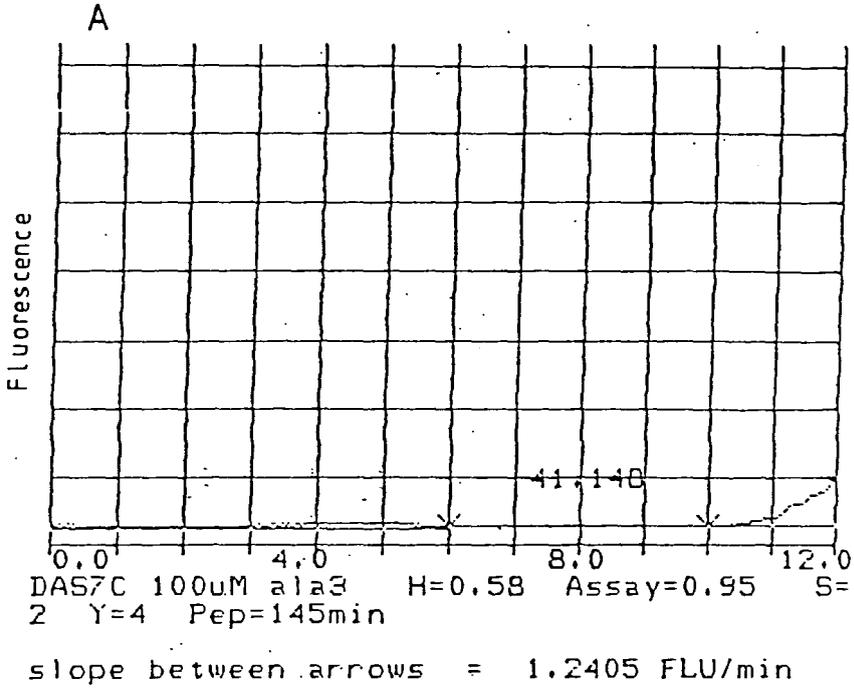
Figure 4.13   Measurement of Base and Plateau Fluorescence

Measurement of base (A) and plateau (B) fluorescence, with the Control

M function (see text).   Plots are fluorescence (arbitrary units)

against time (number of grid squares), for a shrink factor of 1.
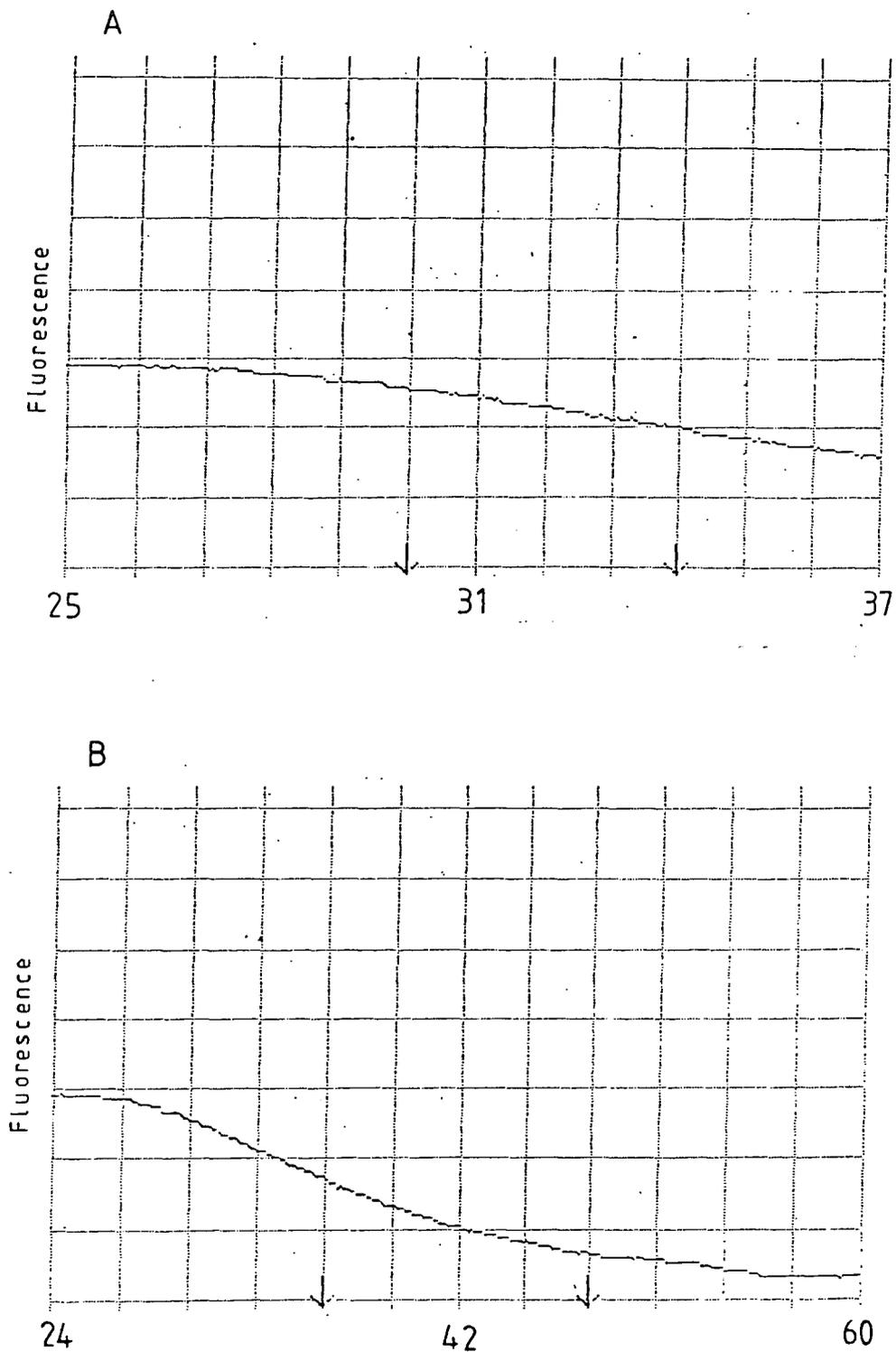
Figure 4.14 Decay Curve for File DAS7C

Decay curve for DAS7C at shrink factor 1 (A), i.e. no compression of data, and at shrink factor 3 (B). Plot is fluorescence (arbitrary units) against time (number of grid squares as indicated).

4)   <u>Editing of Output File</u>. Editing is carried out with a TXED programme. Erroneous data points are removed with a Control K function. Identifying commands to enable the kinetic analysis programme to function are added at this point, in the sequence shown in Table 4.17. An output file is created, followed by a -smear command (see following sections), and a VS or ST identification command to enable the kinetic analysis programme to operate in the correct mode. Following the two columns of data is a -1 command which indicates the end of the data; after which a PR print command is added. If required, the commands for contour analysis can also be added at this point (see following sections). TXED also enables a series of output files to be compiled into a single 'string', which can be analyzed sequentially (usually overnight). Edited files are terminated with a FI stop command.

5.   <u>Kinetic Analysis</u>. Edited files were analyzed by a KINETICS programme, either in a VS or ST mode as directed.

For VS analysis, the data are first plotted as an Eadie Hofstee plot and estimates for Vmax and Km given. This is followed by two V against S plots, on one the line of best fit is plotted, with the gradient; the second VS plot gives the best fit for a Michaelis-Menten hyperbola.

For ST analysis, two S against T plots are given, the first gives the best fit for an exponential curve, the second gives the curve of best fit for a kinetic model.

Output from the KINETICS programme is displayed by entering the output files into a PRINTOUT programme

A

"DAS7C 100uM ala3 H=0.58 Assay=0.95 S=2 Y=4 Pep=145min "
```
0.00000   95.39154
0.26667   89.73923
0.53333   84.08691
0.80000   78.62954
1.06666   73.05929
1.33333   67.97119
1.60000   62.81129
1.86666   58.14381
2.13333   53.12752
2.40000   49.27041
2.66666   45.26970
2.93333   41.51518
3.19999   38.53000
3.46666   35.60640
3.73333   32.88797
3.99999   30.52857
4.26666   28.03582
4.53332   25.94314
4.79999   24.31206
5.06665   23.14263
5.33332   22.14756
5.59998   20.79349
```

-1

B
```
ou=1
-s
st or vs as required

0.00000   95.39154
0.26667   89.73923
0.53333   84.08691
0.80000   78.62954
1.06666   73.05929
1.33333   67.97119
1.60000   62.81129
1.86666   58.14381
2.13333   53.12752
2.40000   49.27041
2.66666   45.26970
2.93333   41.51518
3.19999   38.53000
3.46666   35.60640
3.73333   32.88797
3.99999   30.52857
4.26666   28.03582
4.53332   25.94314
4.79999   24.31206
5.06665   23.14263
5.33332   22.14756
5.59998   20.79349

-1
pr
co
0.95 -1
0 80 200
0 80 200
fi
```

TABLE 4.17 Output Files for DAS7C

A)   Typical output file in ST mode.
      H = Cell density of culture at harvesting, $(mg.dry.wt)ml^{-1}$.
      Assay = Cell density on resuspension in PCG
      S = Scale expansion on fluorimeter

B)   ST output file edited for analysis by KINETICS programme.
      ou = 1   = output file number
      -S   =   indicates omission of desmearing function
      ST or VS = Commands for particular mode of analysis
      -1 = Indicates end of data (stop command).
      pr = Print Command
      co = Contour Command
      0.95 -1 = Confidence limit for CONTOUR (here 95%)
      0 80 200   0 80 defines range of values for Vmax (top)
      0 80 200   and Km (bottom). 200 is the number of
                  estimations which defines size of contour plot

      fi = Finish command, end of job.

Analysis of variance (ANOVAR) is also carried out, the computer calculates whether the data (ST or VS mode) best fits an exponential or Michaelis-Menten progress curve (ST data) or a straight line or Michaelis-Menten hyperbola (VS data). The plotting of all lines and curves is calculated by a least sum of squares method. Values for the various statistical parameters are given in an analysis of variance readout at the end of both the VS and ST plots. Typical output from a KINETICS programme in both VS (Fig. 4.15) and ST (Fig. 4.16) modes, are shown.

6. Contour Analysis. Those output files, which gave good analysis of variance values were used for CONTOUR analysis to determine the significance of the estimates for Vmax and Km. Command functions for CONTOUR are added using TXED (see Table 4.17), the contour limits e.g. 0.95 - 1; are followed by the scale setting for both Vmax and Km. The first two values give the range of Vmax and Km values, the third value (number of evaluations) determines the size of the plot. Where contours are sharp, and closed at a 95% significance level (0.95), within a fairly narrow range of Vmax and Km values; then the Vmax and Km values determined by the KINETICS programme may be regarded as statistically significant. A typical contour plot (Fig. 4.17) for file DAS7C run from grid squares 30 - 53 (Fig. 4.18) is shown

7) Optimisation of ST Analysis. In an attempt to optimise ST data analysis, the effect of the length of the curve to be analyzed was examined by deleting data points from both the top and bottom of the curve for file DAS7C (run from grid squares 30 - 52, 21 data points). Using the TXED editing programme, data points were
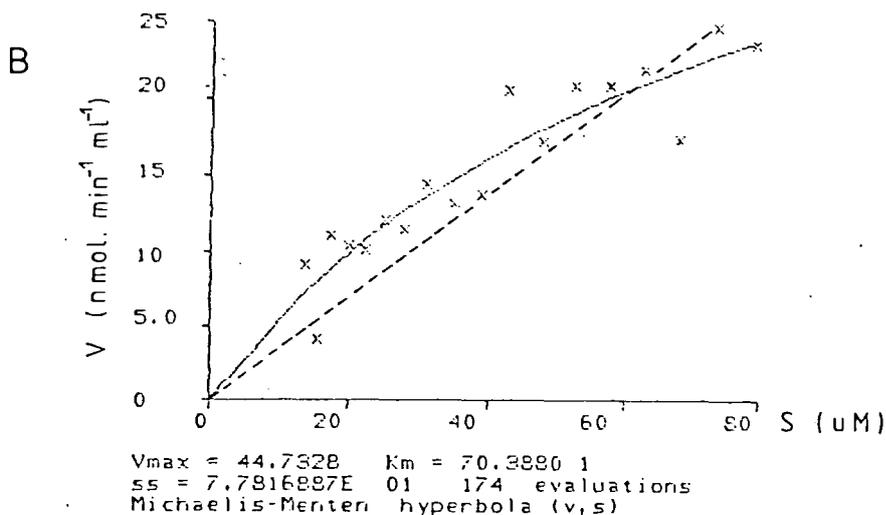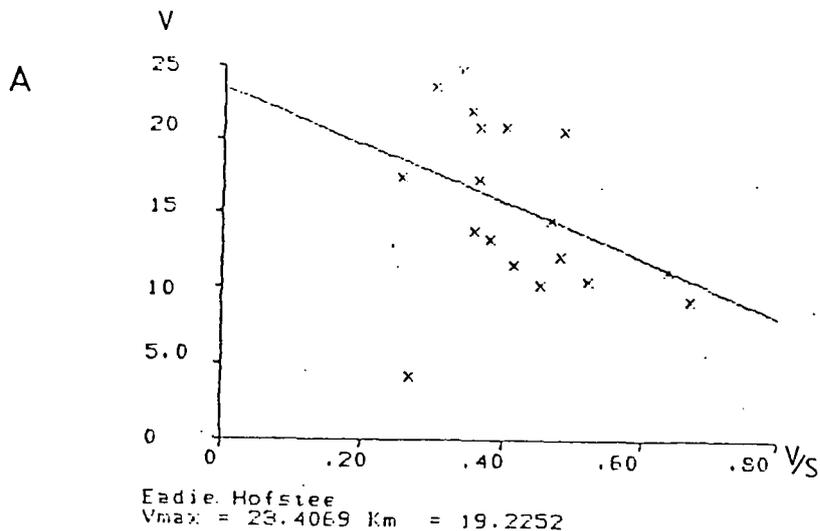
Figure 4.15 Output from KINETICS for VS Analysis (Pointer

Setting of 1 Grid Square)

VS KINETIC analysis output, showing Eadie Hofstee plot (A),

Michaelis-Menten hyperbola (B) and analysis of variance (c), for file

DAS7C run from grid square 31 - 50, with a pointer setting of 1 grid

square (18 data points). The Michaelis-Menten plot shows the straight

line of best fit (dashed line), and the best fit for a Michaelis-Menten

hyperbola (continuous line).

```
S(uM)   90 ─┤×
            │ ╲×
            │   ╲×
       60 ─┤      ×
            │        ×
            │          ×
            │            ×
       30 ─┤              ×  ×
            │                  ×  ×  ×  ×
        0 ─┼──┬────┬────┬────┬────┬──
           0  1.0  2.0  3.0  4.0  5.0  T(min)
```

s0 = 74.8525 base = 15.1162
Vmax = 37.0933   Km = 49.2073
ss = 6.6157532E-01   256 evaluations
Michaelis-Menten progress curve (s,t)
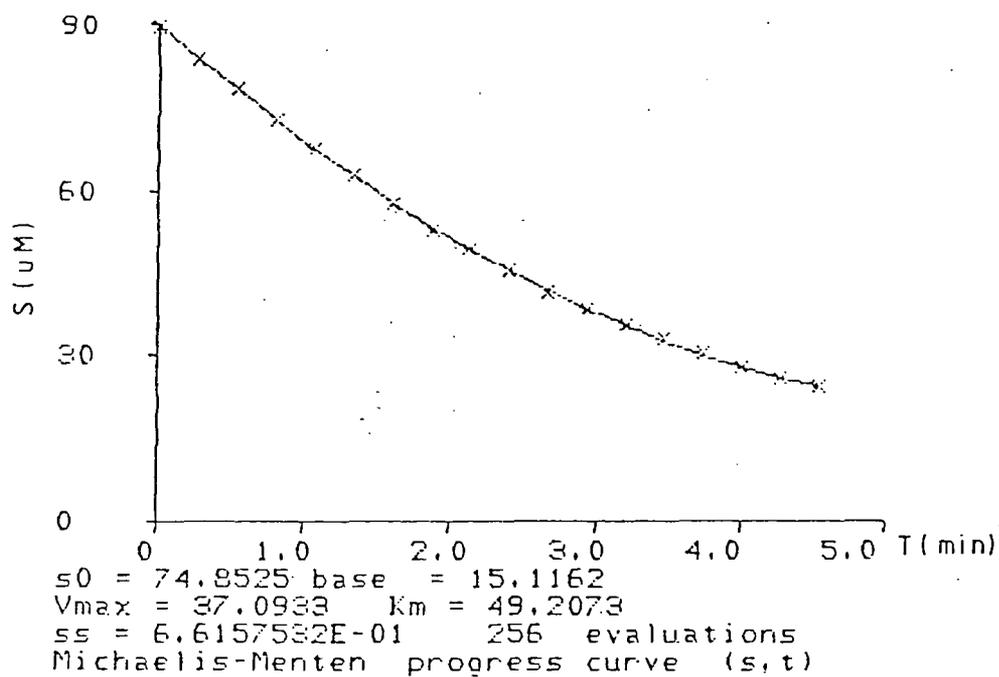
Michaelis-Menten progress curve (s,t)
s0 = 74.85248   base = 15.11621
Vmax = 37.09329   Km = 49.20727
256 evaluations made.

Source	df	SS	MS	F
exp	3	54424.71	18141.57	
kinetic	1	3.578125	3.578125	75.71888
devs	14	0.661575	0.047255	
total	18	54428.94		

Probability = 0.000001

Figure 4.16   Output from KINETICS for ST Analysis of File DAS7C

from Grid Squares 31 - 50

ST KINETIC analysis output, showing Michaelis-Menten progress curve (S, substrate concentration (uM), against T, time (min)), for DAS7C run from grid squares 31 - 50 (18 data points), and analysis of variance.

Vmax =   34.220443
Km =   40.827055
   contours at

P = 0.95000   SS = 1.2827911E 00
P = 0.99000   SS = 1.5339809E 00
P = 0.99900   SS = 1.9811811E 00

        lowest   highest   no. of values

Vmax   20.0000   50.0000   301
Km   20.0000   80.0000   301

Figure 4.17   Contour Plot for ST Analysis of DAS7C from Grid

                Squares 30 - 53

Confidence limits are 95.0, 99.0 and 99.9% from inner to outer contour.

Range limits for Vmax, 20 - 50 $nmol \cdot min^{-1} \cdot ml^{-1}$, Km 20 - 80 uM.   Number

of estimations, 300, defines size of contour plot.

Values for kinetic parameters are obtained from VS analysis of the

decay curve for DAS7C, run from grid squares 30 - 53 (22 data points),

see Fig. 4.18.
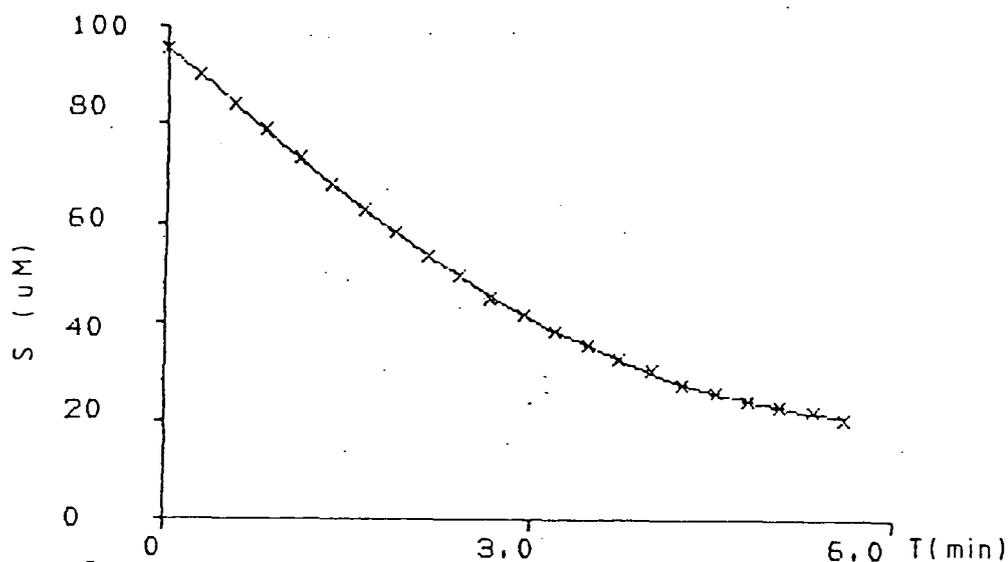
```
s0 = 79.2675 base = 16.4123
Vmax = 34.2204    Km = 40.8271
ss = 9.1959553E-01    314  evaluations
Michaelis-Menten  progress  curve  (s,t)
```

```
Michaelis-Menten progress curve (s,t)
s0 = 79.26753    base = 16.41227
Vmax = 34.22044    Km = 40.82705
314 evaluations made.
```

Source	df	SS	MS	F
exp	3	64971.12	21657.05	
Kinetic	1	14.85156	14.85156	290.7018
devs	18	0.919596	0.051089	
total	22	64986.91		

Probability = 0.000000

Figure 4.18   ST Analysis of DAS7C from Grid Squares 30 - 53

ST KINETIC analysis output, showing Michaelis-Menten progress curve (S,

substrate concentration (uM), against T, time (min)), for DAS7C run

from grid squares, 30 - 53 (22 data points), and analysis of variance.

sequentially removed from both the top and bottom of the output file; each edited file was put through the KINETICS programme, and the values for analysis of variance compared with the parent file (Table 4.18). Deleting upto 6 data points from the top resulted in a small increase in Vmax with a proportionally larger change in Km, whereas deleting upto 6 data points from the bottom resulted in a small decrease in Vmax with a proportionally larger decrease in Km. Deleting data points from the bottom of the curve, had little effect on the values for analysis of variance, probability values remained low, and F values remained high; this resulted in 'tight' contours for all results (results not shown). However deletions from the top of the curve resulted in a sequential increase in the probability value, with an inversely related decrease in the F value; this resulted in poor contours (95% significance limit) for files with 5 and 6 data points removed from the top of the curve (results not shown). When the probability value exceeds 0.001 (F value is approximately 30), then contours tend not to close, even for quite a wide range of Vmax and Km values.

Deletion of data points from both the top and bottom of the Michaelis-Menten decay curve for file DAS7C was thus shown to affect significantly the estimates for the kinetic parameters. The file selected for data examination was arbitarily selected on the basis of visual quality, certainly, other representative files should be examined in this manner, before any definitive statements can be made concerning the optimum length of the curve to be used for data analysis, together with the starting and end

	no.p. deleted	no.dp.	Vmax	Km	ss	est	probability	Fvalue
Parent File	–	21	33.7	39.2	0.9	410	0.000003	232
Delete from	1	20	35.3	43.3	0.7	410	0.000001	170
Top	2	19	37.0	47.6	0.7	310	0.000003	111
	3	18	39.1	53.0	0.6	289	0.000002	66
	4	17	40.1	55.4	0.6	367	0.000038	38
	5	16	43.7	64.3	0.6	311	0.001170	18
	6	15	47.9	60.6	0.6	427	0.008583	10
Delete from	1	20	34.3	41.0	0.9	417	0.000001	160
Bottom	2	19	34.5	41.9	0.8	432	0.000003	108
	3	18	33.5	38.4	0.8	306	0.000001	85
	4	17	31.5	31.5	0.6	344	0.000003	94
	5	16	29.4	24.2	0.3	298	0.000001	137
	6	15	28.3	20.5	0.3	207	0.000003	117

TABLE 4.18   Effect of Deletions from ST Output File for DAS7C

Effect of editing ST output from DAS7C, run from grid squares 30 – 52 (21 data points).   Up to 6 data points were sequentially removed from both the top and bottom of the parent output file.

no p. deleted   Number of points deleted

no d.p.   Number of data points in output file.

ss   Sum of squares.   est   number of estimations

Vmax   in $nmol.min.^{-1}ml.^{-1}$ Km in uM

points for ST analysis. Thus it is essential to have sufficient range of values in which the substrate concentration changes with time, to ensure there is sufficient curvature for ST analysis to be effective.

8)   Optimisation of VS Analysis. In an attempt to optimise VS data analysis, the effect of changing the separation between the moveable pointers (and thus the 'length' of data in the decay curve to be measured for each repeated estimation) was examined for file DAS7C. After calibration with the CONTROL Y command, CONTROL R puts GRAPH into the repeated estimations mode, for producing VS or ST values. For VS estimation, a filename is entered, and GRAPH then goes into the CONTROL S command, the pointers being set a distance apart equal to the shift rate (i.e. the rate at which the data are shifted across the screen, at 25 points per grid square) minus 1. At this point the separation between the pointers can be changed, which changes the shift rate and therefore the size of non-overlapping samples for the repeated estimations. The moveable pointers were positioned at setting of 1 - 5 grid squares minus 1 point apart, and VS data compiled, from grid squares 31 - 50. For ST analysis (see preceeding section) data were obtained by analysis of the decay curve from grid squares 30 - 52, however, when the same length of curve was analyzed in the VS mode, the output file contained clearly erroneous data, due to the curve not being a smooth decay curve. Thus, rate values did not gradually decrease as expected but fluctuated at the top and bottom of the section of the curve examined. Thus, for VS optimisation data were obtained from the

section of the decay curve from grid squares 31 - 50. The
effects of varying the setting of the pointers, and thus the
'length' of non-overlapping samples for repeated estimations on
values for kinetic parameters, are shown in Table 4.19. The
different settings had little effect on either Km or Vmax values
but significantly affected the values obtained from analysis of
variance. A pointer setting of 3 grid squares minus one point
gave the highest F value in the analysis of variance and thus the
tightest contour (95 - 99.9% significance limits) (Fig 4.19).
Output for VS analysis run from grid squares 31 - 50, for a
pointer spacing of 3 grid squares (6 data points) (Fig. 4.20),
and for a pointer setting of 1 grid square (18 data points) (Fig.
4.16) are shown.

9)   Comparison of VS and ST Analysis. The effect of analysing a
portion of the decay curve for DAS7C in both VS and ST modes of
analysis; was carried out to determine which mode of analysis
gave the most reliable values for kinetic parameters, as
evaluated by comparison of values for analysis of variance. The
decay curve from grid squares 31 - 50 (18 data points) was
evaluated in both VS and ST modes, for a moveable pointer setting
of 1 and 3 grid squares respectively. Values for kinetic
parameters and analysis of variance are given in Table 4.20. ST
analysis gave the best values for analysis of variance with a
very low probability value and high F value. Contour plots for
ST (Fig. 4.21) and VS, for an arrow spacing of 3 grid squares
(Fig. 4.19), showed that ST analysis gave the tightest contours,

Distance Between

Moveable Pointers

(Number of Grid

Squares minus

1 point)	no d.p.	Vmax	Km	ss	est	probability	Fvalue
1	18	44.7	70.4	78	174	0.000199	23
2	9	45.2	75.6	8	242	0.000296	44
3	6	43.6	75.0	2	249	0.001024	73
4	4	42.5	70.2	1	279	0.024930	39
5	3	44.6	78.3	0.8	196	0.148670	18

TABLE 4.19   Effect of 'length' of Non-overlapping Samples for the

Repeated Estimations in the VS Mode

Effect of the separation between the moveable pointers (numbers of grid squares minus 1 point), on kinetic parameters and analysis of variance. VS data were collected from grid squares 31 - 50, data were not collected beyond these points to ensure that equivalent data were being compared for each pointer setting.

no d.p.   Number of data points in output file.

ss   Sum of squares.   est   number of estimations.

Vmax, $nmol.min^{-1}ml^{-1}$ Km,   uM

```
Vmax =    43.635798
Km  =    75.011683
    contours at

P =  0.95000    SS =  7.4740276E 00
P =  0.99000    SS =  1.6712458E 01
P =  0.99900    SS =  5.2846622E 01

       lowest  highest    no. of values

Vmax    20.0000  50.0000    301
Km      20.0000  80.0000    301
```
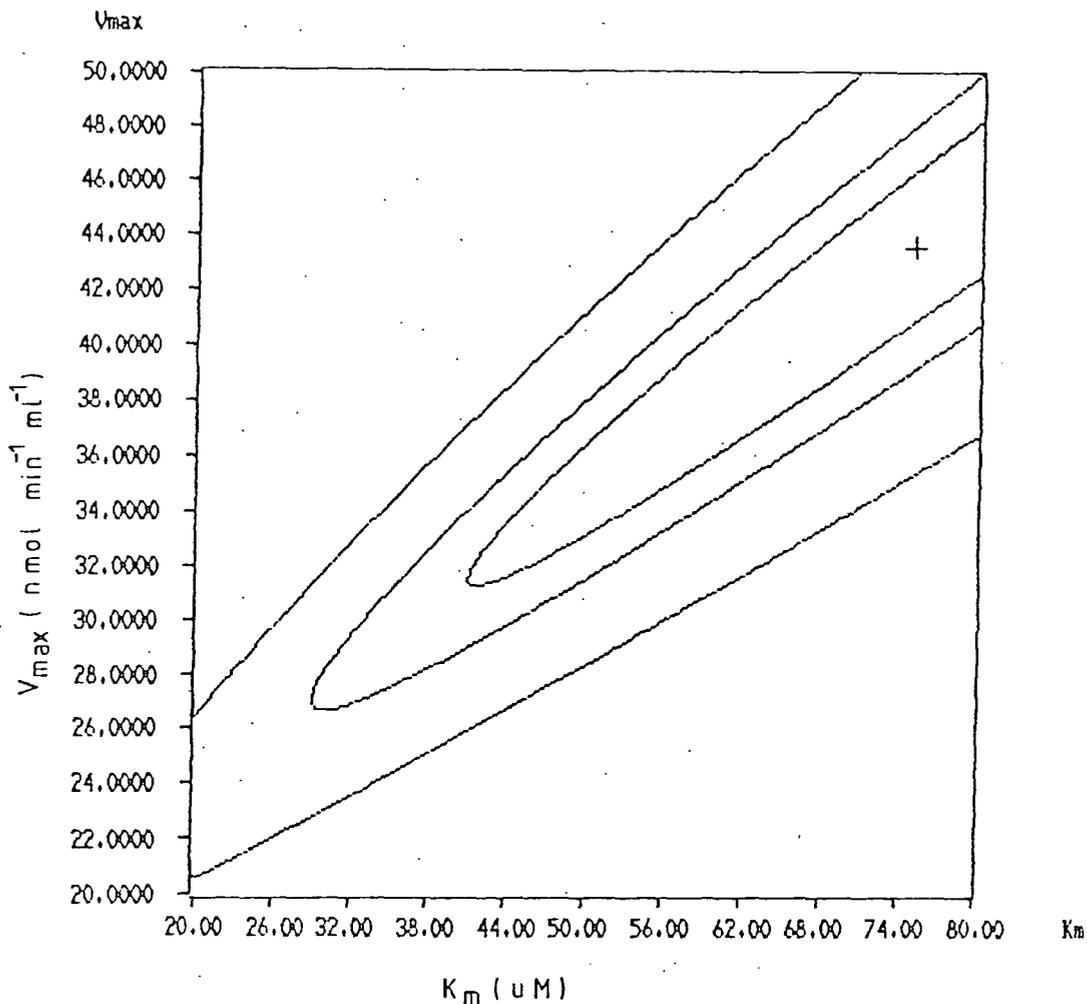

Figure 4.19   Contour Plot for VS Analysis of DAS7C (Pointer

         Setting at 3 Grid Squares).

Confidence limits are 95.0, 99.0 and 99.9% from inner to outer contour.

Range limits for Vmax, 20 - 50 $nmol.min^{-1}.ml^{-1}$, Km 20 - 80 uM. Number

of estimations, 300, defines size of contour plot.

Values for kinetic parameters are obtained from VS analysis of the

decay curve for DAS7C, run from grid squares 31 - 50, with a pointer

setting of 3 grid squares (6 data points), see Fig. 4.20.

A

V
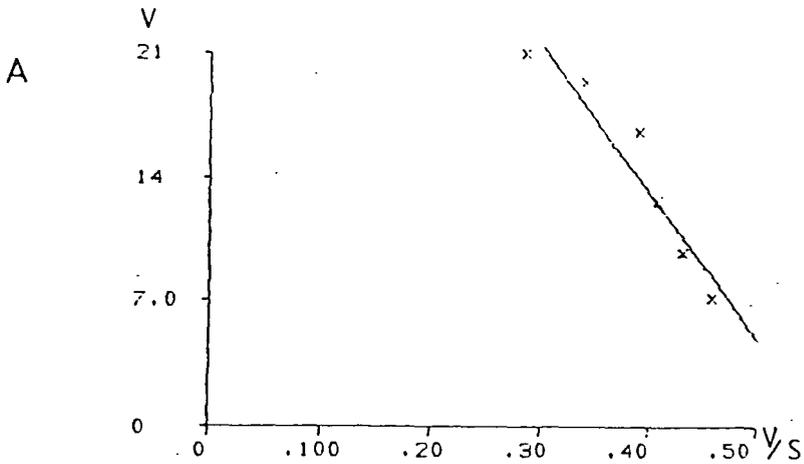
Eadie Hofstee
Vmax = 46.3447 Km = 83.0305

B

V (n mol min⁻¹ ml⁻¹)
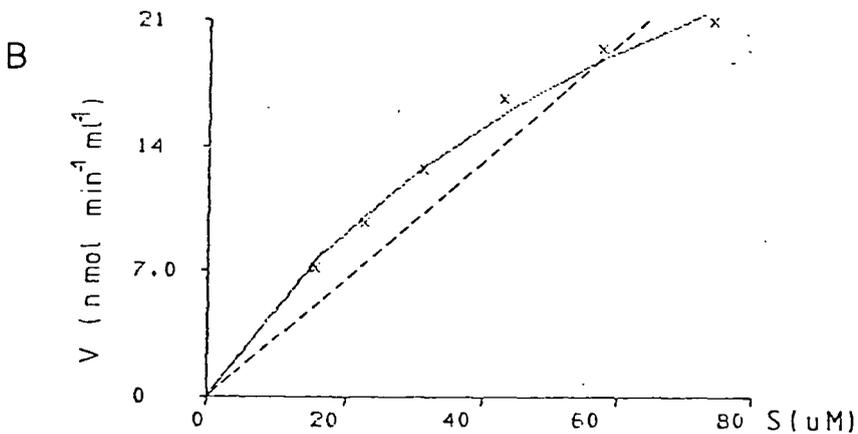
Vmax = 43.6358   Km = 75.0117 8
ss = 1.6712460E 00   249 evaluations
Michaelis-Menten hyperbola (v,s)

Michaelis-Menten hyperbola (v,s)
Vmax = 43.63580   Km = 75.01168
249 evaluations made.

C

Source	df	SS	MS	F
linear	1	1368.308	1368.308	
Kinetic	1	30.60156	30.60156	73.24249
devs	4	1.671246	0.417811	
total	6	1400.580		

Probability = 0.001024

Figure 4.20   Output from KINETICS for VS Analysis (Pointer

Setting of 3 Grid Squares).

VS KINETIC analysis output, showing Eadie Hofstee plot (A),

Michaelis-Menten hyperbola (B) and analysis of variance (C), for file

DAS7C run from grid squares 31 - 50, with a pointer setting of 3 grid

squares (6 data points).   The Michaelis-Menten plot shows the straight

line of best fit (dashed line), and the best fit for a Michaelis-Menten

hyperbola (continuous line).

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Mode of Analy- sis	Distance Between Moveable Pointers (Number of Grid Squares minus 1 point)	no d.p.	Vmax	Km	ss	est	probability	Fvalue
ST	1	18	37.1	49.2	0.7	256	0.000001	76
VS	1	18	44.7	70.4	78	174	0.000199	23
VS	3	6	43.6	75.6	2	249	0.001024	73

TABLE 4.20   Comparison of VS and ST Analysis

The decay curve for file DAS7C was analyzed from grid squares 31 - 50.
in both VS and ST modes, as described in the text.
Abbreviations are as described in Table 4.19.
Vmax, $nmol \cdot min^{-1} \cdot ml^{-1}$ Km, uM

```
    Vmax =      37,093290
    Km =      49,207268
       contours at

    P =   0,95000     SS =   1,0149405E 00
    P =   0,99000     SS =   1,2773048E 00
    P =   0,99900     SS =   1,7747993E 00

          lowest    highest      no, of values

    Vmax      20,0000   50,0000     301
    Km        20,0000   80,0000     301
```
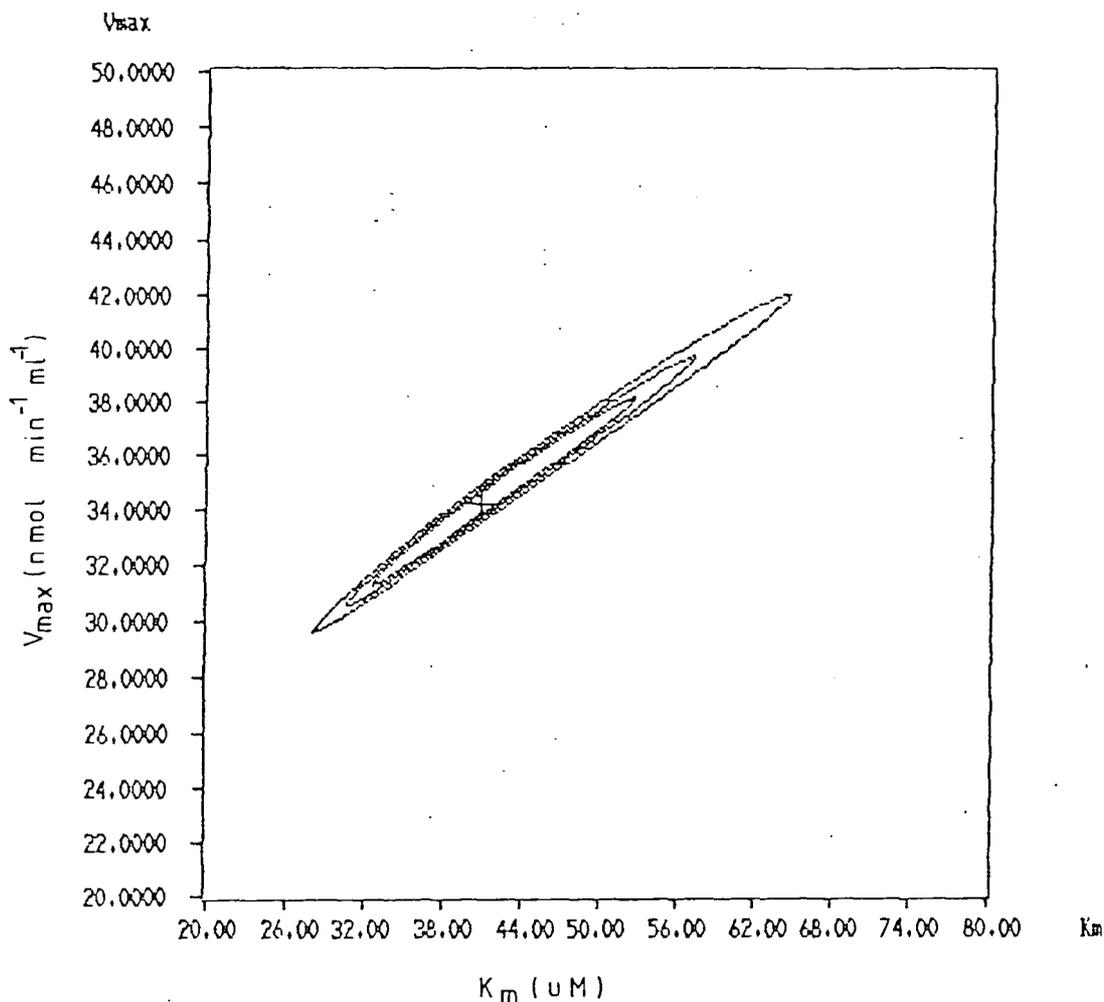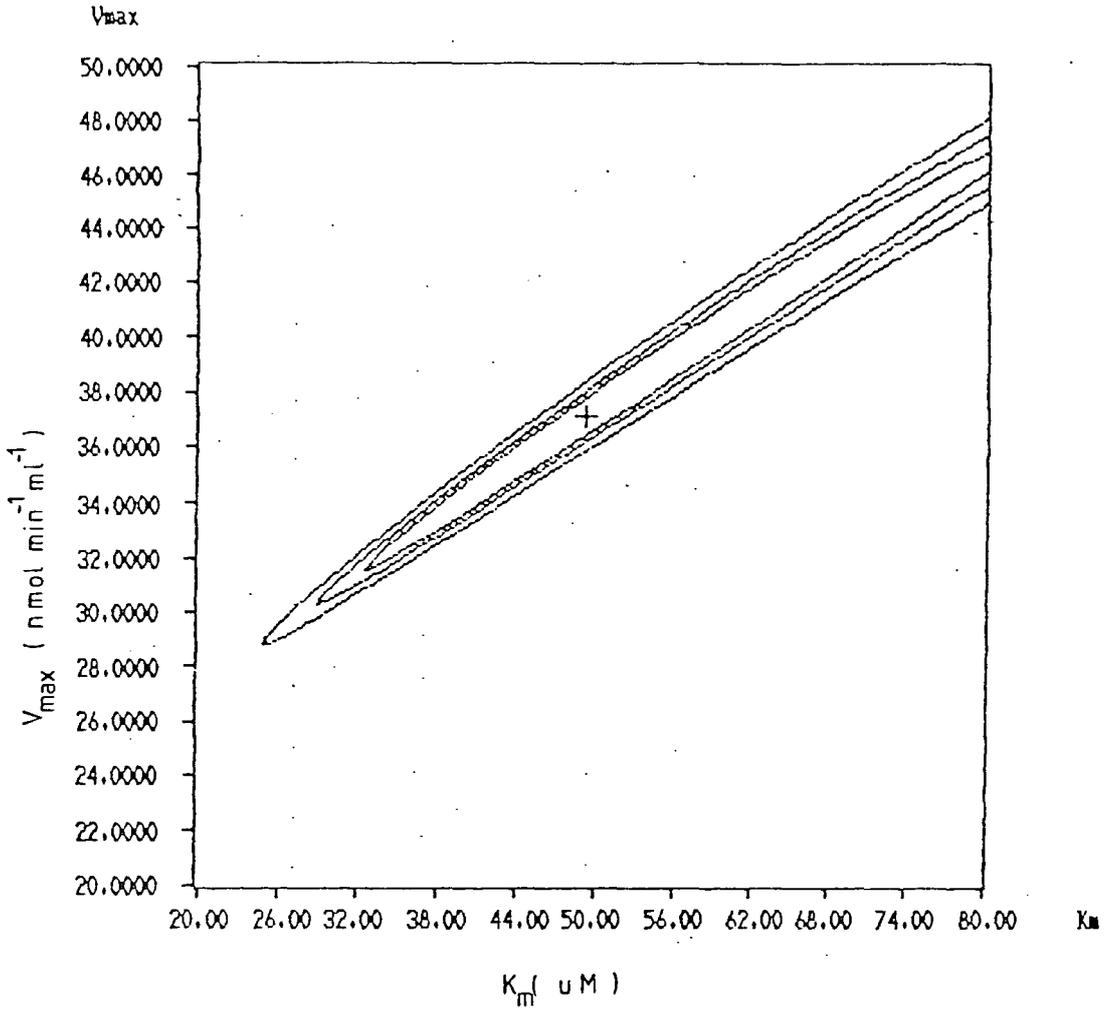

Figure 4.21   Contour Analysis for ST Analysis of DAS7C from

Grid Squares 31 – 50

Confidence limits are 95.0, 99.9 and 99.9% from inner to outer contour.

Range limits for Vmax, 20 – 50 nmol.min^{-1}.ml^{-1}, Km 20 – 80 uM.   Number

of values, 301, defines size of contour plot.

Values for kinetic parameters are obtained from ST analysis of decay

curve for file DAS7C, run from grid squares 31 – 50 (18 data points),

see Fig. 4.16.

these plots are at identical range setting, scale and confidence limits. Thus, ST analysis gives the most statistically significant values for kinetic parameters.

10)   Future Development of the System. The automated system described in these studies has great potential for studies in which a change of fluorescence is generated. The high-powered statistical analysis provides excellent analysis for systems which undergo Michaelis-Menten kinetics. Applications of the system for e.g. enzyme kinetic studies are thus, immediately apparent.

Inherent in the system is a 'lag' (which is most apparent at the start of the traces shown here), because the fluorescence does not instantaneously rise from the base to the plateau on transferring the filter assembly from PCG incubation buffer to the peptide solution, which is due to longitidinal mixing within the decay coil. Deconvolution of the system will permit data analysis (from the start of the decay curve), through introduction of a "desmearing function". A "desmearing function" is currently being developed by Dr. J.T. Gleaves and Dr. J.W. Payne.

4.5.3 Characterisation of Kinetic Parameters for Uptake

The effect of switching cells from Pro- to Pep-Medium on the kinetics of peptide transport was evaluated using the automated fluorescamine assay as described in Section 2.6.2. The transfer of cells from Pro- to Pep-Medium (Step Up) for 100 min has been previously shown to result in a marked increase in the rates of peptide uptake, both for Candida albicans strains B2630 and 6406 (Section 4.4.3).

Cells were grown overnight in Pro-Medium, harvested and resuspended in PCG buffer as described (Section 4.4.3). A portion of the cells were resuspended in Pep-Medium (typically at 0.3 (mg.dry wt.)ml^{-1}), and the remainder assayed for peptide uptake (at a cell density of 0.8 - 1.2 (mg.dry wt.)ml^{-1}) by the automated fluorescamine assay (Section 2.6.2). After a 100 - 160 min incubation in Pep-Medium, cells were reharvested and assayed for peptide uptake. This enables a direct comparison to be made for cells taken from the initial overnight culture.

The effect of incubating cells in different media on peptide transport kinetics is shown in Table 4.21. Cells grown in Pro-Medium were analyzed in the VS mode (arrow spacing of 3 grid squares), whereas cells incubated in Pep-Medium were analyzed in the ST mode. This was because ST analysis of low rates of transport (typically exhibited by cells grown in Pro-Medium) was not feasible with the data available here, as there was too little change in substrate concentration within any one run. The results in Table 4.21 imply that Step Up is accompanied by an increase in both Km and Vmax, though these preliminary analyses do not allow definite statements about the mechanism of Step Up of transport.

In related studies, the kinetics of transport of strain 6406 were examined. This strain has been shown previously to have high rates of peptide transport relative to B2630 (Section 4.3.2) and thus it was hoped that meaningful results from ST analysis, could be obtained with this strain. Cells were grown as described above, and assayed for Ala-Ala and Ala-Ala-Ala uptake. Step Up of Ala-Ala transport involved a 3-fold increase in Vmax, with approximately no change in Km, Table 4.22.

Substrate	Growth Conditions		Initial Substrate Conc.(uM)		Vmax $(nmol \cdot min^{-1} ml^{-1})$	Km (uM)
Ala-Ala-Ala	Pep-	110 min	(A)	50	55	106
	Pep-	145 min	(A)	100	34	41
	Pep-	145 min		200	40	-
Met-Met-Met	Pro-	o/n	(B)	50	2.9	-
	Pro-	o/n	(B)	20	1.9	4.6
	Pep-	150 min	(B)	20	9.3	8.6
Ala-Met	Pro-	o/n	(C)	50	3.6	-
	Pro-	o/n		20	2.7	6.2
	Pep-	120 min	(C)	50	27	56

TABLE 4.21 Effect of Transferring Cells from Pro- to Pep-Medium on Peptide Transport Kinetics

Analysis of kinetics of uptake for Candida albicans strain B2630. Cells grown overnight in Pro-Medium were analyzed in VS mode, cells incubated in Pep-Medium were analyzed in ST mode (A, B or C) cells from the same initial overnight batch, or transferred to Pep-Medium, allowing direct comparison within each group.

Substrate	Growth Medium		Mode of Analysis	Vmax $(nmol \cdot min^{-1}ml^{-1})$	Km (uM)
Ala-Ala	Pro-	o/n	ST	6.7	-
			VS	11.4	40.6
	Pep-	150 min	ST	31.9	32.4
			VS	38.8	50
Ala-Ala-Ala	Pro-	o/n	ST	3.8	-
			VS	5	16

TABLE 4.22   Effect of Step Up on Transport Kinetics for Strain 6406

Analysis of kinetics of uptake for Candida albicans strain 6406, in both ST and VS modes, for each data file.   Initial substrate concentration was 0.05 mM.

However, the kinetics of the Step Up process have not been examined in detail for this strain, so no firm conclusions can be drawn from this result.

In some cases where the initial substrate concentration was high, the transport system would be operating towards Vmax and insufficient kinetic data, (at lower substrate concentrations) would be obtained to give reliable estimates of Km. However, estimates of Vmax obtained in such a manner would be expected to be valid (Dr. J.T. Gleaves, pers. commun.).

4.5.4 Concluding Discussion

These preliminary investigations with strain B2630 imply that Step Up is accompanied by an increase in both Km and Vmax; whereas with strain 6406, Step Up of Ala-Ala involves an increase in Vmax with no change in Km.

Thus, no definite statements can be made at this point concerning the mechanism of Step Up of transport. Clearly, further work is required to define any possible changes in kinetic parameters brought about by the presence of peptides in the media. The use of a strain possessing higher rates of uptake when grown in Pro-Medium should facilitate such studies. The automated fluorescamine assay offers great potential for future studies of this nature.

4.6 Energetics of Peptide and Amino Acid Transport in Candida albicans

4.6.1 Introduction

In order to effect transport, the membrane-bound carrier of the transport system must have an asymmetrical affinity for the substrate, to permit binding of the molecule at one side, with its subsequent

translocation and release, followed by the return of the system to its original state. Thus, the carrier molecule(s) of the peptide transport system would be expected to possess a high affinity for peptides at the extracellular site and a low affinity at the intracellular location. Restoration and maintenance of asymmetry requires energy, either directly by ATP hydrolysis or by the establishment of an electrochemical gradient (proton motive force) across the membrane. This gradient may be generated either through ATP hydrolysis, or through respiratory activity.

Several studies have shown that a metabolisable energy source is required for peptide uptake, and that metabolic poisons inhibit uptake. Peptide transport in E.coli has been shown to be dependent on phosphate bond energy rather than the maintenance of a proton gradient (Payne, 1972a; De Felice et al., 1973; Cowell, 1974; Payne & Bell, 1979), whereas peptide transport has been shown to be dependent on a proton motive force in both Streptococcus faecalis (Payne & Nisbet, 1981; Nisbet & Payne, 1982) and Sacc. cerevisiae (Becker & Naider, 1977; Nisbet & Payne, 1979a; Payne & Nisbet, 1981).

These studies were aimed at a preliminary characterization of the energisation of peptide transport in Candida albicans. Use is made of reagents that are capable of either destroying the proton gradient or inhibiting the membrane-bound ATPases.

4.6.2 Properties of Metabolic Inhibitors and Fungal ATPases

The properties of the metabolic inhibitors used in these studies are reviewed below, after first considering the properties of fungal ATPases.

1)   Fungal ATPases   Yeasts possess a specific plasma membrane ATPase
     which corresponds to the proton pump of the cell. This enzyme is
     distinct from the mitochondrial ATPase in being resistant to
     oligomycin, although both enzymes are sensitive to N,N'-
     dicyclohexylcarbodiimide and diethylstilbestrol (Eddy, 1980;
     Serrano, 1980). The plasma membrane ATPase has been implicated
     in the energization of amino acid transport in yeast (Eddy, 1980)
     Fungal and plant plasma membrane ATPases have similar properties,
     their structure and reaction mechanism are similar to the ATPases
     in animal cells, catalyzing sodium-potassium and proton-potassium
     exchange, and calcium transport. However, fungal and plant
     ATPases are electrogenic proton pumps like the ATPases of
     bacteria, mitochondria and chloroplasts (reviewed by Serrano,
     1983). The plasma membrane ATPase of Neurospora crassa has been
     shown to be distinct from the mitochrondrial and vacuolar
     membrane-bound ATPases (which are very similar) (Bowman et al.,
     1978; Bowman, 1983a), this ATPase has been shown to possess two
     interacting active sites (Bowman, 1983b) The purification and
     reconstitution of fungal plasma membranes has recently been
     reviewed (Serrano, 1983) The properties of bacterial proton-
     ATPases have been reviewed (Amzel & Pedersen, 1983).

2)   N,N'-Dicyclohexylcarbodiimide   N,N'-dicyclohexylcarbodiimide
     (DCCD) is a potent inhibitor of ATPases from various sources,
     including mitochondrial ATPases from Neurospora crassa (Bowman et
     al., 1978) and plasma membrane ATPases from Sacc. cerevisiae
     (Serrano, 1980) and Neurospora crassa (Bowman et al., 1978).
     Yeast produce ATP by fermentation and mitochrondrial oxidative

phosphorylation. DCCD does not inhibit glycolysis, and in energy-starved cells reduces the rate of depletion of the ATP pool (Harold et al., 1969). DCCD was shown to inhibit the ATPase and proton pumping in a respiration-deficient strain of Sacc. cerevisiae, but did not affect ATP levels (Serrano, 1980). The ATPase enzyme has two distinct parts; the hydrophobic, membrane-bound 'F$_o$' moiety that acts as a protonophore, and an 'F$_1$' component allowing ATP hydrolysis (Fillingame, 1980). DCCD inhibits ATPase activity by covalent modification of the carboxyl group of a single glutamic acid residue within the F$_o$ proton channel. However, DCCD can also react with cysteine, tyrosine and amino groups under certain conditions, and thus there is some uncertainty over the specificity of this inhibition. The inhibitory effects of DCCD may be due, in part, to stoichiometric binding with the F$_1$ component (Yoshida et al., 1982; Kopecky et al., 1982; Ceccarelli & Vallejos, 1983), or other proteins involved in translocation (Solioz, 1984). The reactivity of DCCD may also result in the reaction of this reagent with the components of the peptide transport system.

DCCD has been shown to inhibit peptide transport in Sacc. cerevisiae (Payne & Nisbet, 1981; Nisbet, 1980; Becker & Naider, 1977) and Candida albicans (Logan et al., 1979; Davies, 1979).

3)   Diethylstilbestrol   Diethylstilbestrol (DES) is also a potent, specific inhibitor of the plasma membrane ATPase in Neurospora crassa (Bowman et al., 1978). DES is the most specific inhibitor of fungal and plant plasma membrane ATPases (Serrano, 1983). DES has been shown to inhibit peptide transport in Sacc. cerevisiae (Payne & Nisbet, 1981).

4) <u>Carbonylcyanide m-chlorophenylhydrazone</u> Carbonylcyanide m-chlorophenylhydrazone (CCCP) is an uncoupling agent (Harold, 1972) and as such, facilitates diffusion of protons across membranes. CCCP has been shown to cause a 35% reduction of cellular ATP levels in a respiration-deficient strain of <u>Sacc.</u> <u>cerevisiae</u> (Serrano, 1980), this effect being attributed to the partial dissipation of the electrochemical proton gradient, which results in activation of the proton-pumping ATPase. The fact that DES prevents the reduction of ATP caused by another uncoupler, 2-4,dinitrophenol, supports this hypothesis (Serrano, 1980). CCCP may also act as a sulphydryl reagent, inhibiting the activity of certain permeases in <u>E.coli</u> that possess essential -SH groups (Kaback <u>et al.</u>, 1974). However, the half-life of such inhibition was shown to be 20 min, whereas the uncoupling action is complete within 1 min (Cecchini & Koch, 1975). The uncoupling action of CCCP is often very rapid, inhibition of peptide transport in <u>E.coli</u> and <u>Streptococcus faecalis</u> occurring within 15s(J.W. Payne, pers. commun.). CCCP has been shown to inhibit peptide transport in <u>Sacc. cerevisiae</u> (Payne & Nisbet, 1981) and <u>Candida albicans</u> (Davies, 1980).

5) <u>Acetate</u> Acetate is not widely recognised as a metabolic inhibitor, though it is an effective disruptor of the proton gradient (Heuting & Tempest, 1978). The effect of acetate is pH dependent, the undissociated molecule (which is the predominant species below its pK of 4.8) is thought to be mobile within membranes, acting as a proton shuttle to break down proton gradients.

4.6.3 Results

4.6.3.1 Monitoring Substrate Uptake in the Presence of Inhibitors

Candida albicans B2630 was harvested and resuspended in incubation buffer (Section 4.2.2) and preincubated at 28°C in the presence of inhibitors. Stock solutions of DCCD, CCCP and DES were in ethanol, but the final concentration of ethanol in incubations did not exceed 1% (v/v). In all cases where ethanol was introduced, peptide transport was determined relative to a control containing ethanol but no inhibitor. Cells grown in Pep-Medium were preincubated with ethanol (1%, v/v) for 15 min, before addition of Ala-Ala (0.5 mM). Peptide transport was assayed by the standard manual fluorescamine procedure (Section 2.5.2). Preincubation with ethanol was shown to have no effect on the rate of peptide transport, relative to an untreated control (data not shown).

DCCD, DES and CCCP were shown to have no effect on fluorescence yield i.e. did not interfere with the fluorescamine reaction (Nisbet, 1980).

4.6.3.2 Effect of Metabolic Inhibitors on Peptide and Amino Acid Transport

The effect of DCCD, DES and CCCP on peptide and amino transport was examined in Candida albicans B2630 grown overnight in Pro- and Pep-Medium. Cells were harvested and resuspended in PCG incubation buffer (Section 4.2.2), and preincubated at 28°C for 15 min in the presence of inhibitors (0.1 - 1 mM), incubation time being increased to allow permeation of the inhibitors through the cell membrane. Transport of Ala-Ala and Leu was measured by the standard manual fluorescamine assay (Section 2.5.2), transport rates were expressed relative to that of a control determined for each experiment, and results expressed as

percentage inhibition relative to that control without inhibitor (Table4.23).

DES and CCCP were shown to give complete inhibition at a lower concentration (0.1 mM) than DCCD (1 mM). Leakage of fluorescamine-positive material was exhibited by cells incubated with either DES or CCCP.

To examine the effects of acetate on peptide uptake, cells were grown overnight in Pro-Medium and harvested by membrane filtration (Section 4.2.2). However, this procedure differed in that cells were washed on the filter with acetic acid-sodium acetate buffer (2 x 20 ml, 57 mM acetic acid, pH 4.5). Cells were resuspended in this buffer containing glucose (0.8% w/v), the final concentration of acetic acid being 28 mM. Cells were preincubated for 10 min before assaying for uptake of Ala-Ala and Leu-Leu (0.1 mM) by the standard manual fluorescamine assay (Section 2.5.2). Incubation of cells in acetate buffer resulted in the complete inhibition of peptide transport (results not shown).

4.7 Concluding Discussion

4.7.1 General Features of Peptide Transport

At the start of these studies, there had only been two previous publications concerning studies of peptide transport in Candida albicans (Logan et al., 1979; Davies, 1980), both of which followed the uptake of a limited number of radioactively-labelled peptides, together with competition studies using non-labelled substrates. Concurrent with these studies the results of several other studies were published, the work of McCarthy (1983) to some extent paralleling some of the work reported here. The results of these studies are incorporated into this discussion.

% Inhibition of Substrate Uptake

Growth Medium	Substrate		DCCD(0.1)	DCCD(1.0)	DES(0.1)	CCCP(1.0)
Pro-	Ala-Ala	(0.1)	16	100	100(L)	100(L)
	Leu	(0.5)	21	100	100(L)	100(L)
Pep-	Ala-Ala	(0.5)	13	100	100(L)	100(L)
	Leu	(0.5)	15	100	100(L)	100(L)

TABLE 4.23   Effect of Metabolic Inhibitors on Peptide and Amino Acid

Uptake in Candida albicans B2630

Candida albicans B2630 was grown overnight in Pro- or Pep-Medium,

harvested and resuspended in incubation buffer (Section 4.2.2). Cells

were preincubated with inhibitor for 15 min before addition of

substrate.   Transport rates were determined by the standard manual

fluorescamine assay (Section 2.5.2), and inhibition expressed relative

to a control without inhibitor.

(L) Leakage of fluorescamine-positive material.

Initial substrate concentrations (mM), for both substrate and metabolic

inhibitor are given in brackets.

The work presented here utilises novel fluorescent assays developed in these laboratories which offer the considerable advantage of being a direct assay of transport per se, obviating the requirement for competition studies. The majority of the competition studies described in the literature are rather subjective as no account is made of the differing affinities of different substrates for a common transport system. Thus, it is of questionable validity to perform studies with, for example, a radioactively-labelled dipeptide and an unlabelled-dipeptide (both at the same substrate concentration, typically 0.1 mM) without knowledge of their relative Km values. Similarly, absence of demonstrable competition between a labelled-dipeptide and an unlabelled-tripeptide is not conclusive evidence for the existence of separate transport systems, if no account is taken of the respective kinetic parameters for both substrate and competitor.

The use of radioactively-labelled substrates may lead to miscalculation of the true rates of peptide transport (Section 1.3.3.2). These conclusions were endorsed by the results of these studies (Section 4.3.8), which showed that the rates of both radioactively-labelled amino acids and peptides when measured by the radiotracer assay were significantly lower than when measured by the manual fluorescamine assay. This was attributed to the radioactively assayed values representing a net flux, arising from the uptake and concomitant exodus of radioactively-labelled deaminated amino acid derivatives (keto acids and fusel oils) and $[^{14}C]CO_2$ (formed by decarboxylation reactions). These conclusions were supported by the previous studies with Candida albicans. Davies (1980) reported the time-dependent loss of radioactive material (shown to be a deaminated organic derivative) following preincubation of cells with Gly-$[U^{14}C]$Phe. McCarthy (1983) measured the uptake of radioactively-

labelled Ala-Ala by both fluorescamine and label-accumulation assays,

values for kinetic parameters (Km and Vmax) were considerably changed

when estimated by the radiotracer assays. McCarthy (1983) also

demonstrated the release from cells of $[^{14}C]CO_2$ following incubation of

cells with $[U^{14}C]$Ala-Ala.

The problems of metabolism and exodus of secondary metabolites,

and loss of label in the gas phase, apply to all radiotracer assays in

which the substrate is not metabolically inert. Comparative methods to

evaluate the significance of these effects are not normally available.

Clearly, the evaluation of kinetic parameters by radiotracer

techniques and their interpretation, must be subject to an appreciation

of the associated errors involved in such assays. Of particular

relevance to these studies is the fact that the rates of $[^{14}C]$Leu

uptake by cells grown in Pro- and Pep-Medium were significantly

different, whereas when assayed using fluorescamine, no such difference

was observed. This medium-dependence was attributed to differences in

the rate of metabolism, and associated differences in the extent of

exodus of secondary metabolites. Thus, care must be taken when

comparing transport rates by cells grown in different conditions,

claims that transport rates are medium-dependent may, in fact,

represent differences in metabolism with subsequent secondary effects

on observed uptake rate.

Uptake of peptides in Candida albicans was shown not to be

accompanied by amino acid exodus (Section 4.3.3) in contrast to the

usual situation (Section 1.3.3.2). In this respect, Candida albicans

is similar to Sacc. cerevisiae (Nisbet & Payne, 1979a). The lack of

exodus from yeast is attributed to several factors. The intracellular

volume is high relative to the rate of peptide transport, so large

intracellular concentrations are not arrived at very quickly. The free

amino acid pool of Candida albicans cells appears to be similar to that

of other yeast (Shepherd et al., 1985), being of the order 260 - 440

nmol(mg.dry wt.)$^{-1}$ with arginine, aspartate, glutamine, glutamate and

alanine as the most abundant amino acids (Sullivan et al., 1983).

Similarly, Saccharomyces species have been shown to accumulate large

amounts of amino acid intracellularly; Indge et al., (1977) showed

that Sacc. uvarum could accumulate [^{14}C]glycine to a level of 1

nmol(mg.dry wt.)$^{-1}$ with little glycine entering the vacuole. In

addition, rapid intracellular amino acid metabolism in Sacc. cerevisiae

results in the exodus of deaminated secondary metabolites (Woodward &

Cirillo, 1977), this feature having obvious ramifications in the assay

of radioactively-labelled peptides (see above).

Cell extracts taken from cells preincubated with peptides were

shown to have increased intracellular pools of the constituent amino

acids, though accumulation of intact peptide was never detected. This

implies that the rate of intracellular peptide hydrolysis must be very

rapid, indicating that hydrolysis is not the rate limiting step in

transport. Nisbet & Payne, (1979a) demonstrated a similar situation in

Sacc. cerevisiae, and speculated that accumulation of peptide-derived

amino acids may lead to transinhibition of amino acid uptake, as

described by Woodward & Cirillo (1977). The intracellular peptidases

of Candida albicans have yet to be characterized in detail (Section

1.3.5). McCarthy, (1983) reported the identification of eight

cytoplasmic peptidases, six of which possessed overlapping dipeptidase

activity. Logan et al., (1983) have also reported a multiplicity of

peptidases in the yeast and filamentous forms of Candida albicans.

Intact accumulation of the peptidase-resistant peptide, Gly-Sar,

was also demonstrated. These results endorse the principle of intact

uptake of peptides followed by intracellular hydrolysis by peptidase action. Intact accumulation of Gly-Phe and alofosfalin by <u>Candida albicans</u> 6406 has also been reported (Davies, 1980).

However, evidence of intact accumulation of peptide (Gly-Sar) does not necessarily imply active transport at the plasma membrane, as it is possible to envisage a situation where there is passive accumulation across the plasma membrane followed by active accumulation by an organelle such as the vacuole. However, the demonstration that peptide uptake is completely abolished by DES, which is a specific inhibitor of the plasma membrane ATPase (Serrano, 1983), precludes this possibility. Thus, transport across the plasma membrane is an active process. The treatment of the cells required for the examination of cell extracts (Section 4.2.3) would be expected to disrupt vacuolar membranes; thus, the visualization of Gly-Sar in cell extracts does not distinguish whether or not the peptide is compartmentalized. Similar considerations apply when calculating the intracellular concentration of accumulated peptide. Thus, uptake of peptides by organelles such as the vacuole following transport across the plasma membrane cannot be ruled out, though is unlikely because of the high levels of intracellular peptidase activity.

Studies with metabolic inhibitors have demonstrated that peptide uptake requires the maintenance of a proton motive force (Section 4.6), thus resembling amino acid transport in yeast (Eddy, 1980). Preincubation with the protonophore CCCP (0.1 mM) and the ATPase inhibitors DES (0.1 mM) and DCCD (1 mM) resulted in the complete inhibition of peptide transport. However, as cells were preincubated with CCCP for 15 min, secondary effects cannot be discounted (Section 4.6.2). Payne & Nisbet (1981), reported that the addition of CCCP

(also at 0.1 mM) during uptake of Ala-Ala by Sacc. cerevisiae resulted

in inhibition of uptake within 15 sec, which was attributed to CCCP

acting as an uncoupler. Of the ATPase inhibitors, DCCD was the least

toxic, preincubation with 0.1 mM DCCD resulting in only approximately

15% inhibition (relative to controls), complete inhibition being

achieved by preincubation with 1 mM DCCD. This concentration effect is

in agreement with the report that DCCD penetrates the plasma membrane

to the ATPase relatively slowly (Serrano, 1980), and that DCCD was

reported to be relatively slow in inhibiting peptide uptake in Sacc.

cerevisiae (Payne & Nisbet, 1981). Preincubation with both DES and

CCCP resulted in the exodus of fluorescamine-reacting material, which

was taken to be a generalized efflux, similar to that reported by Payne

and Nisbet, (1981).

Peptide transport was shown not to be dependent on an external

glucose supply which is in contrast to the situation in Sacc.

cerevisiae (Nisbet, 1980), where cells could be more easily starved and

uptake rate was shown to increase with glucose concentration up to 0.4%

(w/v). This presumably reflects the presence (under the conditions of

this study) of adequate endogenous energy supplies to drive substrate

uptake. Both glycogen and trehalose have been reported to act as

energy reserves in Candida albicans (Sullivan et al., 1983). It is

also possible that strain B2630 may utilize citrate (in the PCG

incubation buffer) as a metabolizable energy source, utilization of

citrate as a carbon source by this yeast having been reviewed (Odds,

1979).

Thus, these studies have clearly established the principle that

peptide uptake by Candida albicans is by an active process; uptake of

intact peptide occurring via a saturable system which has a requirement

for metabolic energy. Thus, the generally accepted criteria for the demonstration of active transport have been satisfied.

Transport was shown to be pH dependent, with an optimum of pH 4.5. Peptide uptake has been shown to be pH dependent in Sacc. cerevisiae, with a pH optimum of 5.5 for Met-Met-[^{14}C]Met uptake by strain ATCC 9896 (Becker & Naider, 1977) and pH 4.5 for Ala-Ala uptake by strain Σ 1278b (Nisbet, 1980). Peptide uptake by Candida albicans has also been shown to be pH dependent, with reported optima of 3.5 for Met-Met-[^{14}C]Met uptake by strain WD18-4 (Logan et al., 1979), 4.5 for radioactively-labelled Ala-Ala and Gly-Phe by strain 6406 (Davies, 1980) and 5.0 for Ala-Ala uptake by strain 124 (McCarthy, 1983). The ionization of the substrate will be affected by the pH of the medium, and thus affects the affinity of the substrate for the permease. The extracellular pH value will also affect the proton gradient across the plasma membrane, thus affecting the proton motive force, on which transport is dependent.

The optimal temperature for uptake was not determined here. Davies (1979) reported that uptake was temperature dependent between 20 and 40°C, with an optimal value of 37°C and Q_{10} value of 2.7.

Considerable variation in the rates of peptide uptake has been found for different strains of Candida albicans (Section 5.5.2), strains 6406 and A exhibited higher rates of uptake than B2630. This variation may be a result of differing capacities for uptake per se or possibly be due to variations in the porosity of the cell wall. Similar strain variation in Candida albicans has been reported in Candida albicans, McCarthy (1983), whereas uptake rates exhibited by different strains of Sacc. cerevisiae are fairly constant (Nisbet & Payne, 1979a).

In these studies, it was sometimes noted that considerable variation in peptide transport rate was found for batches of cells grown and harvested under nominally identical conditions (Section 4.3.6). Similar variation occurs in Sacc. cerevisiae (Becker & Naider, 1977; Nisbet & Payne, 1979a), though was not found in an earlier study with Candida albicans (McCarthy, 1983) where uptake rates were reported to be remarkably constant. Uptake rates did not correlate with the stage of exponential growth at which cells were harvested (Section 4.3.5). Peptide transport in Candida albicans was shown to be subject to ammonia repression (Section 4.3.2), and therefore it is possible that variations in rate may be due to fairly small variations in the internal and external levels of nitrogenous metabolites (as suggested by Nisbet, 1980)

4.7.2 Specificity of Peptide Uptake - A Basis for the Rational Design of Anticandidal Agents

Much of the interest in peptide transport in Candida albicans has been prompted by the possibilities of utilising the peptide permease(s) as carriers for antifungal agents, i.e. 'illicit transport'. The concepts of 'illicit transport', 'smugglin' of impermeant molecules and 'warhead delivery systems', have been reviewed (Section 1.4.1) together with a review of peptide drugs and their properties. To aid the design of such anticandidal agents, a detailed knowledge of the specificities of the peptide transport systems is required. In these studies, the rates of uptake of a selected range of substrates was determined with the view to defining the structural limitations of transportable peptides.

Peptide transport was found to be highly specific for L-stereoisomers, uptake of peptides containing D-residues being

undetectable, this finding endorsing previous reports (Logan et al.,

1979; Davies, 1980; McCarthy, 1983). Evidence for the tolerance of

D-residues by certain strains has been reviewed (Section 1.3.6.2),

though this feature was not detected in these studies. Stereochemical

selection takes place at the level of binding rather than transport,

i.e. binding is not always accompanied by subsequent uptake (Section

1.3.6.2)

Strain B2630 was able to take up a range of di- to pentapeptides,

the transport rates of tri-, tetra and pentaalanine being higher than

the rate of Ala-Ala uptake. The size limit for transport could not be

determined here as the next oligomer, hexaalanine, is not sufficiently

soluble. Strains A and 6406 were able to take up di- to tetraalanine,

though the possibility that pentaalanine was transported was not

determined (Section 5.5.2). This finding is in contrast to the report

by Davies (1980) that uptake of radioactively-labelled tetraalanine by

6406 was not detected though this peptide was able to compete with the

uptake of di- and tripeptides.

The rates of transport of a representative range of peptides was

determined in order to investigate the effects of different side chains

on transport. Peptides with hydrophobic side chains are generally

better substrates for transport than those containing acidic or basic

residues. Glycyl-peptides were generally poor substrates, transport of

tri- and tetraglycine being undetectable. These results are in

agreement with earlier reports which indicated hydrophobic peptides

have the greatest affinity for transport (Davies, 1979, 1980;

McCarthy, 1983). However, it must be stressed that in all these

studies no account was taken of the kinetic parameters for uptake;

transport was measured at a fixed initial substrate concentration (here

0.1 mM), initial rates of uptake were determined, comparisons and

conclusions were reached. The recent development of the automated fluorescamine assay and the interfacing of a microcomputer for detailed kinetic analysis of transport date (Section 2.6), should enable the kinetic parameters (Km and Vmax) to be determined for a range of representative peptides. The effects of different side chains on uptake could then be determined more precisely. However, studies such as those described here clearly indicate the preference of the transport system for hydrophobic substrates.

The effects of both N- and C-terminal modification have been discussed earlier (Section 1.3.6.2). Esterification and amidation of the C-terminus were shown to prevent uptake (Logan et al., 1979; McCarthy, 1983), though some evidence that substitution of the carboxyl moiety with phosphonate, sulphonate or tetrazole groups was tolerated (Davies, 1980). Thus, there is a requirement for a free acidic group at the C-terminal for transport of the substrate to occur. In contrast, modification of the N-terminal is tolerated, evidence being provided by transport studies with N-terminal-substituted peptides (Logan et al., 1979; McCarthy, 1983) and sensitivity of Candida albicans to N-terminal-substituted toxic peptides (Section 1.4), e.g. N^4-(succinyl Ala-Leu)-5-fluorocytosine (Ti et al., 1980) and N-acylated epoxypeptides (Chmara et al., 1980). However, there is evidence for strain variation as Davies (1980), reported that N-acylated peptides did not compete for uptake with normal radioactively-labelled substrates.

Modification of amino acid side chains is tolerated by the peptide transport system in Candida albicans, evidence being provided by the demonstration that this yeast is sensitive to a range of peptide drugs with unusual side chain moieties (see Sections 1.4 and 5.5). The polyoxins (Section 1.4.4.2), nikkomycins (Section 1.4.4.3) and

bacilysin (Section 1.4.2.1) are toxic peptides with a diverse range of side chain moieties, all inhibit <u>Candida albicans</u> and all have been shown to be transported by the peptide permeases (Chapter 5). All amino acids in these peptides are in the L-configuration, in accordance with the stereospecificity exhibited by the peptide transport system(s). The sensitivity of <u>Candida albicans</u> to m-fluorophenylalanyl-substituted peptides (Section 1.4.2.4), peptide pyrimidine conjugates (Section 1.4.2.6), and α-glycine-substituted peptides (Section 1.4.2.7) is further evidence for broad side chain specificity.

Evidence for a general requirement for an α-linkage has been reviewed (Section 1.3.6.2).

The characterization of the molecular requirements for transport provide the basis for the rational design of synthetic anticandidal peptides. Those molecular determinants essential for transport are summarised below :-

1) Peptides must contain L-stereoisomers, though there is some possibility of positioning D-residues at the C-terminus, but this would be expected to reduce the rate of uptake.

2) Peptides must contain α-linkages.

3) An optimal chain length of 2 - 4 residues is suggested to avoid possible problems with solubility of hydrophobic peptides. Consideration must be made of the hydrodynamic volume of the peptide.

4) Substitution of the C-terminus with a different but similar group is permitted. Certain modifications of the C-terminus are not allowed, e.g. amidation and esterification.

5)   Certain modifications of the N-terminus are acceptable, e.g.

      acetylation and acylation.

6)   Side chain specificity is very broad, linkages to other molecules

      (e.g. toxic warheads) is acceptable.

Recent advances in computer-assisted drug design offer possibilities

for the rational design of anticandidal agents, and appreciation of the

molecular determinants of transport is thus essential for such studies.

4.7.3 Regulation of Peptide Transport in Candida albicans

      Peptide transport has been shown to be subject to ammonia

repression (Section 4.3.2), transport of peptides being undetectable

when cells were grown with ammonium as a sole nitrogen source. Ammonia

repression of peptide transport in Candida albicans has also been

demonstrated using radioactively-labelled substrates (Logan et al.,

1979 Davies, 1980) and by fluorescamine analysis (McCarthy, 1983);

and in Sacc. cerevisiae (Becker & Naider, 1977; Nisbet & Payne,

1979a).

      In addition to regulation of peptide transport by ammonia

repression, a second mechanism of regulation dependent on the presence

of substrate (peptides), has been demonstrated by these studies.

      There have been other reports in the literature that growth of

cells in rich media (containing peptones), leads to higher rates of

peptide uptake (Davies, 1979; McCarthy, 1983). Here it has been

clearly shown that peptide transport activity can be expressed at three

levels dependent on growth medium. Cells were grown overnight in

simple defined media (all contained Yeast Nitrogen Base without amino

acids or ammonium sulphate, and glucose), supplemented with different

nitrogen sources (ammonium sulphate, Casein Acid Hydrolysate, proline

and Bacto-Peptone). The presence of peptone was shown to result in high rates of Ala-Ala transport for strains B2630, A, and 6406 (Table 4.2). Further examination of this phenomenon showed that uptake of a representative range of di- and tripeptides was increased by overnight growth in Pep-Medium (relative to cells grown overnight in Pro-Medium) (Table 4.8). Interestingly, when rates were expressed relative to that of an Ala-Ala control (i.e. as a percentage of the rate of uptake for Ala-Ala), the ratios were fairly similar for uptake of a particular peptide by cells grown in either Pro- or Pep-Medium.

More detailed studies were carried out with strain B2630 in an attempt to determine the mechanism of this 'substrate' regulation. The effect of overnight growth in different defined media supplemented with different nitrogen sources was examined. Three levels of peptide transport were demonstrated, a minimal level due to ammonia repression, an enhanced level exhibited by cells grown on a poor nitrogen source (i.e. amino acids, either in a single amino acid (proline) or a mix of amino acids (Casein Acid Hydrolysate)) and a high level due to the stimulatory effect of peptides (Pep-Medium)(Table 4.9). The presence of peptides was shown to override ammonium repression of Ala-Ala transport (Table 4.9, Experiment 6). Overnight growth in a medium containing both Bacto-Peptone and proline, resulted in the same rate of Ala-Ala transport as by cells grown overnight in Bacto-Peptone alone (Table 4.9, Experiment 7).

The timing of the changes in peptide transport rates was examined by switching cells from Pro- to Pep-Medium (Step Up) and from Pep- to Pro-Medium (Step Down). Step Up of cells from NH_4-, Casein Acid Hydrolysate- or Pro-Medium to Pep-Medium (for a 100 min incubation)

resulted in an increase in peptide transport, equivalent to the rate exhibited by cells grown overnight in Pep-Medium (Table 4.10). Cells transferred from overnight growth in Pro-Medium to medium containing Bacto-Peptone and ammonium sulphate (Pep- + NH_4-Medium, Table 10, Experiment 3), also exhibited high rates of Ala-Ala transport. Step Down of cells from Pep- to Pro-Medium resulted in a decrease in peptide transport, equivalent to the rate exhibited by cells grown overnight in Pro-Medium (Table 4.10, Experiment 7).

Experiments in which cells were transferred from Pro- to Pep-Medium and assayed at 100, 200 and 300 min showed no increase in the rate of Ala-Ala transport after the initial assay at 100 min. (Table 4.11). A more stringent analysis of both Step Up (Table 4.12) and Step Down (Table 4.13), showed that both processes occur within 30 min of transfer from one medium to another. Step Up and Step Down was clearly demonstrated by monitoring Gly-Phe transport (Table 4.14). However, transfer of cells from overnight growth in Pro-Medium to a medium containing Ala-Ala (0.2% w/v) as the sole nitrogen source, for 100 min, did not result in an increase in Ala-Ala or Ala-Ala-Ala transport, conditions in which Step Up of transport for both substrates was shown to occur for cells transferred to Pep-Medium (0.2% w/v peptone). Thus in one test case, exposure to a single defined peptide (Ala-Ala) by itself cannot elicit the Step Up mechanism (at least during a 100 min. incubation), suggesting that the increase in transport rates is more likely dependent on a combination of peptides such as are present in Bacto-Peptone or Trypticase-Peptone. Cells grown overnight in Casein Acid Hydrolysate exhibited peptide transport rates equivalent to those exhibited by cells grown in Pro-Medium (Table 4.9). Thus, the

molecular form of the supplemented nitrogen source is important, the total amino acid content of Casein Acid Hydrolysate and Trypticase-Peptone (both digests of Casein) would be expected to be very similar. The peptide composition of the two brands of peptone used in these studies were not specified, giving no indication as to the type (chain length ?) of peptide that is responsible for eliciting the Step Up phenomenon. Isolation of the component peptides from Bacto-Peptone on the basis of molecular weight, and testing their capacity to elicit Step Up of transport would thus be of considerable interest.

Step Up was shown to be independent of protein synthesis (Section 4.4.5). Treatment of cells with the protein synthesis inhibitor, cycloheximide; was shown to have no effect on the peptone induced increase in the rates of uptake of the dipeptides Ala-Ala and Gly-Phe (Table 4.16). In related control experiments, cycloheximide was shown to inhibit the growth of Candida albicans B2630 (Fig. 4.9), and to inhibit the incorporation of radioactively-labelled amino acids (both Phe and Leu, Figs. 4.10 and 4.11 respectively) into trichloroacetic acid-precipitable material. These results were attributed to the inhibition of protein synthesis by cycloheximide. Thus, it was concluded that the observed Step Up in peptide transport activity observed when cells are transferred to peptone-based medium, does not require de novo synthesis of cycloheximide-inhibitable proteins.

In further studies, the stimulatory effect of peptone on transport was shown to be specific for peptides, cells grown overnight in Pro- or Pep-Medium were shown to have equivalent rates of amino acid (Gln and Leu) uptake (Fig. 4.6).

Thus, the mechanism of regulation of peptide transport in <u>Candida</u> <u>albicans</u> is clearly distinct from the mechanism of amino acid transport in yeast (Section 1.3.2.3), though is still subject to ammonia repression in the absence of peptone. The isolation of a bacilysin-resistant mutant that was shown to be defective in dipeptide transport whilst retaining typical wild-type rates of oligopeptide uptake, and was unable to utilise dipeptides as a sole nitrogen source (Chapter 5), clearly indicates the existence of separate transport systems for dipeptides and oligopeptides in <u>Candida</u> <u>albicans</u>. Discussions concerning the regulation of peptide transport must take into account the existence of separate systems. These studies were concerned principally with the effects of peptone on the kinetics of Step Up and Step Down of the transport of dipeptides (Ala-Ala and Gly-Phe), therefore comparable statements concerning the kinetics of regulation of the oligopeptide transport system cannot be made with the same degree of precision. Future studies are required to determine whether identical kinetics hold for regulation of the oligopeptide transport system.

Before speculating on possible mechanisms whereby these interacting regulatory circuits might be controlled and activated it is pertinent to consider related studies in the literature on regulation of amino acid transport in yeasts. Early studies on the regulation of amino acid transport have been reviewed (Section 1.3.2.3). Regulation of amino acid transport in yeasts has been widely studied, particularly the activity of the general amino acid transport system (Gap). The Gap system is synthesised when <u>Sacc.</u> <u>cerevisiae</u> is grown on a medium containing a poor nitrogen source, such as proline, but not when it is

grown in a medium containing a good nitrogen source such as ammonium ions (Grenson et al., 1970; Grenson & Hou, 1972; Grenson, 1983b). The Gap permease has been shown to be regulated by control of synthesis and control of activity (Courchesne & Magasanik, 1983; Grenson, 1983a,b). Grenson (1983a), demonstrated a reversible-inactivation process which progressively develops upon addition of ammonium ions to a proline-grown culture, completely suppressing transport activity within one hour. Repression of the formation of active permease was also demonstrated in the presence of ammonium ions (Grenson, 1983a). However, organisms grown in ammonia-containing media can be derepressed for Gap activity (Woodward & Cirillo, 1977), though for derepression to occur, organisms were required to be phosphate-sufficient.

The degree of plasma-membrane phospholipid unsaturation has also been shown to affect expression of the GAP permease in Sacc. cerevisiae (Calderbank et al., 1985), and the activity of several other amino acid permeases (Calderbank et al., 1984; Keenan et al., 1982). The GAP permease in Sacc. cerevisiae consists of a plasma membrane-bound complex of probably three proteins, which effects transmembrane transport of a number of amino acids, together with a loosely-bound, periplasmic binding protein (Woodward & Kornberg, 1980). A number of proteins present in the periplasmic and plasma-membrane fractions of wild-type cells that are derepressed for the GAP system, are absent from a Gap mutant (isolated on the basis of resistance to D-amino acids), and are absent from wild-type cells grown under conditions in which the GAP system is repressed (Woodward & Kornberg, 1980). Activity of the Gap system has been shown to be inhibited by the uptake and accumulation of its substrate amino acids, i.e. transinhibition

(Woodward & Cirillo 1977). Cells in which uptake of amino acids has been inhibited by the prior uptake of valine, remain inhibited for some time even after the valine initially taken up has been metabolized (Woodward & Cirillo, 1977; Woodward & Kornberg, 1981). Under conditions in which the intracellular amino acid 'pools' can be shown to have no direct inhibition of Gap activity, Woodward & Kornberg (1981), demonstrated that inhibition is associated with a change in the membrane component of the Gap system, and that recovery from the inhibition involves the resynthesis of this protein.

The exact mechanism for controlling the activity of preformed permease remains unclear. Activities of the Gap and proline-specific permease (PUT4), are inactivated by addition of ammonia or glutamate, lowering the activity to that found during steady-state growth on these nitrogen sources (Courchesne & Magasanik, 1983). These workers isolated mutants altered in the regulation of the PUT4 permease; certain of these mutations were pleiotropic, affecting other permeases, though they had no direct effect on various cytoplasmic enzymes involved in nitrogen assimilation. In one class of mutations, ammonia inactivation of Gap and PUT4 did not occur, whereas inactivation by glutamine did, indicating two independent systems. The inactivation systems were shown to be constitutive, and not to require transport of the effector molecules per se, apparently operating on the inside of the cytoplasmic membrane (Courchesne & Magasanik, 1983). Grenson (1983a), proposed that two distinct regulatory mechanisms are responsible for the absence of Gap activity in cells grown in the presence of ammonium ions. One is by a reversible inactivation process which progressively develops on addition of ammonium ions to a proline-

grown culture. and the second is by repression of formulation of active

permease. The second regulatory mechanism is absent in mutants

affected at the GDHCR locus (mutations at this locus suppress nitrogen

catabolite repression of all nitrogen catabolic enzymes tested, and

most of the ammonia-sensitive permeases are active), which might code

for a repressor molecule; and in the presence of a Gln^{ts} mutation

(temperature sensitive glutamine synthetase) suggesting glutamine as an

effector. Grenson (1983b), proposed a tentative scheme the regulation

of the GAP permease through a double regulatory mechanism involving

inactivation-reactivation and repression of the uptake system. The Gap

permease is also subject to feed-back inhibition by its amino acid

transport substrates (Crabeel & Grenson, 1970; Woodward & Cirillo,

1977). Thus, regulation of amino acid transport systems in Sacc.

cerevisiae is very complex, and awaits full characterization.

Other aspects of regulation of nitrogen metabolism and gene

expression have recently been reviewed (Marzluf 1981 Harder &

Dijkhuizen, 1983 Cooper 1982a,b). Cooper & Sumadra (1983), have

proposed that nitrogen catabolite repression is functionally a long-

term response to rather stable changes in the environment, short-term

responses to more subtle environmental variations probably occurring by

one or more distinct mechanisms Control of transport accounts for

differing proportions of the overall level of control for metabolism of

various substrates (Cooper & Sumrada, 1983)

Finally, therefore, what mechanism might be operative with

regard to regulation of peptide transport in Candida albicans ? We

have shown that the peptide transport activity increases very rapidly

on transfer from Pro- to Pep-Medium This increase could not be

attributed to de novo synthesis of permease, as the Step Up of

transport was not inhibited by treatment of cycloheximide. Thus, the increase in transport rates presumably must occur by an activation process of preformed transport components. Step Down of transport was also shown to be very rapid, which must presumably be due to inactivation of the system, rapid repression of synthesis alone would still leave 'active' permeases in the plasma membrane, and transport activity would be expected to decrease exponentially with each generation as permeases are diluted out with each cell division. The rapid reversible-activation process is therefore analagous to the mechanism of control of Gap activity proposed by Grenson (1983a), though it is clearly distinct.

A possible mechanism of regulation of peptide transport that is particularly appealing and appears to satisfy many of the experimental observations, is one involving an exocytotic insertion and endocytic retrieval of preformed transporters (reviewed by Leinhard, 1983). This mechanism of control has been invoked to explain insulin stimulation of glucose transport in fat and mucosa cells, histamine stimulation of acid secretion in the stomach mucosa, and vasopressin enhancement of water permeability across bladder and kidney epithelia. Whether a similar mechanism can account for substrate regulated peptide transport activity in Candida albicans remains to be seen. Experimental validation would at the least require demonstration that already synthesised peptide transport proteins are somehow mobilised from their 'dormant location' in membrane vesicles and inserted into the plasma membrane. Confirmation or otherwise of this mechanism appears an experimentally obtainable goal.

In summary, therefore, these studies have shown that peptide transport in <u>Candida</u> <u>albicans</u> is regulated at several levels, ammonia repression, general nitrogen status and by substrate availability. The stimulatory effect of peptides overrides control by ammonia repression, and involves a rapid reversible-activation of transport activity, which does not involve <u>de</u> <u>novo</u> synthesis of permease.

CHAPTER 5

ISOLATION AND CHARACTERIZATION OF MUTANTS RESISTANT

TO TOXIC PEPTIDES

CHAPTER 5   ISOLATION AND CHARACTERIZATION OF MUTANTS RESISTANT TO

TOXIC PEPTIDES

5.1   Introduction

As part of the study aimed at optimising the carrier structure

for anticandidal drugs, it was essential to define more clearly the

number and nature of the peptide permeases in Candida albicans. To

this aim a series of representative strains was tested for sensitivity

to polyoxins, the most sensitive strain was then used as a test-strain

for attempts to isolate and characterize drug-resistant mutants.   The

isolation of such mutants is an essential part of the determination of

the number of systems present, for competition studies may give

misleading results unless the kinetic parameters of the competing

substrates have been well defined, and even then different systems may

have overlapping specificities.   The isolation of specific transport-

deficient mutants and their characterization should in principle

clarify the nature of the systems present in Candida albicans.

5.2   Materials and Methods

5.2.1   Organisms and Growth Conditions

Candida albicans and Saccharomyces cerevisiae strains used in

these sensitivity tests and mutant isolation studies were as described

(Section 2.8.2.1).   Candida albicans B2630 was used as the parental

wild type for mutant isolation studies.

Organisms were maintained as master streaks on Pro-Medium plates,

as described (Section 2.8.2.1), and in liquid Pro-Medium for

subculturing as required.

Strains were lyophilized for storage, cells were grown in Pro-

Medium overnight (150 ml) to mid-exponential phase, harvested by

centrifugation (6000 rpm for 30 min at 10°C, MSE-18 Centrifuge), and the supernatant discarded. The cells were resuspended in sterile nutrient cryoprotectant (500 ul, containing foetal calf serum, 60% v/v, and glucose, 8% w/v). Aliquots of the suspension (4 x 100 ul) were transferred to sterile soda glass tubes, the suspension frozen by suspending the tubes in liquid nitrogen, and then freeze-dried overnight before sealing the tubes under vacuum. Viability of cells was not markedly affected by this treatment.

5.2.2 Toxic Peptides

Dipeptidyl polyoxin B and D, and tripeptidyl A (approximately 8 mg of each) were a gift from Dr. K Isono, Institute of Physical and Chemical Research, Wako-Shi, Japan, a sample of polyoxin D (3 mg) was purchased from Calbiochem. Basic and acidic polyoxin fractions were prepared from a crude mixture (Polyoxin Z) as described (Chapter 3). Bacilysin (2 mg) was a gift from Prof. E.P. Abraham, Oxford University and Nikkomycin (X + Z mixture, approximately 10 mg) was from Prof. G.W. Gooday, Aberdeen University, m-fluoro-phenylalanylalanine and m-fluoro-phenylalanylalanylalanine (approximately 10 mg of each) were from Dr. W.D. Kingsbury, Smith Kline & French, Philadelphia, and the corresponding fluoro-amino acid, m-fluorophenylalanine was from Sigma Chemical Company Ltd.

The limited availability of these materials precluded extensive drug sensitivity screening, and necessitated the development of micro-assays (Section 2.9) to minimise peptide use.

5.2.3 Isolation of Mutants Resistant to Toxic Peptides

Candida albicans B2630 was grown in Pro-Medium to about 3 - 7 x 10^7 cells ml^{-1}, and harvested by centrifugation (M.S.E., Microcentaur) in sterile plastic Eppendorf centrifuge tubes (1 x 1 ml). Cells were

diluted with PCG incubation buffer pH 4.5 (Section 4.2.2) to approximately $5 \times 10^6 - 2 \times 10^7$ cells ml^{-1} and 0.1 ml samples were spread onto Pro-Medium plates to produce an even lawn of cells ($5 \times 10^5 - 2 \times 10^6$ colony forming units plate^{-1}).

Drug sensitivity discs containing basic polyoxin fraction (5 mg), bacilysin (0.2 mg), nikkomycin (0.4 mg) or m-fluoro-phenylalanylalanylalanine (FPAA, 0.6 mg) were placed at the centre of the plates, which were incubated at 30°C for 22 – 45 h. Colonies growing within the inhibition zones were picked, streaked for single colonies, incubated for 45 h at 30°C, and from representative single colonies master streaks were produced (on Pro-Medium plates). Presumptive mutants were then screened for peptide sensitivity using the radial streak assay (Section 2.9.4). Strains were also maintained in liquid Pro-Medium for subculturing as required. Resistant mutants had the same growth rate as B2630 in liquid Pro-Medium (160 min). A list of isolated mutants with their designated strain numbers is given in Appendix II.

5.2.4 Patch-Plate Assay for Nitrogen Assimilation Studies

The ability of parental wild-type strains, peptide-resistant mutants and their corresponding revertants to utilize Ala, Ala-Ala, and Ala-Ala-Ala (all 1 mg ml^{-1}) as a sole nitrogen source, was examined relative to growth on Pro-Medium plates (proline, 4 mg ml^{-1}) and a plate with no supplemented nitrogen source. (All plates contained glucose, 2% w/v, Yeast Nitrogen Base without amino acids and ammonium sulphate, 1.7 mg ml^{-1}, and Bacto-Agar 1.5% w/v). To minimise the use of expensive di- and tripeptides, small petri dishes (10 ml agar, 5 cm diam) were used for some of these studies.

Routinely, a grid pattern (1 cm^2 for standard plates, 0.8 cm^2 for small plates) was marked onto the back of each plate to facilitate patching. Cells were picked from fresh master streaks and patched onto the plates using a fine platinum needle (excess cells being removed by a preliminary streak onto an agar plate with no supplemented nitrogen source). Plates were incubated at 30°C for 45 h before assessing growth. Parental strains and the original drug-resistant parent of revertant strains were always included on the plates.

5.2.5 Methods for Mutagenesis of Candida albicans

5.2.5.1 Introduction

In an attempt to increase both the frequency and to produce different classes of drug resistant mutants, Candida albicans was mutagenised before exposing the cells to the toxic peptides, mutants being selected for by the standard procedure (Section 5.2.3). Although the frequency of transport-deficient mutants is high (c.a. one in 2 x 10^4) it was hoped that mutagenesis would produce different classes of resistant mutants for biochemical characterization.

Yeast (Saccharomyces cerevisiae) are commonly treated with such mutagens as ultraviolet radiation (UV), nitrous acid, ethylmethanesulphonate (EMS), diethyl sulphate and 1-methyl-1-nitro-nitrosoguanidine in order to enhance the frequency of mutations. These mutagens are remarkably efficient and can induce mutations at a frequency of 5 x 10^{-4} to 10^{-2} per gene without a great deal of killing (Sherman et al., 1983). Candida albicans B2630 was treated with both UV and EMS according to established methodology (Sherman et al., 1983; Lindegren et al., 1965), and polyoxin-resistant mutants selected for and isolated by the standard method. EMS mutagenesis methodology, the only procedure proving to be useful, is presented in the following section.

5.2.5.2 EMS Mutagenesis

The alkylating agent EMS was used here to mutagenise <u>Candida</u>

<u>albicans</u> B2630 prior to selection of polyoxin-resistant mutants. The

efficiency of EMS as a mutagen of <u>Candida</u> <u>albicans</u> was first

investigated in order to optimise incubation time with the agent. In

<u>Sacc</u>. <u>cerevisiae</u> optimal mutagenic effect was achieved by a 70 min

incubation with EMS (3% v/v), during which 90% of the original cell

population was killed (Lindegren <u>et</u> <u>al</u>., 1965).

The 'killing curve' of <u>Candida</u> <u>albicans</u> strain B2630 by EMS

(2.5%) treatment is shown in Fig 5.1. 5 ml of an overnight Pro-Medium

grown culture (approx. 3.2×10^7 cells ml^{-1}) was added to fresh Pro-

Medium (5 ml), containing EMS (5% v/v) pre-equilibrated at $28^{\circ}C$, and

the suspension incubated at this temperature for up to 100 min.

Samples (0.2 ml) were removed periodically, diluted as required with

PCG incubation buffer, pH 4.5, and spread onto Pro-Medium plates for

viable counting. A plot of viable count (cells $ml^{-1} \times 10^6$) against

period of incubation with EMS (2.5 % v/v) indicated that a 90% kill was

achieved after an approximately 90 min incubation period with EMS.

<u>Candida</u> <u>albicans</u> B2630 was pretreated with EMS (2.5% v/v) prior

to selection of presumptive polyoxin-resistant mutants by the following

procedure. Cells (5 ml, 4×10^7 cells ml^{-1}) from an overnight Pro-

Medium grown culture were added to fresh Pro-Medium (5 ml), containing

EMS (5% v/v). Cells were preincubated at $28^{\circ}C$ for 90 min, after which

time a sample (0.4 ml) was added to PCG incubation buffer pH 4.5 (3.6

ml) Cells (4 x 1 ml) were harvested by centrifugation (M.S.E.

Microcentaur), the supernatant discarded, and the pellets combined and

resuspended in PCG incubation buffer (1 ml total). Cells were

reharvested, the supernatant discarded and the pellet resuspended in
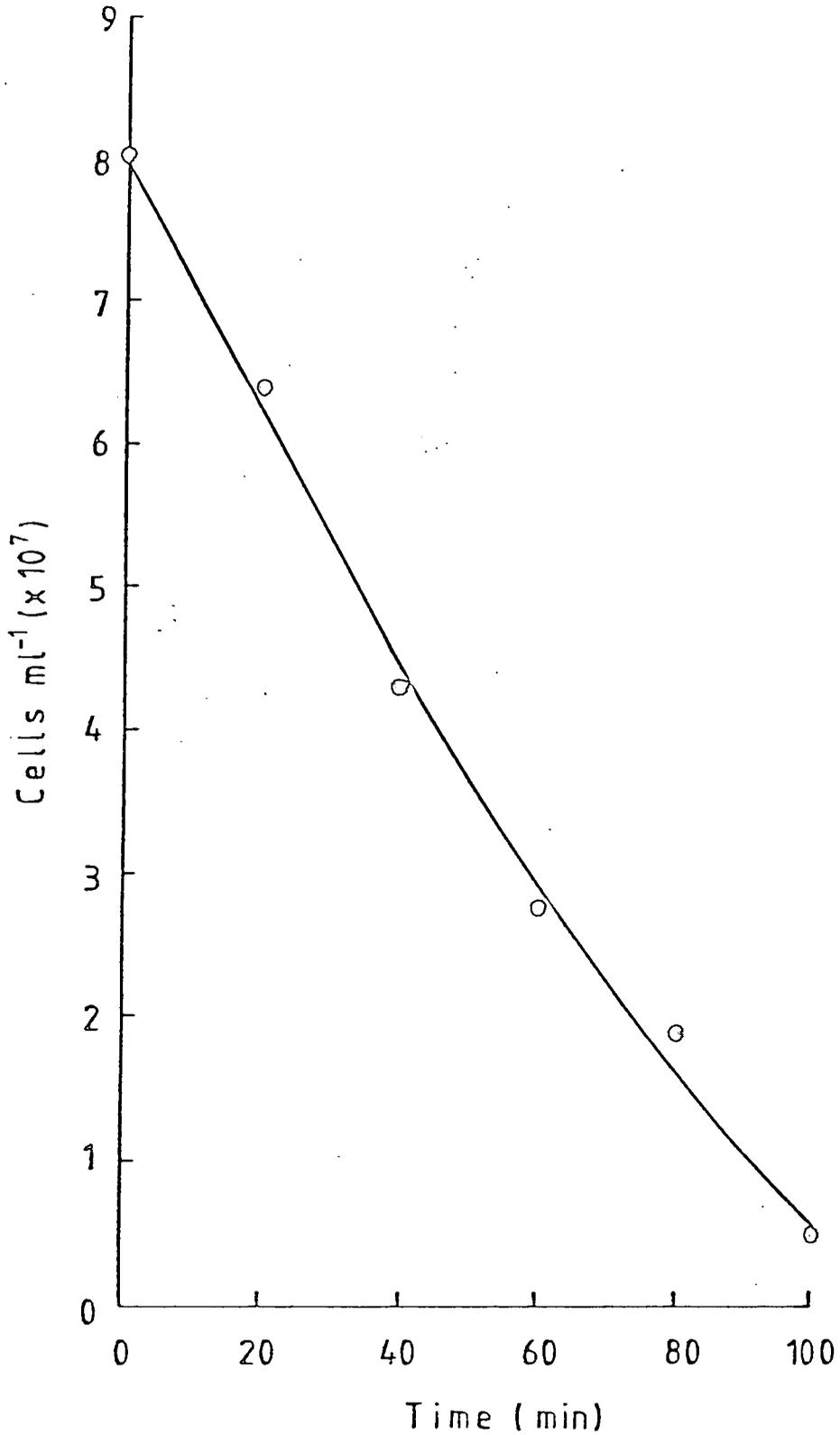
Figure 5.1   The Lethal Effect of EMS on Candida albicans B2630

Candida albicans B2630 (10 ml, 1.6 x 10^7 cells ml^{-1}) was incubated with

EMS (2.5% v/v) for up to 100 min.   Samples (0.2 ml) were taken

periodically for dilution and determination of viable counts, according

to the text (Section 5.2.5.2).

PCG incubation buffer (0.1 ml). A sample (90 ul) was then immediately spread onto Pro-Medium plates for selection of polyoxin-resistant mutants (Section 5.4.2). A 90% kill is expected from the 90 min incubation with EMS, therefore the above procedure would be expected to produce a lawn of 7.2 x 10^5 cells plate^{-1}, i.e. equivalent to the numbers of cells laid down in the standard method (Section 5.2.3).

The three dilution and washing stages in the above procedure, would be expected to remove most of the EMS. Such a large dilution factor removed the need to inactivate the EMS with sodium thiosulphate (Lindegren et al., 1965).

5.3 Sensitivity of Yeast Strains to Polyoxins

The sensitivity of Candida albicans strains B2630, A and 6406 to the different polyoxins was determined using a microtitre plate assay (Section 2.9.3).

Into the wells of a tissue culture plate was added 2x concentrated Pro-Medium (50 ul), inoculum (25 ul, 4 x 10^5 cells ml^{-1}) and solutions of polyoxins A, B and D and the basic polyoxin fraction (25 ul, 10, 2, 0.4, 0.08 mg ml^{-1}). Final polyoxin concentrations were 2.5, 0.5, 0.1 and 0.02 mg ml^{-1}, inoculum was 1 x 10^5 cells ml^{-1}. Plates were incubated for 45 h at 30°C and assessed for growth (deposition of a white cellular sediment) relative to a control containing no polyoxin (Fig. 5.2). A sensitivity profile for the yeasts was obtained, together with MIC values (Table 5.1).

In this assay the basic polyoxin fraction was shown to have an approximately equivalent toxicity to the purified samples and was subsequently used in further studies to conserve the limited quantities of the purified components.
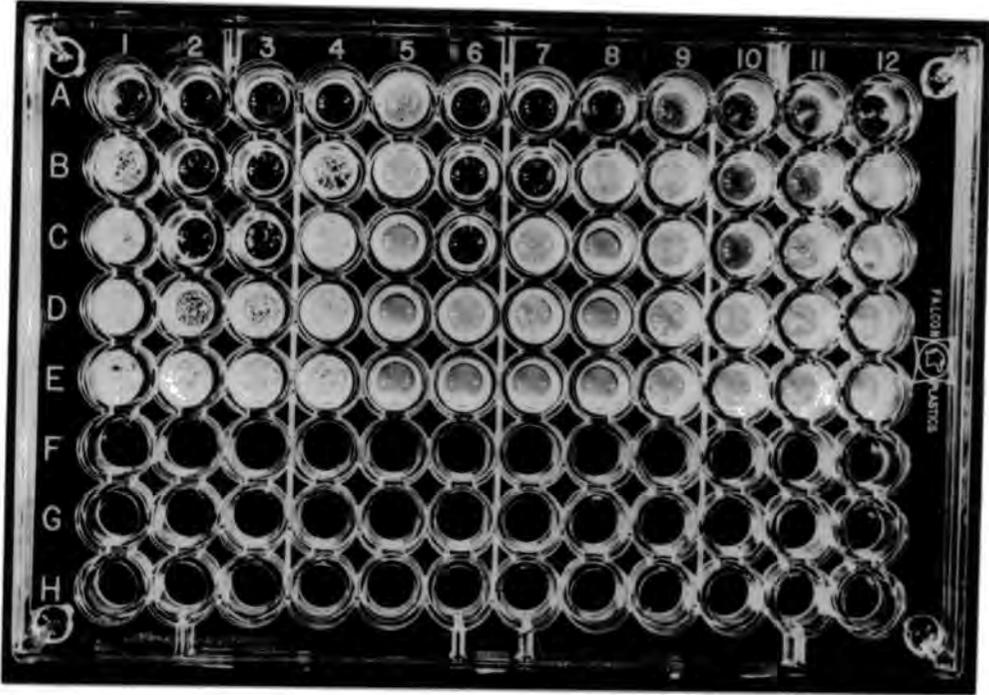
Figure 5.2   Sensitivity of Various Strains of Candida albicans
            to Polyoxins

A microtitre plate assay of the sensitivity of Candida albicans strains
B2630 (lines 1 - 4), A (lines 5 - 8) and 6406 (lines 9 - 12) to
polyoxins A (lines 1, 5, 9), B (lines 2, 6, 10), D (lines 3, 7, 11) and
basic polyoxin fraction (lines 4   8, 12).   Final polyoxin
concentrations were 2.5 (line A), 0.5 (line B), 0.1 (line C) and 0.02
$mg.ml^{-1}$. (line D), inoculum being 4×10^4 cells ml^{-1}.   A control with
no added polyoxin (line E) was included.

POLYOXIN

Strain	A	B	D	Basic Polyoxin Fraction
B2630	2.5	0.1	0.1	2.5
A	N.D	0.1	0.5	2.5
6406	2.5	0.5	2 5	2.5

TABLE 5.1 Sensitivities of Various Strains of Candida albicans to
Polyoxins

Candida albicans strains B2630, A and 6406 were grown in the presence
of polyoxins A, B, D and basic fraction as described in the text
(Section 5.3). Values given are MIC (mg ml^{-1}) sufficient to inhibit
approximately 1×10^4 cells per well in the microtitre plate assay
(Section 2.9.3). N.D. no detectable inhibition at highest
concentration tested (2.5 mg ml^{-1}).

The sensitivities of several strains of yeast to the basic polyoxin fraction was examined by an agar dilution assay (Section 2.9.2). A sensitivity profile (Fig. 5.3) for the yeasts was obtained, together with MIC values (Table 5.2). Candida albicans strain B2630 was shown to be more sensitive to basic polyoxin fraction than strain A, the MIC value obtained by this assay being lower than that obtained by the microtitre plate assay previously described.

To investigate the toxicity of basic polyoxin fraction cells (2 ul, 5×10^3 cells ml^{-1}) were surface inoculated onto Yeast Morphology Agar, (containing amino acids) containing basic polyoxin fraction (0.1 mg ml^{-1}). Plates were incubated at $28^{\circ}C$ for 96 h before assessing for growth relative to an untreated control. Certain strains showed slight sensitivity i.e. produced a smaller zone of growth (Table 5.3)

Candida albicans strain B2630 was shown by these assays to be the most sensitive strain to basic polyoxin fraction and was thus chosen as the parental wild-type for isolation of mutants resistant to toxic peptides (following sections), and for transport studies (Chapters 4 and 6)

5.4 Isolation of Mutants Resistant to Toxic Peptides

5.4.1 Isolation of Polyoxin-Resistant Mutants

Polyoxin-resistant mutants were isolated by picking colonies growing within an inhibition zone (2.5 cm diam., 7×10^5 cells $plate^{-1}$) around a drug sensitivity disc impregnated with basic polyoxin fraction (5 mg) according to the standard method (Section 5.2.3); 6 presumptive mutants were picked after 22 h incubation and a further 11 after 45 h. These mutants were screened against basic polyoxin (4 mg) by the radial streak technique (Section 2.9.4), and 3 showed up as resistant (all 3
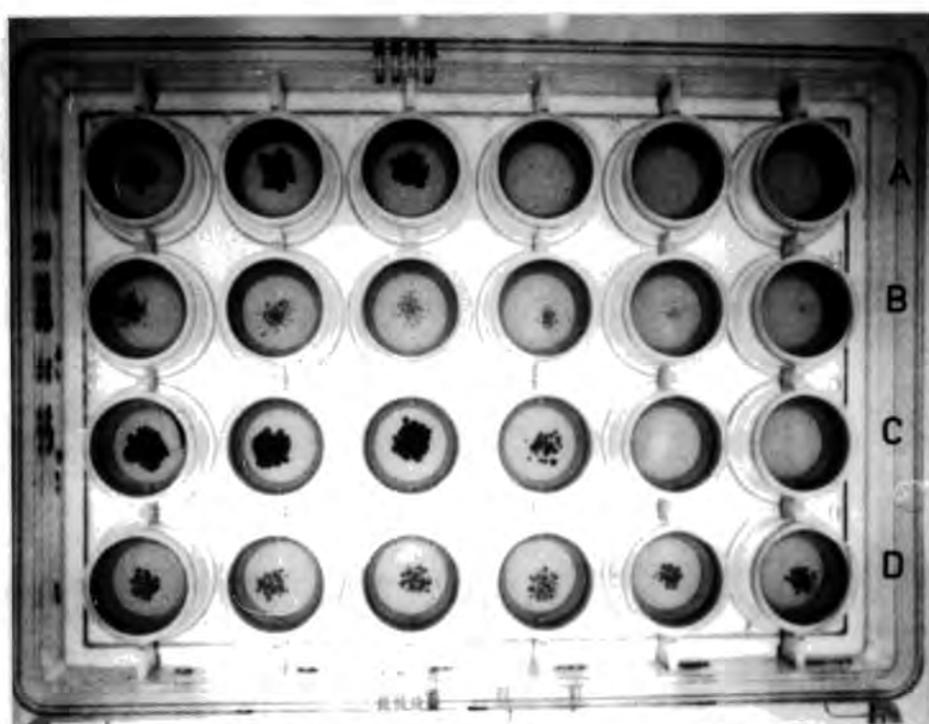
Figure 5.3. Sensitivity of Various Yeasts to Basic Polyoxin

Fraction

An agar dilution assay of the sensitivity of selected yeast, [Candida albicans B2630 (line A), Candida parapsilosis (line B), Candida albicans A (line C) and a Sacc. cerevisiae (line D)] to basic polyoxin fraction 0.062, 0.125, 0.25, 0.5, 1.0 and 2.0 mg.ml^{-1}, from left to right. Plates were prepared and incubated as described in the text.

	MIC (mg ml^{-1})
Candida albicans B2630	0.5
Candida parapsilosis	1.0
Candida albicans A	1.0
Sacc. cerevisiae	N.D.

TABLE 5.2   Sensitivity of Various Yeasts to Basic Polyoxin Fraction

Selected yeasts were grown in the presence of basic polyoxin fraction as described in the text (Section 5.3). MIC values quoted as final concentration (mg ml^{-1}) sufficient to inhibit growth of approximately 1 x 10^3 cells ml^{-1} in the agar dilution assay (Section 2.9.2).

N.D., no detectable inhibition at highest concentration tested (2.0 mg ml^{1}).

Strain	Sensitivity
Candida albicans A	S
Candida albicans B	ND
Candida albicans B2630	S
Candida albicans 6406/8 (Amphotericin B resistant)	ND
Candida albicans 500/8/1 (5-fluorocytosine resistant)	S
Candida tropicalis	ND
Candida parapsilosis (Leech)	ND
Candida parapsilosis (Worme & Lambert)	ND

TABLE 5.3   Sensitivity of Various Candida Species to Basic Polyoxin

Fraction

10^3 Cells were surface inoculated onto Yeast Morphology Agar (containing amino acids) containing basic polyoxin fraction (0.1 mg ml^{-1}). Plates were incubated at 28°C for 96 h before assessing for growth relative to an untreated control performed at the same time.

S,   Partial sensitivity

ND,   No detectable inhibition

were picked after 22 h incubation). A resistance frequency of at least

1 in 1 x 10^4 was calculated. Presumptive mutants were also screened

against polyoxin D, nikkomycin and bacilysin by the radial streak

assay. The 3 mutants resistant to the basic polyoxin fraction

exhibited cross-resistance to these compounds (Figs. 5.4 and 5.5).

5.4 2   Isolation of Polyoxin-Resistant Mutants from EMS-Treated Candida albicans B2630

Candida albicans was preincubated with EMS (2.5% v/v) for 90 min

before harvesting, washing, and resuspending in PCG incubation buffer,

pH 4.5 (Section 5.2.5.2). A sample (90 ul) of this suspension was

spread onto a Pro-Medium plate, to produce a lawn of approximately 7 x

10^5 viable cells plate^{-1} (assuming 90% of the cells pretreated with EMS

were killed during the 90 min incubation period).

Two polyoxin-impregnated drug sensitivity discs (4 mg basic

polyoxin fraction disc^{-1}) were placed on the plate 16 mm apart. In

selection for presumptive mutants from untreated cells (Section 5.2.3)

a single polyoxin impregnated disc produced a 12 mm radius inhibition

zone. Thus, placing two discs 16 mm apart should produce an

overlapping, i.e. reinforced inhibition zone. Plates were incubated at

28°C for 45 h, after which time 96 presumptive mutants were picked,

streaked for single colonies, and master plate produced. Most of the

presumptive mutants were picked from periphery of the inhibition zone

(Fig. 5.6).

One of the isolates, PA8024, was shown to be resistant to

polyoxin D and nikkomycin (Fig. 5.4) and to bacilysin (Fig. 5.5), and

to the basic polyoxin fraction (data not shown) This mutant,

initially isolated as a single smooth colony, produced wrinkled

colonies and a wrinkled master streak, this can be clearly seen on the

Figure 5.4   Sensitivity of Presumptive Polyoxin-Resistant Mutants
             to Polyoxin D

Sensitivity of presumptive basic polyoxin fraction-resistant mutants to
polyoxin D (3 mg.disc^{-1}), relative to the parent strain Candida
albicans B2630.

Strains in a clockwise direction are B2630 (at 12 o'clock), 6406,
PA8001, PA8005, PA8006, PA8007 (6 o'clock), PA8020, PA8021, PA8024 (EMS
treated), Sacc. cerevisiae Σ 1278b and its bacilysin-resistant mutant
Σ1278b gpp.
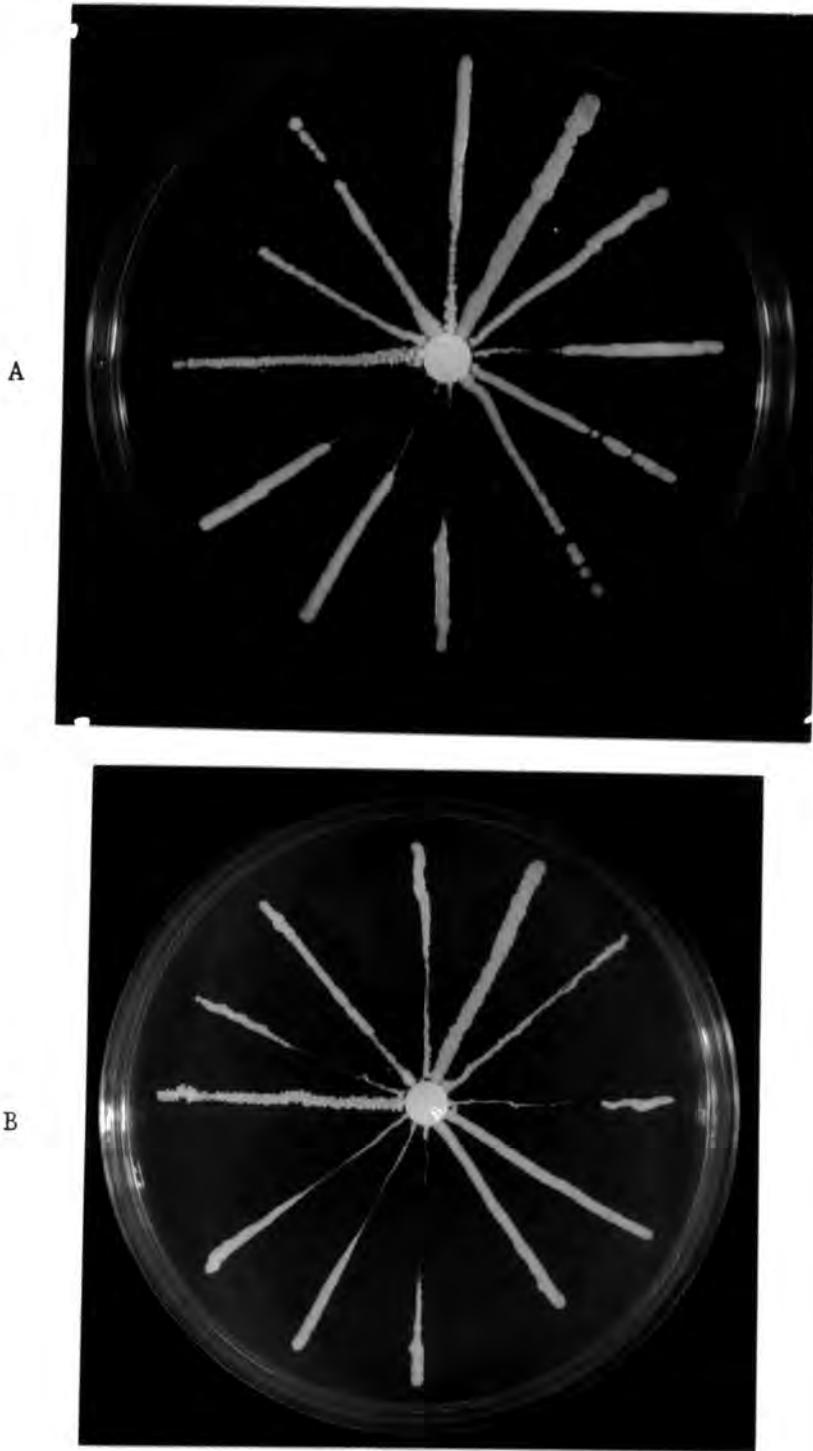
Figure 5.5   Sensitivity of Presumptive Polyoxin-Resistant Mutants

to Nikkomycin and Bacilysin

Sensitivity of presumptive basic polyoxin fraction-resistant mutants to

(A), nikkomycin (0.2 mg.disc^{-1}); and to (B), bacilysin (0.1 mg.disc^{-1}).

Strains are as described in Fig. 5.4.

Figure 5.6   Isolation of Presumptive Polyoxin Resistant Mutants

From EMS-Treated Candida albicans B2630

Inhibition zone produced by two discs containing basic polyoxin fraction (4 mg.disc^{-1}), on a lawn of Candida albicans B2630 (7.2 x 10^5 viable cells plate^{-1}), after incubation at 30°C for 45 h.

radial streak assay plates (Figs 5.4 and 5.5). On repeated subculturing this mutant lost its wrinkled streak appearance.

A resistance frequency value was not calculated here, because the exact number of cells spread onto the plate was unknown, and also most of the mutants were not screened for polyoxin resistance.

5.4.3 Isolation of Nikkomycin-Resistant Mutants

Nikkomycin-resistant mutants were isolated by picking colonies growing within an inhibition zone (2.8 cm diam, 8×10^5 cells plate^{-1}) around a nikkomycin-impregnated drug sensitivity disc (0.4 mg), according to the standard method (Section 5.2.3), 40 colonies being picked after a 45 h incubation. These presumptive mutants were screened against nikkomycin (0.2 mg) by the radial streak technique (Section 2.9.4), and 4 showed up as fully resistant. A resistance frequency of at least 1 in 2×10^4 was calculated. Three of these mutants were then screened against basic polyoxin fraction and bacilysin, again using the radial streak assay; all 3 mutants exhibited cross-resistance to both these compounds (Fig. 5.7).

5.4.4 Isolation of Bacilysin-Resistant Mutants

Bacilysin-resistant mutants were isolated by picking colonies growing within an inhibition zone (4.4 cm diam, 2×10^6 cells plate^{-1}) around a bacilysin-impregnated disc (0.2 mg), according to the standard method (Section 5.2.3); 70 colonies being picked after a 45 h incubation. 59 presumptive mutants were screened against nikkomycin (0.1 mg) by the radial streak assay (Section 2.9.4), and 33 showed up as cross-resistant (Fig. 5.8). Nikkomycin was used in the initial screen to conserve the limited supply of bacilysin. A resistance frequency of at least 1 in 2×10^4 was calculated. Selected mutants,

A   B   C
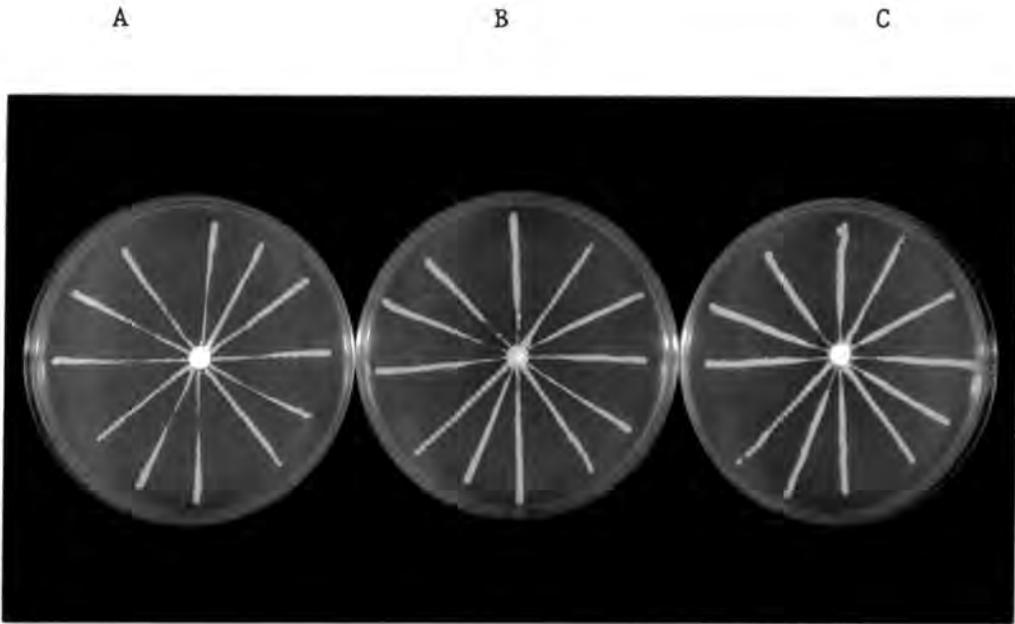
Figure 5.7 Sensitivity of Presumptive Nikkomycin-Resistant Mutants

to Bacilysin, Nikkomycin and Polyoxin

Sensitivity of 12 presumptive nikkomycin-resistant mutants to (A),
bacilysin (0.1 mg.disc^{-1}); (B), nikkomycin (0.2 mg.disc^{-1}); and to (C),
basic polyoxin fraction (4 mg.disc^{-1}); relative to the parent Candida
albicans B2630.

Strains in a clockwise direction are B2630 (at 12 o'clock), PA8026 (1
o'clock), PA8027 (5 o'clock) and PA8028 (8 o'clock). PA8026, PA8027
and PA8028 are clearly cross-resistant to all three toxic peptides.
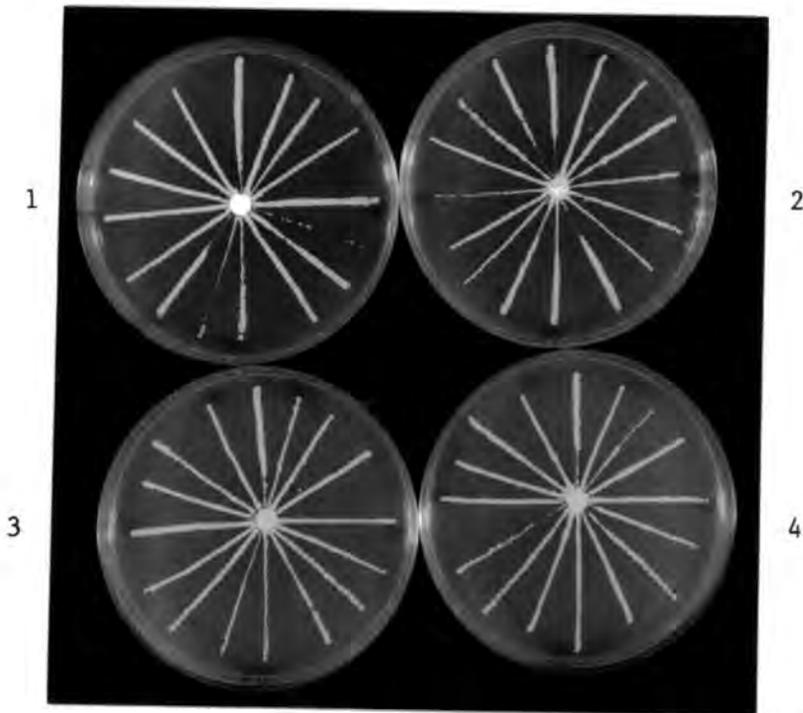
Figure 5.8 Sensitivity of Presumptive Bacilysin-Resistant Mutants

                to Nikkomycin

Sensitivity of presumptive bacilysin-resistant mutants to nikkomycin

(0.1 mg.disc), relative to the parent strain Candida albicans B2630.

Strain B2630 is positioned at 12 o'clock on each plate. Of particular

interest is PA8035 positioned at 9 o'clock on plate 3, and the two

strains below this, PA8033 and PA8031 (in this order), all three of

which are clearly cross-resistant to nikkomycin.

exhibiting cross-resistance to nikkomycin, were examined for sensitivity to bacilysin; all showed up as bacilysin-resistant (data not shown).

5.4.5 Morphology of Bacilysin-Resistant Mutants

Amongst the bacilysin-resistant isolates, in addition to normal wild-type morphology, some produced rough, wrinkled colonies, and others gave variable colony type, e.g., some produced mixed colony size on restreaking, whereas others uniformly produced either 'large' or 'small' colonies, there was no discernible pattern between resistance pattern and colony type. Several isolates, producing rough wrinkled colonies on initial restreaking, produced rough wrinkled master streaks. After several routine sub-culturings several mutants lost this rough, wrinkled streak phenotype and produced wild-type smooth streaks.

A bacilysin-resistant mutant, PA8031, was shown to produce a mix of standard-sized and small colonies on a Pro-Medium plate (Fig. 5.9). On initial isolation the colony was picked from the bacilysin inhibition zone and streaked onto a Pro-Medium plate producing a mix of standard-sized and small colonies. A typical standard-sized colony was picked and a master streak produced and designated PA8031. An overnight Pro-Medium grown culture was produced from an inoculum taken from this master streak, and a sample (0.1 ml) of this culture was taken and diluted so that a 0.1 ml aliquot gave approximately 100 colonies plate^{-1}. Again, a mix of standard-sized and small colonies was produced, representative colonies were picked, master streaks produced and at the same time restreaked for single colonies. The standard-sized colonies were designated PA8031-L1 and PA8031-L2 and the small colonies PA8031-S1 and PA8031-S2   PA8031-L1 and

B2630   PA8031   PA8035
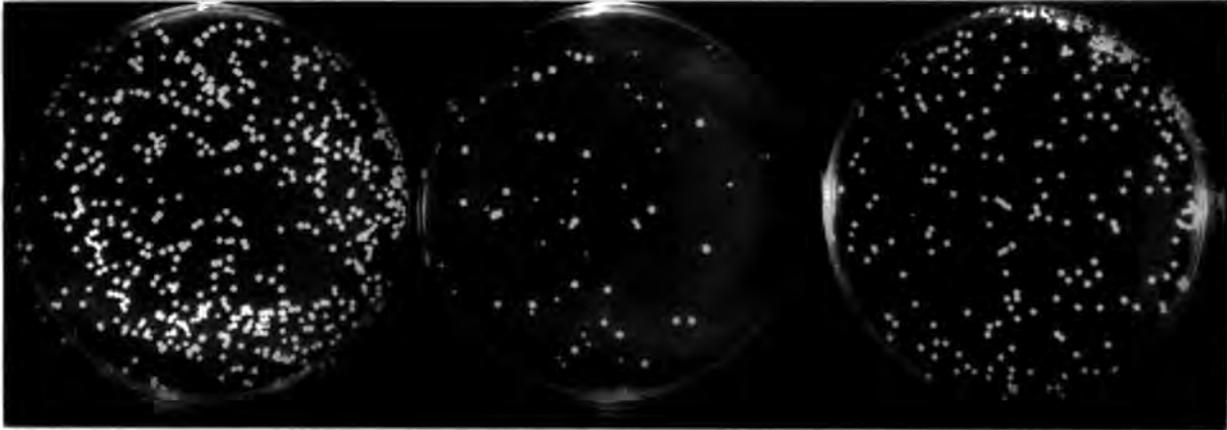
Figure 5.9 Colony Appearance of Bacilysin-Resistant Mutants

Overnight Pro-Medium grown cells were serially diluted, and spread on
Pro-Medium plates to give 50 - 100 cells plate^{-1}. Strain PA8031
(middle plate) clearly produced a heterogeneous colony population,
relative to B2630 and PA8035.

PA8031-S1 were restreaked for single colonies, PA8031-L1 produced uniformly standard-sized colonies, whereas PA8031-S1 produced a mix of both standard-sized and small colonies, representative colonies again being picked for master streaks. The pattern of sensitivity to bacilysin (0.1 mg disc^{-1}) was determined for these isolates, each streak on the radial streak assay being taken directly from the original colony from which these sub-strains were taken. A flow diagram showing the sub-division and resistance profile of these strains is given (Fig. 5.10).

No discernible correlation between resistance profile and colony size was found. Thus, the significance of the production of both large and small colonies, and the alternate production of a homogeneous colony population by these mutants, with regard to the pattern of resistance remains uncertain.

5.4.6   Isolation of m-fluoro-phenylalanylalanylalanine-Resistant Mutants

FPAA-resistant mutants were isolated by picking colonies growing within an inhibition zone (4.2 cm diam, 5×10^5 cells plate^{-1}) around a FPAA-impregnated disc (0.6 mg), according to the standard method (Section 5.2.3), 16 colonies being picked after a 45 h incubation. The inhibition zone produced by FPAA was rather diffuse (Fig. 5.11), with no clear-cut boundary, more a gradation of cell density away from the disc. All colonies picked were larger than the background growth, care was taken to pick only isolated colonies. The diffuse nature of the inhibition zone produced by FPAA probably reflects the lack of toxicity of this compound.

Candida albicans B2630 (S)

Bacilysin Selection

PA8031 (R)

Overnight Culture grown in Pro·Medium

PA8031-L1(S)   PA8031-L2(S)   PA8031-S1(R)   PA8031-S2(R)

directly streaked   directly streaked

PA8031-L1-L1(S)   PA8031-L2-L2(S)   PA8031-S1-L1(R)   PA8031-S1-S1(S)

PA8031-S1-L2(R)   PA8031-S1-S2(S)
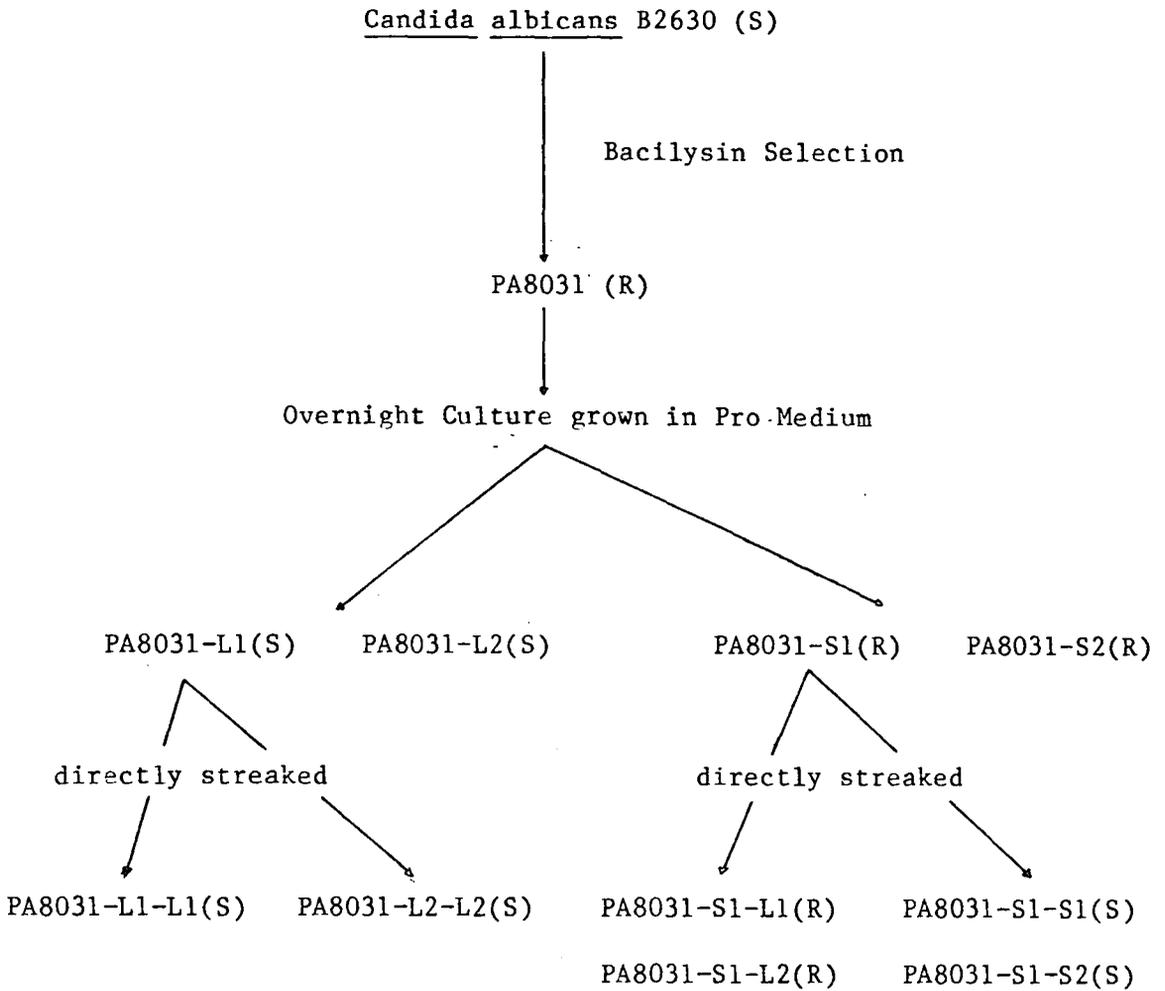
Figure 5.10   Flow-Diagram Showing the Sub-Division and Resistance

Profile of Strains Derived from the Bacilysin-Resistant

Mutant PA8031

A series of sub-strains was derived from the bacilysin-resistant mutant

PA8031, as described in the text (Section 5.4.5). Strains were tested

for sensitivity to bacilysin by the radial streak assay (Section

2.9.4), strains being designated as either resistant, (R); or

sensitive, (S).
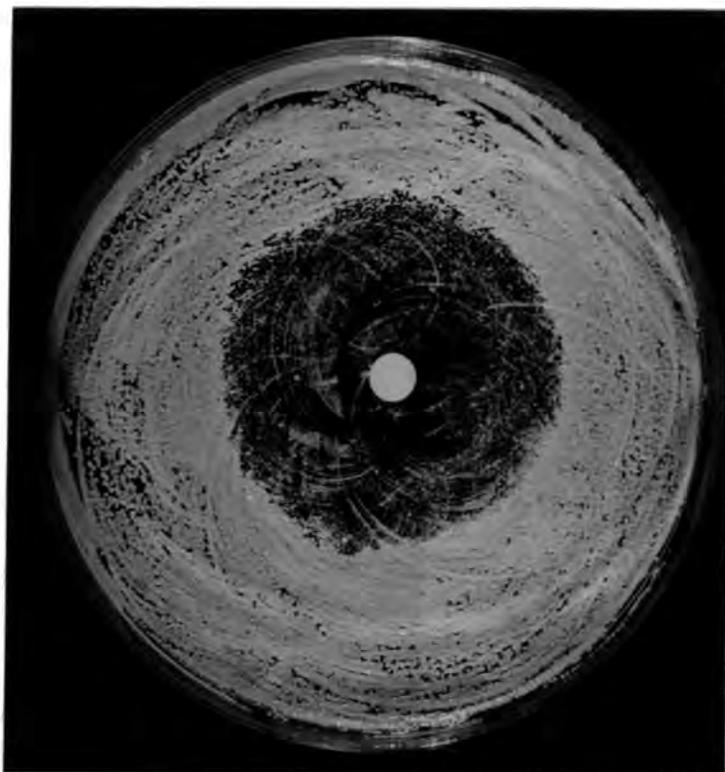
Figure 5.11   Inhibition Zone Produced by FPAA on a Lawn of

Candida albicans B2630

Inhibition zone produced by FPAA (0.6 mg.disc^{-1}) on a lawn of Candida albicans B2630 (5 x 10^5 cells plate^{-1}), after incubation at 30°C for 45 h.

On streaking for single colonies, 9 of the presumptive mutants produced a mixture of large and small colonies, representatives of which were restreaked for single colonies (to test colony homogeneity), and also master colonies for each representative colony were streaked at the same time The larger colonies consistently produced a mixed colony size on secondary streaking, whereas small colonies tended to produce either totally small colonies or a mixed colony population on secondary streaking.

Presumptive FPAA-resistant mutants were screened against FP, FPA and FPAA (Fig. 5.13), a photographic enlargement of the FPAA plate is also given (Fig. 5.12) Four of the 16 presumptive mutants examined showed up as resistant to FPAA, whereas all presumptive mutants exhibited the same sensitivity to FPA and to FP as the parent, B2630. The fact that these mutants are only resistant to the tripeptide form (but not to the dipeptide or amino acid form), raises the possibility that the mutants may be defective in transport of the tripeptide form. A resistance frequency was not calculated because of the heavy background growth within the inhibition zone.

5.4.7 Isolation of Revertants of Peptide-Resistant Mutants

To explore the molecular genetic basis for resistance, attempts were made to isolate revertants of peptide-resistant mutants. For example revertants of the bacilysin-resistant mutant PA8035 were isolated on the basis of enhanced dipeptide utilisation. Thus cells (5×10^5) were spread onto a minimal agar plate containing Ala-Ala (1 mg. ml^{-1}) as a sole nitrogen source, plates were incubated for 45 h at $30^\circ C$ before picking large colonies (relative to background) and preparation of master streaks. Presumptive revertants were screened for regained sensitivity to the peptide used in the initial isolation

Figure 5.12   Sensitivity of Presumptive FPAA-Resistant Mutants

to FPAA

Sensitivity of presumptive FPAA-resistant mutants (0.3 mg.disc^{-1}),
relative to the parent strain <u>Candida</u> <u>albicans</u> B2630.   Strains in a
clockwise direction are B2630 (12 o'clock), PA8110, PA8112, PA8114,
PA8116, PA8035 (bacilysin-resistant, 3 o'clock), PA8118, PA8119,
PA8120, PA8121, B2630 (6 o'clock), PA8123, PA8124, PA8126, PA8127,
PA8001 (polyoxin-resistant, 9 o'clock), PA8130, PA8132, PA8133.
Strains PA8119, PA8120, PA8123 and PA8127 are clearly resistant to
FPAA.

Figure 5.13 Sensitivity of Presumptive FPAA-Resistant Mutants
            to FP, FPA, and FPAA

Sensitivity of presumptive FPAA-resistant mutants to (1), FP (0.6
mg.disc^{-1}); (2), FPA (0.3 mg.disc^{-1}); and (3), FPAA (0.3 mg.disc^{-1}),
relative to the parent strain Candida albicans B2630.
Strains are as described in Fig. 5.12

of the resistant mutant. Of 15 presumptive revertants, 3 regained

sensitivity to bacilysin (0.1 mg), as shown by the radial streak assay.

Presumptive revertants were also tested for their capacity to utilise

peptides as their sole nitrogen source relative to their bacilysin-

resistant parent PA8035 (Section 5 5.5)

An attempt to select revertants of the polyoxin-resistant mutant

PA8001, by the above methodology, proved unsuccessful. Several

slightly larger colonies were picked, but did not exhibit regained

sensitivity to bacilysin (0.1 mg) when screened using the radial streak

assay.

5.5 Characterization of Peptide-Resistant Mutants

5.5.1 Introduction

Peptide resistant mutants were further characterized for possible

patterns of cross-resistance to other toxic peptides, ability to

transport a range of representative peptides and for utilization of

peptides as a sole nitrogen source. These studies were performed in an

attempt to determine the number of peptide transport systems present in

Candida albicans

5.5.2 Transport Properties of Resistant Mutants and Revertants

Uptake rates for several peptides and amino acids were measured

in the wild-type strains and the peptide-resistant mutants. Transport

was assayed using both the manual fluorescamine assay (Section 2 5 2)

and by using radioactively-labelled substrates (Section 2.7.2). Uptake

rates for wild-type strains, the bacilysin-resistant strains and

corresponding revertants are given in Table 5.4, rates for polyoxin-,

nikkomycin-, and FPAA-resistant strains and their parental strain are

given in Table 5 5.

Rates of Transport $(nmol.min^{-1}(mg.dry\ wt)^{-1})$

	B2630	6406	A	Bac. R.		Bac. R. REVS.	
				PA8030	PA8035	PA8096	PA8100
Ala–Ala	1.25	3.05	3.3	ND	ND	1.2	1.16
Ala–Ala–Ala	1.6	5.37	4.2	1.57	1.42	1.81	1.94
Ala–Ala–Ala–Ala	2.36	6.88	4.4	2.9	2.58	2	2.52
Gln	19.8				16.6		20.5
Leu	8.24				7.9		9.1

TABLE 5.4   Transport Rates in Wild Types and Mutant Strains of Candida albicans

All strains were grown in Pro-Medium overnight, harvested and resuspended as described (Section 4.2.2), and uptake rates determined by the standard manual fluorescamine assay (Section 2.5.2).
Initial substrate concentrations were 0.1 mM for peptides 0 5 mM for amino acids.

Bac.R.   :   Bacilysin-Resistant

Bac.R REVS :   Revertants of Bacilysin-Resistant Mutants

N.D.   :   Not detectable

$$\underline{\text{Rates of Transport}}\ (\text{nmol.min}^{-1}(\text{mg.dry wt})^{-1})$$

	B2630	P.R.		NIK.R		FPAA.R	
		PA8001	PA8005	PA8026	PA8027	PA8119	PA8123
Ala–Ala(F)	1.25	ND	ND	1.0	0.9	1.0	1.0
Ala–Ala–Ala(F)	1.6	0.29	ND	1.75	2.22	1.56	1.29
Ala–Ala–Ala–Ala(F)	2.36	ND	ND	3.0	2.29	1.73	1.36
Met–Met–Met(F)	2.15	ND	0.49	2.73	2.38		
Ala–Ala(R)	0.41	0.0135					
Ala–Ala–Ala(R)	0.65	0.22					
Gln(F)	19.8	6.8	7.3				
Leu(F)	8.24	2.2	3.4				
Leu(R)	2.8	1.2					

TABLE 5.5   Transport Rates in Candida albicans B2630 and Mutant Strains

All strains were grown in Pro-Medium overnight harvested and resuspended as described (Section 4.2.2), and uptake rates determined by the standard manual fluorescamine assay (F), and by using radioactively-labelled substrates (R).

Initial substrate concentrations were 0.1 mM for peptides, 0.5 mM for amino acids

P.R.   Polyoxin Resistant   NIK R.   Nikkomycin Resistant

FPAA.R   m-fluorophenylalanylalanylalanine Resistant

N.D.   Not detectable

Transport properties of other isolated mutants are summarised below :-

1) <u>Bacilysin-Resistant Mutants</u> : 9 of the strains exhibiting cross-resistance to nikkomycin (Section 5.4.4) were examined for Ala-Ala uptake. Of these, 5 were Ala-Ala transport deficient, 3 exhibited low rates of uptake (0.3 - 0.5 nmol.min.$^{-1}$(mg.dry wt)$^{-1}$) and one exhibited the typical parental rate of Ala-Ala uptake. Of the 2 strains further characterized, PA8030 produced rough wrinkled colonies and wrinkled master streaks (Section 5.4.5) whereas PA8035 produced standard 'wild type' colony and streak morphology. PA8035 also exhibited typical parental rates of amino acid uptake.

2) <u>Revertants of Bacilysin-Resistant Mutants</u> : Of the 15 presumptive revertants exhibiting growth on Ala-Ala equivalent to the parent B2630 (Section 5.4.7), only 3 regained sensitivity to bacilysin Of these, 2 were examined for peptide uptake and shown to exhibit the parental rates for Ala-Ala. The revertant PA8100 exhibited typical parental rates of amino acid uptake.

3) <u>Polyoxin-Resistant Mutants</u> : All three mutants isolated as resistant to polyoxin and exhibiting cross-resistance to nikkomycin and bacilysin (Section 5.4.1), were shown to be deficient in, or exhibit very low rates of, peptide transport of Ala-Ala Ala-Ala-Ala, Ala-Ala-Ala-Ala and Met-Met-Met. Two of the mutants were characterized further and shown to exhibit low rates of amino acid transport.

4) <u>Nikkomycin-Resistant Mutants</u> : 3 of the 4 strains isolated as being resistant to nikkomycin, and showing cross-resistant to polyoxin and bacilysin, (Section 5.4.3) were shown to have parental rates of peptide uptake.

5)   FPAA-Resistant Mutants : 3 of the 4 strains isolated as being

resistant to FPAA were examined for peptide uptake. One strain

(PA8123) was shown to have reduced tri-, and tetra-peptide

uptake; the values for uptake rate lying below the range of

values exhibited by the parent. The other strains produced low

values for Ala-Ala-Ala and Ala-Ala-Ala-Ala transport rates, some

values lying below the values exhibited by the parent strain

B2630.

Thus, the transport properties of the isolated mutants and

revertants revealed a range of phenotypes. The bacilysin-resistant

mutants provide the first clear evidence for the existence of separate

systems for uptake of di, and oligopeptides in Candida albicans, as is

the case for several other microbial species (Matthews & Payne, 1980).

This mutation was revertable and the cross-resistance pattern and

peptide uptake capacity were strictly associated. The complementary

properties of the FPAA-resistant mutants endorse these conclusions.

5.5.3 Sensitivity of Mutants to m-fluorophenylalanyl-Peptides

Towards the end of these mutant studies, synthetic m-fluor-

phenylalanyl-substituted peptides became available. The sensitivity of

the various resistant mutants and their corresponding revertants to the

dipeptide, m-fluoro-phenylalanylalanine (FPA, 0.3 mg), the tripeptide

m-fluoro-phenylalanylalanylalanine (FPAA, 0.3 mg), and to the

corresponding amino acid, D,L-m-fluoro-phenylalanine (FP, 0.6 mg) was

determined by the radial streak assay (Section 2.9.4). Results are

tabulated in the following section.

Sensitivity to m-fluoro-phenylalanyl-substituted peptides was

also examined by the seeded soft-agar overlay assay (Section 2.8.5).

The bacilysin-resistant mutant PA8035 was shown also to have become resistant to FPA (relative to its parent B2630) whilst retaining sensitivity towards FPAA. The revertant, PA8100, exhibited normal wild-type sensitivity to the dipeptidyl compounds (see Fig. 5.14).

Mutants showing resistance to FPAA in the radial streak assay, nevertheless showed sensitivity to FPAA in the seeded soft-agar overlay assay (results not shown).

5.5.4   Summary of Cross-Resistance Studies

Several independent isolates from each selection procedure were tested for cross-resistance using the radial streak assay (Section 2.9 4). Representative results are shown in Table 5.6.

Polyoxin-, and nikkomycin-resistant strains were cross-resistant to polyoxin, nikkomycin and bacilysin, but were still sensitive to FPA and FPAA. The bacilysin-resistant mutant was cross-resistant to polyoxin, nikkomycin and FPA, but retained sensitivity to FPAA. Finally, a mutant isolated from the inhibition zone around FPAA (Section 5.4.6) showed increased (but not complete) resistance, whilst retaining wild-type sensitivity to FPA.

In related studies, Candida albicans B2630 and the polyoxin-resistant mutants PA8001 and PA8005, were shown not to be sensitive to the aminoxy peptide Ala-Ala-OAla (98 ug disc^{-1}, equivalent to 400 nmol).

5.5.5   Utilisation of Peptides as a Sole Nitrogen Source

The utilisation of peptides as a sole nitrogen source by Candida albicans B2630 and peptide-resistant mutants was examined by the patch-plate assay (Section 5.2.4). All strains showed equivalent growth on Ala, Ala-Ala-Ala and Pro-Medium plates (Fig. 5.16), differential growth being exhibited on the Ala-Ala plate, an enlargement of which is shown
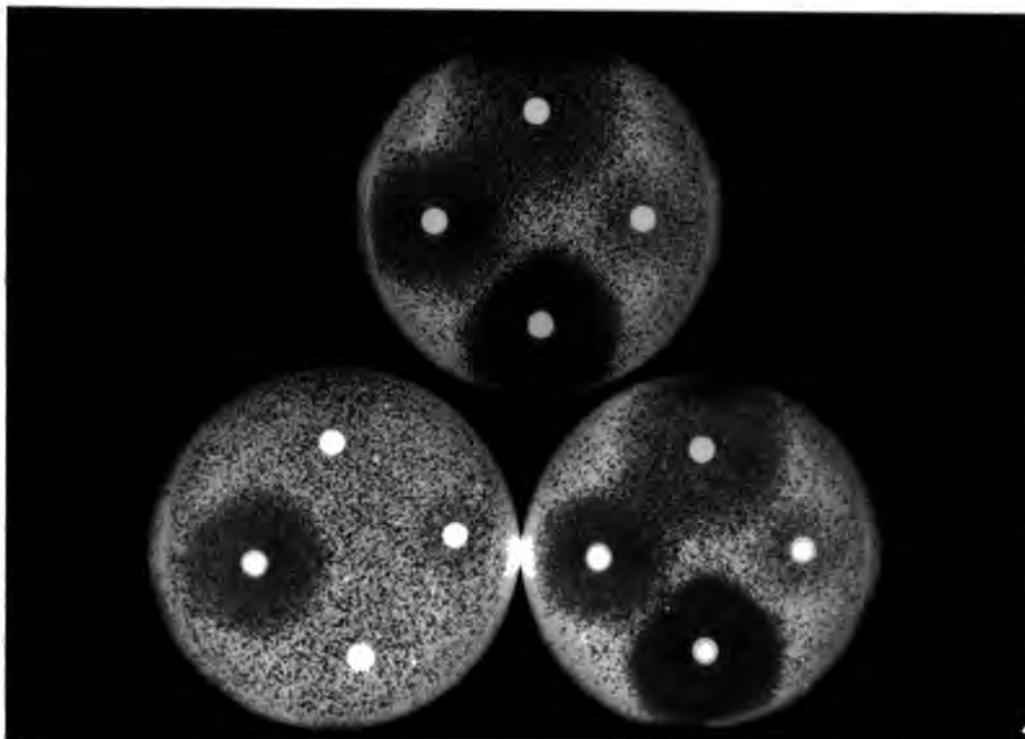
Figure 5.14 Sensitivity of Wild Type and Mutant Strains of

Candida albicans to Toxic Peptides

Soft-agar overlays were seeded with the following strains   Top, B2630;

bottom left, PA8035 (bacilysin-resistant);   bottom right, PA8100

(revertant of PA8035).

Peptide analogues were applied to the discs, clockwise from the top as

follows : FPA (0.3 mg.disc^{-1}), FPAA (0.025 mg.disc^{-1}), bacilysin (0.02

mg.disc^{-1}) and FPAA (0.1 mg.disc^{-1}).   Plates were incubated as

described in the text.

			Strain			
Peptide analogue			Bac.R	Bac.R.REV	P R.	N.R.
(mg disc^{-1})	B2630	6406	PA8035	PA8100	PA8001	PA8027
Polyoxin (Basic Mix)(4)	11	0	0	0	0	0
Nikkomycin (0.1)	12	0	0	0	0	0
Bacilysin (0.1)	27	0	0	27	0	0
FPA (0.3)	20	23	0	19	20	18
FPAA (0.3)	19	23	18	19	20	19

TABLE 5.6   Sensitivities of Candida albicans to Toxic Peptide Analogues

Sensitivities were determined using the radial streak assay (Section 2.9.4), values are radii of inhibition zones (mm) from centre of disc to start of streak growth.

Bac.R   Bacilysin Resistant

Bac.R.REV   Bacilysin Resistant Revertant

P.R.   Polyoxin Resistant

N.R.   Nikkomycin Resistant
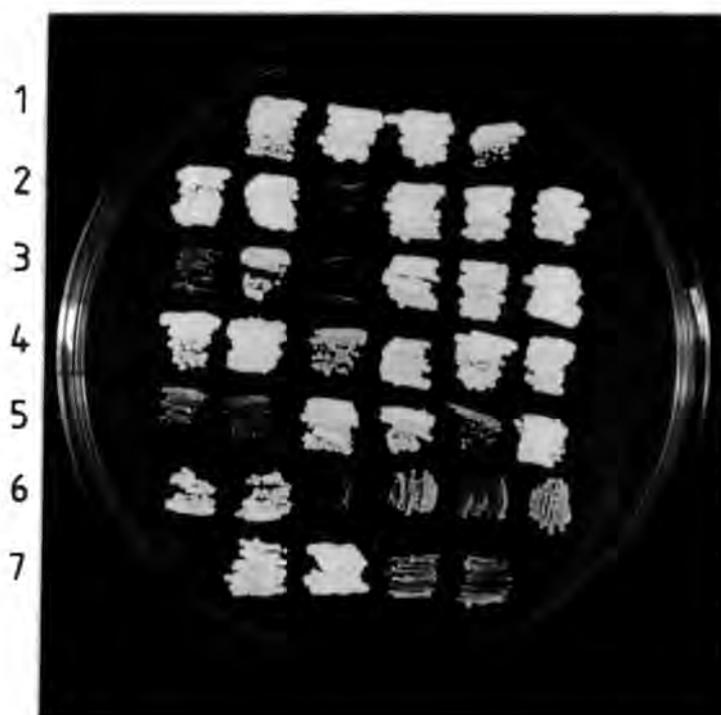
Figure 5.15   Utilisation of Ala-Ala as a Sole Nitrogen Source
by Selected Mutants

Utilisation of Ala-Ala as a sole nitrogen source by Candida albicans
B2630 and selected mutants, was examined by the patch-plate assay
(Section 5.2.4).   Strains were laid down as follows (from left to
right)

1.   B2630, PA8005, PA8001 (both P.R.) B2630.

2.   PA8088, PA8086 (Bac.R.Rev.), PA8035 (Bac.R), PA8084, PA8082,
PA8080 (Bac.R.Rev.).

3.   PA8104, PA8102 (Bac.R.Rev.), PA8035 (Bac.R.), PA8094, PA8092,
PA8090 (Bac.R.Rev.).

4.   B2630, PA8108, PA8106, PA8100, PA8098, PA8096 (Bac.R.Rev.).

5.   PA8031, PA8030 (Bac.R.), B2630, PA8028, PA8027, PA8026 (N.R.).

6.   PA8036 (Bac.R.), B2630, PA8035, PA8034, PA8033, PA8032 (Bac.R.).

7.   B2630, PA8039, PA8038, PA8037 (Bac.R.)

Abbreviations as described in Table 5.6.
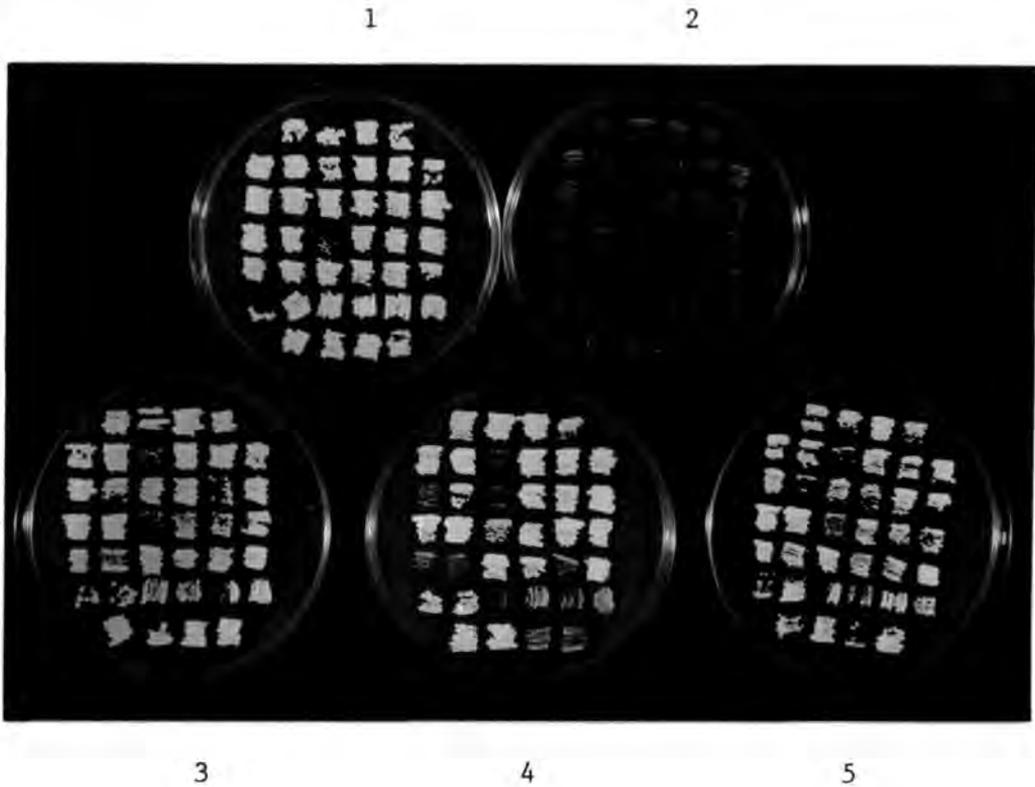
Figure 5.16   Utilisation of Various Sole Nitrogen Sources
          by Selected Mutants

Utilisation of various sole nitrogen sources by Candida albicans B2630 and selected mutants was examined by the patch-plate assay. (1), proline (4 mg·ml^{-1});   (2), no supplemented nitrogen source;   (3), Ala (1 mg·ml^{-1});   (4), Ala-Ala (1 mg·ml^{-1});   (5) Ala-Ala-Ala (1 mg·ml^{-1}). Strains are laid down as described in Fig. 5.15.

in Fig. 5.15. It should be noted that even on the control (minus nitrogen) plate (Fig. 5.16, 2), faint growth was observed in all cases. This feature should be considered in interpreting the differential growth on the Ala-Ala plate. It is possible that this background growth might be minimalised if, e.g., Noble Agar were to be used. The utilisation of Ala-Ala by the various mutants is described below :-

1)   Polyoxin-Resistant : growth equivalent to Candida albicans B2630

2)   Nikkomycin-Resistant : growth equivalent to Candida albicans B2630

3)   Bacilysin-Resistant : 8 strains shown to be bacilysin-resistant in the radial streak assay, grew poorly on the Ala-Ala plate. Another strain PA8039 shown to be bacilysin-resistant exhibited equivalent growth to B2630.

4)   Revertants of Bacilysin-Resistant Mutants : of the 15 presumptive revertants, 14 exhibited growth equivalent to B2630.

In early related studies, attempts were made to devise a screen for peptide transport deficient mutants using a somewhat modified version of the radial streak assay In this study, agar plates containing agar (1.5% w/v), glucose (2% w/v) and yeast nitrogen base without ammonium sulphate or amino acids (1.7 mg ml^{-1}) were prepared. Sole nitrogen sources were concentrated onto drug sensitivity discs, which were placed in the centre of the agar plates. Upto 16 cultures were streaked from the periphery of the plate, inwards to the disc, and plates incubated for 45 h at 28°C. It was hoped to pick up a growth differential between the wild-type B2630 and the polyoxin-resistant strains PA8001 and PA8005 for growth on a peptide, e.g. Pro-Val (4 mg) and the corresponding free amino acids (Pro, 2 mg; Val, 2 mg) concentrated together. However, such a differential was never detected

for a wide range of peptides, which is consistent with the observation that B2630 and PA8001 utilize Ala-Ala with the same growth efficiency. Following the development of the patch plate assay and detection of a growth differential between wild-type and bacilysin-resistant strains, no further attempts were made to develop this modified streak assay as the patch plate assay was more time-saving and inexpensive. However, such a differential would be expected if one were to compare the utilization of wild-type and bacilysin-resistant, peptide transport-deficient strains·

Of interest was the observation that a growth differential was observed for B2630 streaked on plates with discs containing Casein Acid Hydrolysate or with Trypticase Peptone (a pancreatic digest of casein) or Bacto-Peptone (all 4 mg disc^{-1}), growth being greater when peptone is the sole nitrogen source. This is consistent with the observation that peptide transport rates are higher for cells grown in peptone relative to Casein Acid Hydrolysate (Section 4.4.2), and indicates that peptides are more nutritionally advantageous than the corresponding amino acids supplied in a free form.

5 5.6 Effects of Polyoxin D on Cell Growth

The effect of Polyoxin D (2.5 mg ml^{-1}) on the growth of Candida albicans B2630, and the polyoxin-resistant mutant PA8001, was followed over a 12 h period, as described (Section 2.9.6).

Polyoxin D was shown to be fungicidal against B2630 (Fig. 5·17), the viable count falling from 4.6 x 10^4 to 1.8 x 10^3 during a 12 h incubation with polyoxin D in Pro-Medium The polyoxin-resistant mutant PA8001 grew in the presence of polyoxin D (2.5 mg ml^{-1}), at the same rate as its parent B2630 in an untreated control.
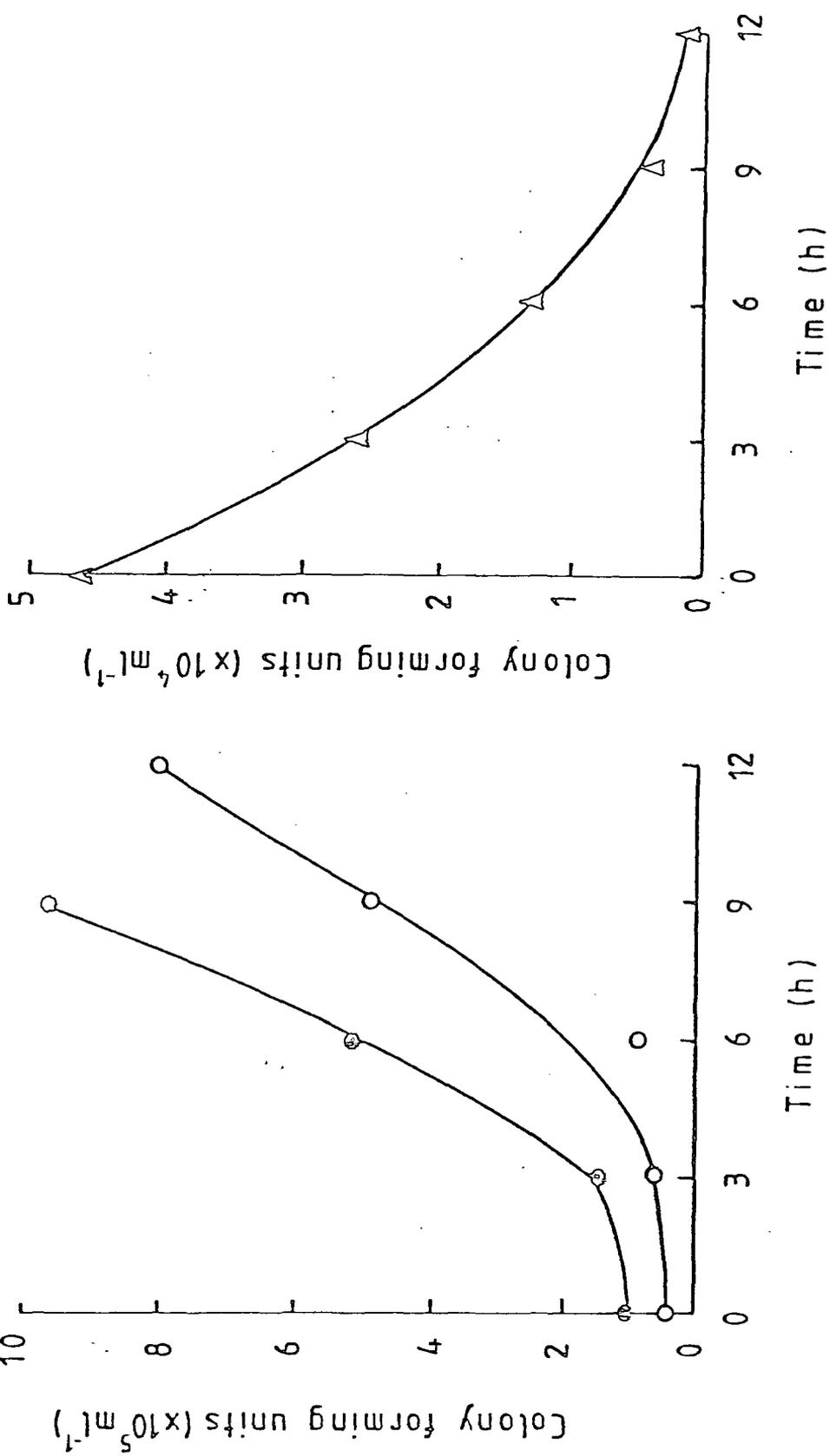
Figure 5.17 Growth Effects of Polyoxin D

Effect of Polyoxin D (2.5 mg ml $^{-1}$) on the growth of <u>Candida albicans</u>

B2630 and the polyoxin-resistant mutant PA8001.

(O) PA8001 + polyoxin,   (O) B2630, untreated control,

(△) B2630 + polyoxin.

These results show clearly that polyoxin D is fungicidal to Candida albicans.

5.5.7 Characterization of Chitin Synthetase Activity in Polyoxin-, and Nikkomycin-Resistant Mutants

To ensure that the polyoxin-, and nikkomycin-resistant mutants did not possess an altered chitin synthetase (in addition to the transport defect exhibited by the polyoxin-resistant mutants), the sensitivity of the isolated enzyme to polyoxin D was determined according to the methods described in Section 3.6. These studies were performed by workers at the Bioscience II Laboratories, I.C.I. Pharmaceuticals Division, Alderly Park, Cheshire, as part of a routine chitin synthetase screen. Values of kinetic parameters for substrate affinity (Km) and inhibition of chitin synthetase by polyoxin D (Ki) for both polyoxin-resistant (PA8001 and PA8005) and nikkomycin-resistant strains (PA8026 and PA8027), were comparable with those values obtained for the wild-type enzyme (results not shown). Thus, for those mutants characterized for cross-resistance and transport activity, there was no alteration in their chitin synthetase activity relative to the wild-type.

5.5.8 Laboratory Tests for the Presumptive Identification of Candida albicans

Because of the variations in colony morphology in certain of the isolated mutants (Sections 5.4.2 and 5.4.5), a series of representative mutants was taken through some diagnostic tests for the identification of Candida albicans (reviewed by Odds, 1979). These studies were

carried out by Mr. R.G. Wilson of Bioscience I, I.C.I. Pharmaceuticals Division, Alderly Park, Cheshire.

Each organism was tested for germ-tube production in foetal calf serum, for the ability to assimilate sucrose (SAM test), for sugar assimilation using an API 20C diagnostic kit for yeast identification, and for morphology when cultured on BIGGY and Corn-meal-Tween 80 agar. A double positive result for the germ-tube and SAM tests is taken as confirmation for <u>Candida albicans</u>.

Mutants PA8024 (EMS-treated, polyoxin-resistant), PA8026 - 28 (nikkomycin-resistant), PA8030 - 35 (bacilysin-resistant), PA8097, PA8100 and PA8102 (revertants of bacilysin-resistant PA8035) were all germ-tube positive and SAM positive and in all respects behaved as typical <u>Candida albicans</u>. Mutant PA8027 produced two distinct colony types on BIGGY agar, but each behaved identically in all the tests.

Mutants PA8001 and PA8005 (polyoxin-resistant) behaved similarly, being germ-tube variable but SAM positive. The cells were consistently smaller than the parent B2630 and gave a profile indicative of <u>Torulopsis candida</u> in the sugar assimilation test. The significance of these findings is unclear; <u>Torulopsis candida</u> is not a common yeast (R.G. Wilson pers. commun.) and would be an unlikely contaminant. The possibility of selecting a polyoxin-resistant contaminant within a specific inhibition zone is remote.

5.6 Concluding Discussion

Throughout the course of these studies a series of toxic peptides became available, polyoxins (see Section 1.4.4.2), nikkomycin (X and Z mixture, see Section 1.4.4.3), bacilysin (see Section 1.4.2.1) and synthetic m-fluorophenylalanine-peptide conjugates (see Section

1.4.2.4). As part of the study aimed at optimising the carrier structure for anticandidal drugs, it was essential to define more clearly the number and nature of the peptide permeases in Candida albicans. To this end a series of peptide-resistant mutants was isolated and characterized for cross-resistance, utilisation of peptides as a sole nitrogen source and peptide transport. Their properties clearly indicate the existence of separate systems for the uptake of dipeptides and oligopeptides.

Early studies of peptide transport in Candida albicans gave conflicting conclusions regarding the number of permeases in this yeast, though these conclusions were based only on competition and growth studies and not on mutant studies. Davies (1980) reported that di- and tripeptides competed for transport, and proposed the presence of a single permease. Logan et al., (1979) reported that Met-Met did not compete for uptake of radioactively-labelled Met-Met-Met and concluded that di- and tripeptides did not share a common permease in Candida albicans WD 18-4.

Early reports in the literature (reviewed in Section 1.3.3) indicated the presence of multiple permeases in bacteria and fungi. For example, resistance to a toxic dipeptide can be associated with a loss of dipeptide transport (via a system analagous system to the dpp in E.coli), with no effect on oligopeptide transport activity or on the intracellular target site. Resistance through loss of transport activity would also prevent utilization of that particular class of peptide as a source of required nitrogen or amino acid assuming that the various permeases do not have overlapping specificities. Thus, the studies described here attempted to delineate the nature of the peptide permeases in this yeast, by employing these 'classical' methods of mutant characterization

Mutants Resistant to Bacilysin and m-fluorophenylalanylalanylalanine

The bacilysin resistant mutants provide the first clear evidence
for the existence of separate systems for the uptake of dipeptides and
oligopeptides in Candida albicans. This mutation was revertible, and
the cross-resistance pattern and peptide transport capacity were
strictly associated Oligopeptide transport was unaffected in these
mutants, transport rates being equivalent to those exhibited by the
parent strain B2630. Similarly transport of amino acids (for those
substrates examined) was again equivalent to that in the parent strain.
These mutants were unable to utilize Ala-Ala as a sole nitrogen source
whereas utilization of the tripeptide was equivalent to that of the
parent strain. The existence of separate transport systems for di- and
oligopeptides was clearly demonstrated by the demonstration of cross-
resistance to FPA whilst retaining sensitivity to FPAA. The revertants
of the bacilysin-resistant mutant PA8035 regained sensitivity to
bacilysin and FPA, exhibited parental rates of Ala-Ala transport and
regained the ability to utilize Ala-Ala as a sole nitrogen source.

However, several of the bacilysin-resistant mutants exhibited
unexpected properties, e.g. varied morphological forms were detected,
the exact significance of which is uncertain. This is reminiscent of
earlier reports in which Candida albicans mutants have been obtained in
high yield, which produce large and small colonies, show pleiotropic
defects in transport and auxotrophy etc., and it has been suggested
that the natural heterozygosity of some genes may be responsible
(Whelan et al., 1981; Whelan & Magee, 1981). No discernible
correlation between resistance profile and colony size was found, and
thus the significance of these colonies with altered colony morphology
is unclear. Certain of the bacilysin-resistant mutants showed only a

partial reduction of Ala-Ala transport (approximately 30% of the transport rate exhibited by the parent). One of these mutants PA8034, was also shown to have decreased ability to grow on Ala-Ala as a sole nitrogen source. This class of 'partial' mutants was not examined further.

Of the 14 colonies regaining a wild-type ability to utilize Ala-Ala as a sole nitrogen source, only 3 showed wild-type sensitivity to bacilysin. Of these, 2 were examined for peptide uptake and both were shown to exhibit the same rates as the wild-type. The exact mechanism by which the other 11 mutants acquired the ability to utilize Ala-Ala and yet remained resistant to bacilysin remains unclear; one possibility is the release of extracellular peptido-hydrolytic activity to produce free amino acids for utilization.

Thus, although several types of mutant phenotype were found, studies with these bacilysin-resistant mutants and their corresponding revertants, have demonstrated the existence of separate systems for uptake of dipeptides and oligopeptides in Candida albicans. The complementary properties of the FPAA-resistant mutants endorse these conclusions. These mutants were demonstrably resistant to FPAA in the radial streak assay though still showed sensitivity in the seeded soft-agar overlay assay. These mutants exhibited reduced oligopeptide (Ala-Ala-Ala and Ala-Ala-Ala-Ala) transport but retained dipeptide transport activity at the same level as that exhibited by the parent B2630. In both of the sensitivity assays the cells encounter a variable zone of drug concentration diffusing radially from a drug reservoir the seeded agar assay being the more sensitive as the cells are suspended at a lower effective concentration in a soft-overlay containing the toxic peptide. Thus, partial resistance (reduced sensitivity) may possibly be easier to detect by the radial streak assay.

Cross-resistance to bacilysin by both polyoxin- and nikkomycin-resistant mutants has been reported both in this study and in recent publications (see following pages). Bacilysin has previously been shown to be transported by peptide permeases in both bacteria and fungi (Section 1.4.2.1), through studies involving selection and characterization of bacilysin-resistant mutants and antagonism of bacilysin action by various dipeptides.

Mutants Resistant to Polyoxin and Nikkomycin

Early reports in the literature indicated that polyoxins were inactive against zoopathogenic fungi including Candida albicans (Chiew et al., 1980; Endo et al., 1970; Isono et al., 1965, 1967, 1971; Ohta et al., 1970). However, convincing evidence has recently been presented for polyoxins inhibiting several strains of Candida albicans (Naider et al., 1983; Becker et al., 1983; Shenbagamurthi et al., 1983; Mehta et al., 1982, 1984). Similarly, inhibition of Candida albicans by the structurally related nikkomycins has also been reported (Yadan et al., 1984; McCarthy et al., 1985a; Furter & Rast, 1985; Adams & Gooday, 1983), all of these reports being published during the course of these studies.

In accordance with these recent reports, varied sensitivities to polyoxins amongst several strains of Candida albicans were found in these studies. Sensitivity to polyoxins (A, B, D and basic polyoxin fraction) were determined by dilution assays in both liquid (microtitre plate assay) and on solid media (agar dilution assay). MIC values for strains B2630 and A were lower when determined by the agar dilution assay. Considerable variation was found in the sensitivity of the strains tested to the various polyoxins (Tables 5.1 and 5.2) with B2630

being the most sensitive and the polyoxins being fungicidal against this strain; for this reason B2630 was chosen for further characterization

Polyoxins have long been known to be inactive in complex, peptide-based media (Bowers et al., 1974; Mitane & Inoue, 1968; Endo & Misato, 1969) implying that their entry into the cell may be by a peptide transport system(s). The media-dependence of polyoxin action has recently been further investigated; the effects of polyoxin D (1 mM, i.e. about 0.5 mg.ml^{-1}) on Candida albicans were shown to be inhibited by the presence of tryptone and to a lesser extent by yeast extract and peptone (all 1% w/v) (Becker et al., 1983). Similarly, polyoxins B and D did not exhibit any growth inhibition when tested in Sabouraud-glucose broth or Trypticase soy broth (Mehta et al., 1984) These authors examined the effects of medium composition on polyoxin activity, and MIC values as low as 0.1 ug ml^{-1} were obtained for a medium containing ribose, yeast nitrogen base (without added carbon and nitrogen source) with aspartate, glutamate or lysine as a sole nitrogen source. Alanyl-, and methionyl-, di-, and tripeptides were shown to antagonize the activity of dipeptidyl polyoxin B (Mehta et al., 1984).

Three mutants isolated on the basis of resistance to basic polyoxin fraction (which is predominantly a mix of tripeptidyl polyoxin A and dipeptidyl polyoxin B) were shown to exhibit cross-resistance to the structurally-related nikkomycin (nikkomycin X and Z mixture), and also to bacilysin whose mechanism of toxicity is different; but they retained sensitivity to synthetic fluoro-phenylalanyl di- and tripeptides. Transport of di-, tri- and tetrapeptides was not detectable in these mutants. Two mutants were characterized further and both exhibited reduced rates of amino acid transport. Utilization

of Ala-Ala and Ala-Ala-Ala as a sole nitrogen source was equivalent to that of the parent B2630. These properties are incompatible with the 'classic' mechanism of drug-resistance through loss of a single transport system, and as such, are difficult to explain.

There have been only two other reports in the literature of the isolation of polyoxin-resistant mutants of Candida albicans. Mehta et al., (1984) reported the isolation of three mutants resistant to polyoxin B, which were cross-resistant to FPA and bacilysin; two of these mutants were cross-resistant to FPAA whilst the third exhibited partial sensitivity to the tripeptide. The other report concerned a mutant with an altered chitin synthetase, and will be discussed later.

The nikkomycin-resistant mutants described in these studies were cross-resistant to both polyoxin and bacilysin. Peptide transport was not affected in the nikkomycin-resistant mutants, rates being equivalent to that of the parent B2630 (i.e. within the range of values exhibited by B2630, though in some cases the mean value for tri- and tetrapeptide uptake was actually higher than that of B2630). These mutants utilized Ala-Ala and Ala-Ala-Ala with the same efficiency as B2630 and showed equivalent sensitivity to FPA and FPAA. Clearly, these mutants differ from the polyoxin-resistant ones.

Nikkomycin-resistant mutants of Candida albicans have also been described in the literature during the course of this study (McCarthy, 1983; McCarthy et al., 1985a; Yadan et al., 1984).

Of six nikkomycin-resistant mutants of Candida albicans 124, only one, NIK5, was defective in Ala-Ala transport. This mutant was cross-resistant to polyoxin and to bacilysin, and had greatly reduced dipeptide transport. However, it had markedly increased rates of tri- and tetrapeptide uptake (upto 253% of the rate exhibited by the wild-type), though transport of tripeptidyl FPAA was reduced in this strain

with a corresponding reduction in sensitivity to this peptide (McCarthy, 1983; McCarthy et al., 1985a). These authors also showed that in a similar manner to studies with polyoxins, the activity of nikkomycin was antagonised by the inclusion of peptone (2% w/v) or Ala-Ala (10 mM equivalent to 1.6 mg.ml^{-1}) and Leu-Gly (also at 10 mM). Certain apparently non-transported dipeptides (e.g. Gly-Tyr and Gly-Gly), and Ala-Ala-Ala and Ala-Ala-Ala-Ala (both transported) did not antagonize the activity of nikkomycin. In contrast, Leu-Leu, which apparently is not transported by strain 124, competed with nikkomycin activity, and also inhibited the uptake of radioactively-labelled Ala-Ala thus it was proposed that Leu-Leu competes at a binding level (McCarthy, 1983). The uptake of radioactively-labelled Ala-Ala was shown to be inhibited by both nikkomycin (Ki, 4.85 ug ml^{-1}) and polyoxin (Ki 195 ug ml^{-1}). The fact that nikkomycin resistance was associated with a defect in peptide transport was taken as evidence for the existence of at least two separate peptide permeases, system I having high affinity for dipeptides including the dipeptide nikkomycins, and system II having high affinity for oligopeptides (McCarthy, 1983; McCarthy et al., 1985a).

In contrast to other studies, Yadan et al., (1984) reported that nikkomycin Z was active against Candida albicans ATCC 26278 in both peptone and minimal media (at 10 ug ml^{-1} and 5 ug ml^{-1}, respectively). However, they reported that the activity of polyoxin was media-dependent, MIC values being 5 ug ml^{-1} in minimal media (isoleucine as the sole nitrogen source) and 200 ug ml^{-1} in peptone-based media. These workers described the isolation of a spontaneous nikkomycin-resistant mutant (designated ATCC 26278 Nik), by growing cells in peptone-media containing nikkomycin Z (50 ug ml^{-1}) and spreading

dilutions of resistant cell suspensions onto peptone-agar plates containing nikkomycin. The Nik mutant lost its capacity to take up radioactively-labelled nikkomycin, and had reduced uptake of dipeptide (Met-Met) but greatly increased rates of uptake of tripeptide (Met-Met-Met). These results, coupled with the results of competition studies between nikkomycin, Met-Met and Met-Met-Met, were taken as evidence for the existence of multiple peptide transport systems in Candida albicans (Yadan et al., 1984) the increased activity of the tripeptide system in the Nik mutant being considered to be an adjustment to environmental conditions (i.e. growth in peptone-based media).

However, the nikkomycin-resistant mutants described in both of these studies were not obtained using a rigorous, spontaneous, single-step isolation procedure (e.g. isolation from a drug inhibition zone) and are thus potentially suspect as to the exact nature of the genetic basis of their resistance. Thus, the mutants described by McCarthy (1983) were UV-irradiated prior to selection on a plate containing nikkomycin and therefore there is a possibility that the NIK5 mutant may contain several mutations. Certainly, the fact that both the Nik and NIK5 mutants described in these studies have elevated levels of oligopeptide transport, despite being isolated on the basis of resistance to a toxic dipeptide (with a corresponding loss of dipeptide transport) is unexpected and difficult to explain.

The evidence for the multiplicity of peptide transport systems in Candida albicans is further supported by results from studies with the novel chromophoric peptides (alanyl-2-thiophenylglycine, (Ala-α-TPG); and alanyl-2-thiophenylglycylalanine, (Ala-α-TPG-Ala)) (McCarthy et al., 1985b). Subsequent to transport into Candida albicans, intracellular enzymic hydrolysis of this peptide leads to the efflux of

thiophenol, which is quantified by using Ellman's reagent. Thiophenol release was shown to be correlated with peptide transport and hydrolysis, transport being the rate limiting step. Candida albicans 124 was able to transport and hydrolyse both Ala-α-TPG and Ala-α-TPG-Ala, whereas the mutant NIK5 was able to transport only the tripeptide, providing supporting evidence for the existence of multiple peptide transport systems in this yeast.

The polyoxin- and nikkomycin-resistant mutants isolated in the studies here, did not possess an altered chitin synthetase, the sensitivity of isolated chitin synthetase preparations to polyoxin D being equivalent to that exhibited by the parent strain B2630 (Section 5.5.7). Similarly, the nikkomycin-resistant mutant described by McCarthy (1983), was also shown not to possess an altered chitin synthetase. There has been only one report of an altered chitin synthetase associated with resistance to peptide-nucleoside drugs; a 'streak' morphological mutant of Schizophyllum commune isolated as resistant to polyoxin D (Ki values were 16 uM for the wild-type and 100 uM for the mutant) (MacGruder, 1979).

Thus, the exact nature of the mechanism of resistance of the nikkomycin-resistant mutants remains unclear. Cross-resistance of these mutants to bacilysin (in addition to polyoxin) makes any possible explanation complicated. Thus, a change in the intracellular cleavage leading to nikkomycin breakdown is precluded; as bacilysin in addition to being structurally distinct, actually requires peptidase cleavage for activity. It is possible that the nikkomycin-resistant mutants described in these studies are unable to concentrate these toxic peptides due to an enhanced efflux of transported peptides. Thus, it may not be possible for these mutants to concentrate these toxic

peptides intact to a sufficiently high intracellular concentration for to exert their normal inhibitory effects

Thus, these studies and others have established that resistance to polyoxin and nikkomycin can be demonstrably associated with a defect in peptide transport, though somewhat bizarre phenotypes have been reported in each study to date. The general unavailability of pure antibiotic samples in sufficient quantities, and the variety of assay and growth conditions used, have so far precluded any definitive statements as to the mechanism of resistance. Clearly, the possible mechanisms of resistance are more complex than was initially envisaged. This yeast has been shown to possess two distinct peptide transport systems, though the exact specificities of these systems remains unclear A detailed characterization of the transport capacities of the mutant strains for a wide range of peptide substrates together with sensitivity studies with a range of defined toxic peptides, should resolve both the number and nature of peptide transport systems in Candida albicans.

It has been proposed recently that uptake of polyoxins and nikkomycins is brought about by the transpeptidase reaction of the γ-glutamyl cycle (Furter & Rast, 1985), the antagonism between di- and tripeptides and nikkomycin action being attributed to competition for a common carrier. However, recent reports (e.g. Payne & Payne, 1984) have precluded a major direct role for the γ-glutamyl in the bulk uptake of peptides (and amino acids) in yeast (see Section 1.3.5), thereby precluding a significant role for this cycle in the uptake of nikkomycins and polyoxins.

Gooday et al (1985), reported that nikkomycin inhibited the formation of β-chitin spines by the diatom Thalassiosira fluviatilis and slowed its growth rate. (Inhibition of chitin formation resulted in sedimentation of the cells, as the chitin spines act as a flotation device). These authors reported that the action of nikkomycin was not antagonised by certain dipeptides, which were shown to be taken up by this organism; though this may be due to the presence of multiple peptide transport systems in this organism, with nikkomycin and the non-competing peptides utilising different systems for their uptake. Also, the optimal conditions for peptide uptake were not determined in this study; higher rates of uptake of peptides or nikkomycin may result if this organism were to be grown in a medium containing a poor nitrogen source (analagous to the regulation of peptide transport in yeast; Sections 1.3.3.4, 4.4).

One unexpected result reported in this study, was that amongst the strains of Candida albicans tested, there was no correlation between their sensitivity to polyoxin (Section 5.3) and their rates of peptide uptake (Table 5.4). Strain 6406 was the least sensitive strain to polyoxins (Table 5.1) though exhibited the highest rates of peptide transport, whereas strain B2630 proved to be the most sensitive strain to polyoxins though exhibited the lowest rates of peptide transport. This aspect clearly requires further study, though it may reflect strain variation in either the accessability to the peptide transport system, through differences in permeability at the cell wall, or differences in affinities of these peptide-nucleosides for the peptide transport systems which is not directly reflected with the affinities of natural peptides for the systems.

The effects of the polyoxins and nikkomycins on cell morphology were not examined in this study having been previously well documented (Sections 1.4.4.2 and 1.4.4 3). Becker et al., (1983) showed that polyoxins at millimolar concentrations caused marked morphological alterations of Candida albicans and Cryptococcus neoformans. Candida albicans incubated in the presence of polyoxin D grew in long chains that were severly swollen, prolonged incubation leading to cell death of both yeast and hyphal forms. Again, some strain variation to polyoxin sensitivity was noted. Shenbagamurthi et al., (1983) reported that analogues of polyoxin L caused severe distortions of Candida albicans in culture. Similarly, tripeptidyl derivatives of polyoxins D and C affected cell morphology (Naider et al., 1983). Nikkomycin (X and Z mixture) was shown to affect seriously cell wall morphology in Candida albicans 124 through inhibition of primary septum formation, a protoplasting effect in growing cells due to a weakening of the cell wall, and finally, the lysis of the cell wall at the point adjacent to the septum, resulting in cell death (McCarthy, 1983; McCarthy et al., 1985a). Similarly, Yadan et al., (1984) reported that incubation of Candida albicans with nikkomycin Z resulted in morphological distortion.

The Future Potential of Inhibitors of Chitin Synthetase as Anticandidal Agents

Recent studies have concentrated on designing and synthesising analogues of polyoxins (Shenbagamurthi et al., 1983; Naider et al., 1983) and uridine 5'-diphosphate-N-acetylglucosamine (UDPNAG) (Adams & Gooday, 1983), with a view to producing more effective inhibitors of

chitin synthetase (Section 1.4.4 1). Shenbagamurthi et al., (1983) reported the synthesis of 6 analogues of polyoxin L all of which inhibited chitin synthetase. However, these analogues were not effective against Candida albicans presumably because of their low affinity for the peptide transport system and their rapid intracellular hydrolysis to uracil-polyoxin C (which is inactive against chitin synthetase). Polyoxin D was also reported to have a low affinity for the peptide transport system, but was resistant to intracellular hydrolysis and therefore was the most effective at killing the cells. All the analogues caused morphological alterations but a longer period of incubation with the intact drug was required for cell death. Naider et al., (1983) reported the synthesis of three tripeptidyl polyoxin prodrugs, leucyl-polyoxin D (Leu-POD), norleucyl-uracilpolyoxin C-leucine (Nle-UPOC-Leu) and leucyl-norleucyl-uracilpolyoxin C (Leu-Nle-UPOC). These tripeptidyl polyoxins were poor inhibitors of chitin synthetase but Leu-POD and Leu-Nle-UPOC were active against intact cells. Leu-POD and Leu-Nle-UPOC were hydrolyzed to POD and Nle-UPOC respectively, both products being active against chitin synthetase, whereas Nle-UPOC-Leu was hydrolyzed to the inactive UPOC-Leu. Further hydrolysis of Nle-UPOC and UPOC-Leu led to the production of inactive UPOC. Leu-Nle-UPOC was shown to inhibit the uptake of radioactively-labelled Met-Met-Met, suggesting that it enters the cell by the peptide transport system. Adams & Gooday (1983) reported that synthetic derivatives of UDPNAG inhibited a chitin synthetase preparation from Coprinus cinereus, although they were not as inhibitory as polyoxins and nikkomycins.

An important consideration in the design of analogues of peptide-nucleoside drugs (and UDPNAG) for targeting against chitin synthetase is that these compounds must be resistant to intracellular peptidase

action (which cleaves these analogues to inactive moieties), whilst retaining sufficient affinity for the peptide transport system(s). In this way an optimal intracellular concentration can be attained which will result in cell death. Tripeptidyl analogues of POD with a high affinity for the peptide system, which are still resistant to cleavage by intracellular peptidases (to release the active moiety POD), therefore offer potential as effective prodrugs. Thus, it is clear that chitin synthetase inhibitors have potential as anticandidal agents.

Conclusion

The availability of pure samples of di- and tripeptidyl polyoxins and/or nikkomycins in reasonable amounts would greatly facilitate further characterization of the transport systems in Candida albicans. In this study, the limited amount of available tripeptidyl polyoxin A precluded any attempts to isolate mutants resistant to this drug. Unfortunately, the toxicity of FPAA for B2630 was low, however, the mutants isolated as FPAA-resistant (as indicated by the radial streak assay) did exhibit reduced rates of oligopeptide transport, offering some hope for future work. The recent description of peptide-photoaffinity labels (Sarthou et al., 1983; Becker et al., 1982) also offers promise for future studies. If photo inactivation of individual systems proves feasible, it could allow detailed characterization of the kinetic parameters for the individual systems without the complication presented by possible overlapping substrate specificities. In addition, the possibility of labelling specific permeases with radioactively-labelled photoaffinity labels would aid the characterization of the molecular components of the various permeases.

CHAPTER 6

FURTHER CHARACTERIZATION OF THE TRANSPORT SYSTEMS

IN CANDIDA ALBICANS

THE EFFECTS OF CHEMICAL MODIFICATION OF MEMBRANE PROTEINS UPON PEPTIDE AND AMINO ACID TRANSPORT

6.1 Introduction

Specific chemical modification of amino acid side chains is a valuable technique in the determination of those regions of a protein which are essential to its continued integrity and activity, and has been the subject of several reviews (Cohen, 1968; Glazer, 1970; Sigman & Mooser, 1975; Rakitzis, 1984).

In any modification study, care must be taken in the interpretation of an inhibition. Inactivation of an enzyme or transport protein could be due to not only the direct modification of catalytically important residues, but also indirectly to the alteration of conformation of the active site, either through steric hindrance by a large reagent moiety, or by an "allosteric" distortion of the protein molecule. Furthermore in energy-coupled processes such as transport, suitable control experiments must be performed to ensure that a direct effect is being observed and not simply on interference with the electrochemical gradient, membrane potential or ATP supply. The possibility that the observed effects maybe due to a secondary mode of inhibition may be partially eliminated by the ability to 'substrate screen', in which substrate molecules of the transport-system confer protection against a modifying reagent, presumably by covering or preventing access to the susceptible target amino acid side chains of the active site of the protein. Such protection will not necessarily restrict group modification of non-catalytic molecules, located elsewhere in the protein.

There is a large number of potein-modification reagents commercially available but although several of them are highly

selective, absolute specificity is exceptional. Several of these reagents, being available as either radioactively-labelled or chromophore/fluorescent-labelled reagents, have applications in the identification and isolation of specific transport proteins, the label acting as a 'handle' for presumptive identification of the protein. Whereas, several of the available reagents are highly selective, the nature of the micro-environment of the potential reactive residues in an active site, may preclude modification of particular residues. The chemical derivatization of a functional group may be prevented by steric "protection" at the site by other residues, or a particularly hydrophobic or ionic environment may preclude successful modification. Membrane-bound, transport proteins pose additional considerations regarding accessability of functional residues, because factors such as the penetrability of the reagent to sites inside the membrane come into play, and, in addition, the possible lipophilic nature of the carrier proteins may exclude particularly polar or charged reagents from their vicinity. Thus, though many reagents may be pronounced specific or non-specific with regard to their activity towards simple molecules in a test system, care must be taken in extrapolating this apparent selectivity to the situation in a protein molecule in vivo, where functional groups may have very different reactivities as a result of their particular micro-environment.

In the studies presented here, the effects of a range of chemical modification reagents on the transport of representative peptides and amino acids is described, with a view to the selective inhibition and ultimate isolation of specific carrier protein(s). The possibility of specific labelling, coupled with the availability of various transport-deficient, peptide drug-resistant mutants (Chapter 5), offers great

potential for the isolation and identification of the protein component(s) of the peptide transport system(s) in <u>Candida albicans</u>

6.2 <u>Methods</u>

6.2.1 <u>Preincubation with Inhibitors and Reharvesting of Cells</u>

Cells were grown overnight in liquid Pro- or Pep-Medium and harvested by membrane filtration as described (Section 4.2.2). Organisms were resuspended in incubation buffer (phosphate-citrate-glucose, (PCG) pH 4.5, Section 4.2.2) to 1 - 2 (mg dry wt)ml^{-1} (2 x 5 ml), and preincubated at 28°C for 5 min, cells being gently agitated by means of a small magnetic stirrer-bar.

After 5 min preincubation, inhibitors (1 - 10 mM final concentration) were added and incubation continued for the desired period (2 - 30 min). Cells were reharvested by centrifugation (MSE Microcentaur) in plastic Eppendorf centrifuge tubes (2 - 4 x 1 ml, depending on the number of cells required for transport assays), inhibitor-treated cells always being reharvested before the untreated control Cells were centrifuged at high speed for 1 min, the supernatant solution was discarded, and the cells were resuspended in PCG incubation buffer, pH 4.5 (1 ml) by vortexing, before recentrifuging (1 min at high speed). Cells were resuspended in PCG incubation buffer (approx. 0.4 ml tube^{-1}), aliquots combined and diluted to the required cell density for each particular transport assay (2 - 4 ml, 0.7   2.5 (mg dry wt)ml^{1-}), cell densities being measured on a Bausch and Lomb Spectronic 20 spectrophotometer as described (Section 4.2.1). Cells were preincubated for a further 10 min at 28°C before addition of peptide or amino acid (0.1 - 0.5 mM), transport being assayed as previously described (Section 4.2.2).

The actual process of reharvesting by centrifugation was shown to actually inhibit transport per se (Fig. 6.1), relative to cells harvested and resuspended by the standard method (Section 4.2.2) i.e. harvesting by membrane filtration and resuspension into PCG incubation buffer.

For preincubation of cells with phenylglyoxal (Section 6.4.2.2) and diethylpyrocarbonate (Section 6.4.3.2), cells were resuspended in phosphate-citrate incubation buffer, either pH 7 (38 mM phosphate) or pH 6 5 (10 mM phosphate) respectively, containing glucose (0.8% w/v). Organisms were preincubated with inhibitor and reharvested by centrifugation as previously described, before assay of peptide and amino acid transport at pH 4.5, according to the standard method (Section 4.2.2). In control experiments, cells were grown in Pep-Medium overnight, harvested by membrane filtration and resuspended in PCG incubation buffer at pH 4.5 and at pH 7 (1.4 - 1.8 (mg dry wt.) ml^{-1}), and preincubated for 40 min before reharvesting by centrifugation and assaying for transport of Ala-Ala (0.2 mM) at pH 4.5. No marked difference in measured transport rate was detected (5.31 nmol min^{-1} (mg dry wt)$^{-1}$ for cells preincubated at pH 4.5, and 5.24 nmol min^{-1} (mg dry wt)$^{-1}$ for cells preincubated at pH 7), showing that the preincubation of cells at pH 7 had no effect on measured transport rate. For all experiments involving preincubation at pH 7, batches of control cells were always preincubated at pH 7, at the same time as the inhibitor-treated cells.

6.2.2 Addition of Inhibitor During Substrate Uptake

In addition to preincubation studies (Section 6.2.1), inhibitors were also added during uptake of substrate. Organisms were grown in Pep-Medium overnight, harvested by membrane filtration and resuspended
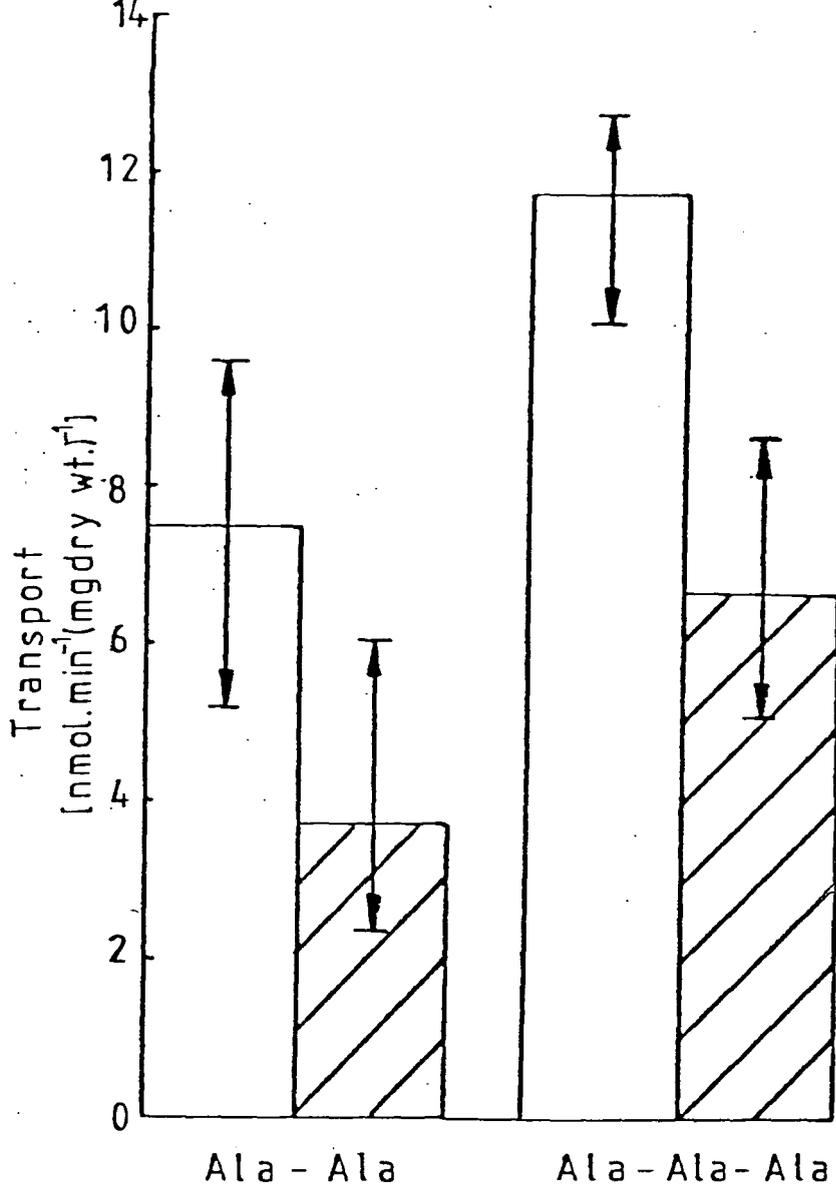
Figure 6.1 Effect of Reharvesting by Centrifugation on Peptide

Transport Activity

Candida albicans B2630 was grown overnight in Pep-Medium, harvested and

resuspended according to the standard method (Section 4.2.2). For

inhibitor preincubation studies, untreated control cells were

reharvested by centrifugation (Section 6.2.1).

Transport of peptide (0.2 mM) was assayed by the standard manual

fluorescamine assay (Section 2.5.2), for both standard treated (□)

and those cells reharvested by centrifugation (▨).

Values represent the mean and range of at least 3 separate

determinations.

in PCG incubation buffer, pH 4.5, typically 0.6 - 1.6 (mg dry wt)ml^{-1}

(2.4 ml), according to the substrate under examination (Section 4.2.2).

Cells were preincubated at 28°C for 10 min before addition of substrate

(0.2 mM). Samples (0.3 ml) were taken periodically (typically at 1, 4,

8, 12 and 16 min after addition of substrate) using an automated

pipette, and immediately freed of cells by filtration (Section 4.2.2).

The substrate concentration of the filtrate (2 x 100 ul) was assayed by

the standard fluorescamine procedure (Section 2.5.2) and results

plotted against time, allowing determination of initial rates of

transport.

In this manner the time scale for the effect of inhibitors (0.1 -

5 mM) on the uptake of substrate can be determined, for those

inhibitors which do not interfere with the standard fluorescence assay

(Section 6.2.3). Routinely, inhibitors were added 7 min after addition

of substrate, the required volume of inhibitor (of known concentration)

being calculated from the residual volume of the cell suspension. A

further 3 samples (0.3 ml) were removed for fluorescence assay.

Inhibitor studies were always performed with an untreated control for

each transport substrate examined, for cells taken from the same

overnight batch.

6.2.3 Effects of Inhibitors on Fluorescence Assays

A prerequisite for determination of any time-dependant effects of

protein-modification reagents on transport activity (Section 6.2.2) is

that the reagent should not interfere with the transport assay per se.

The effects of the various protein modification reagents on the

standard manual fluorescamine assay (Section 2.5.2) was therefore

determined.

Throughout the course of the protein modification studies, initial rates of peptide transport (typically Ala-Ala and Ala-Ala-Ala) were determined at an initial substrate concentration of 0.2 mM. Assay conditions were as follows :- filtrate (0.1 ml) in duplicate, K_2HPO_4 (0.25 ml, 0.2 M), fluorescamine (100 ul, 1 mg ml^{-1} in Analar acetone) plus K_2HPO_4 buffer (2 ml) to make upto a readable volume. With an initial substrate concentration of 0.2 M, 20 nmol per tube was typically assayed by the modified manual fluorescamine procedure. For chemical modification studies, reagent was typically at 5 mM, i.e. present at a 25 fold excess relative to peptide or amino acid substrate. Therefore, though most of the reagents examined contain no free primary amino groups, it was essential to check for any possible effects on the manual fluorescamine assay.

To determine whether the protein modification reagents had any effect on the manual fluorescamine assay, Ala-Ala solutions (0.05 - 0.2 mM) was preincubated with inhibitor (5 mM) in PCG incubation buffer pH 4.5 for 20 min (28°C), before performing the standard manual fluorescamine assay. Results were plotted as fluorescence (arbitary units) against concentration of Ala-Ala assayed (5 - 20 nmol) (Fig. 6.2). Protein modification reagents tested in this way were N-ethylmaleimide (NEM), p-chloromercuribenzenesulphonic acid (PCMBS), phenylarsineoxide (PAO) and N-ethyl-5-phenylisoxazolium-3'-sulphonate (Woodwards Reagent K, or WRK)

Of the inhibitors examined, only WRK interfered with the fluorescence assay; NEM, PCMBS and PAO all had no detectable effect on the fluorescent yield of the standard Ala-Ala solutions. Interference by WRK, thus precludes its use in experiments in which inhibitors are
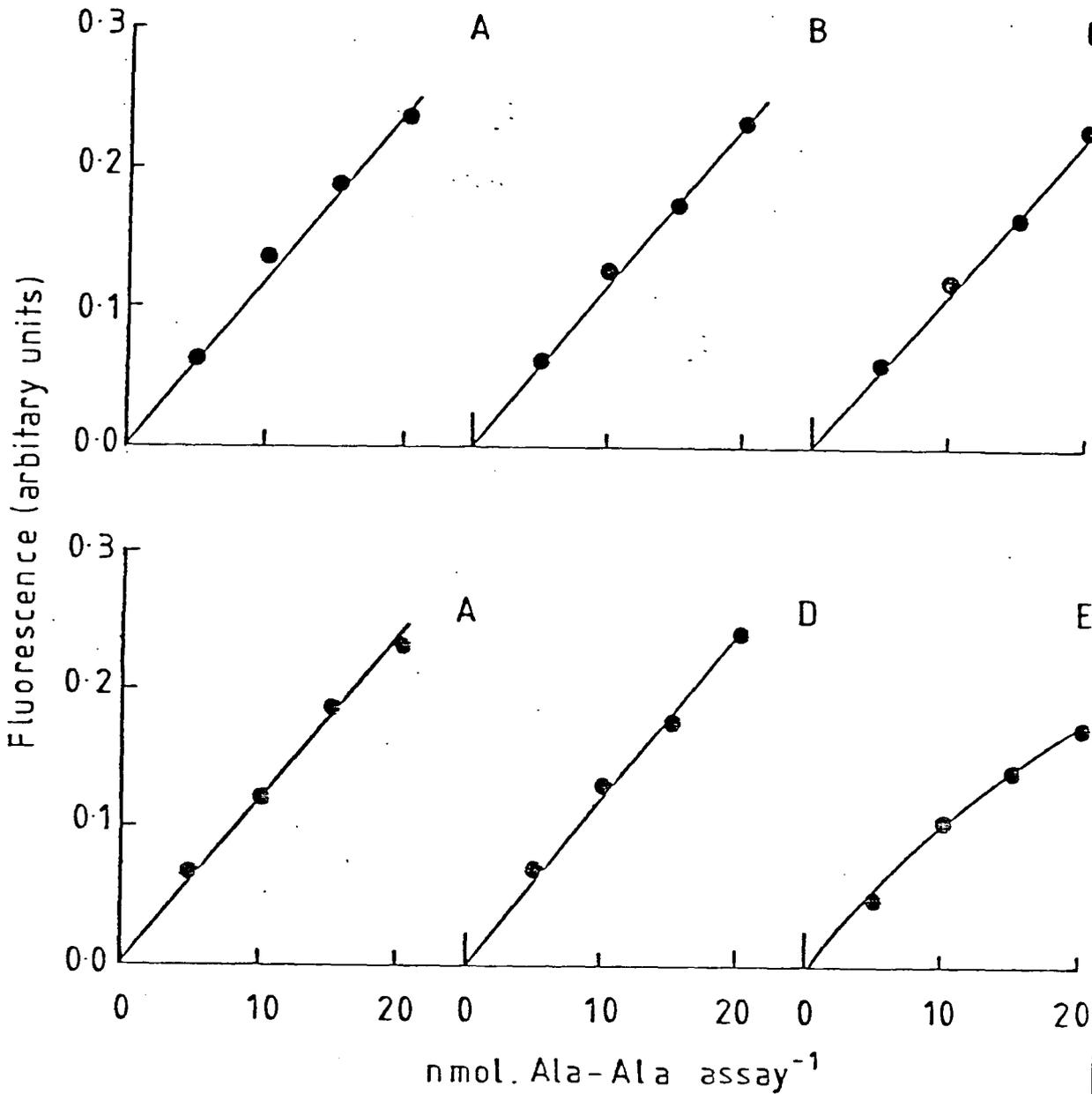
Figure 6.2   Effect of Protein-Modification Reagents on Fluorescence Assay

Fluorescence of Ala-Ala solutions (0.05 - 0.2 mM) preincubated with protein-modification reagents, NEM (B), PCMBS (C), PAO (D), and WRK (E) relative to that obtained for an untreated control (A).   Abbreviations and assay conditions are described in the text (Section 6.2.3)

added to the cell suspensions undergoing substrate uptake i.e. pulse experiments (Section 6.2.2). Conversely, the use of NEM, PCMBS and PAO in the assays is legitimised.

Having demonstrated that the protein-modification reagents NEM, PCMBS and PAO do not interfere with the fluorescence assay of Ala-Ala it may be assumed that they will not interfere with the fluorescence assay of other peptides or amino acid substrates, since the assay is based on a similar reaction of fluorescamine with the primary, unprotonated α-amino group of these substrates (Section 2.5.1). However, controls similar to those described here should perhaps be performed for peptide or amino acid substrates containing additional amino groups, e.g Lys containing peptides.

The application of phenylglyoxal and diethylpyrocarbonate in these pulse studies is precluded because of their requirement for a high pH (pH 7 and pH 6.5 respectively) for activity, whereas peptide transport is optimal at pH 4.5 (Section 4.3 4), and all uptake experiments were performed at this pH. Thus, the effects of these two reagents were examined by preincubation studies only (Section 6.2.1).

6.3 Effect of Sulphydryl Modification on Transport Activity

6.3.1 Effect of N-Ethylmaleimide on Transport Activity

6.3.1.1 N-Ethylmaleimide - Chemical Properties

N-substituted maleimides, form a family of compounds that react readily with sulphydryl groups. These reagents consist of a chemically reactive maleimide moiety, bonded to a side chain substituent whose structure can be manipulated to confer different physical properties Thus, impermeant maleimides carrying hydrophilic bulky side chains have been synthesised, which are specific for only those SH groups exposed

at the surface. In contrast, N-ethyl-, N-butyl- and N-butyl-substituted maleimides can successfully derivatize SH groups within hydrophobic membrane environments (Le-Quoc et al., 1981). Fluorescent derivatives e.g. N-(1-pyrene)maleimide (Wu et al., 1976) and coloured maleimides (Witter & Tuppy, 1960) have also been developed.

The most widely used reagent is N-ethylmaleimide (NEM), whose chemical reactivity has been studied in some detail (Smyth et al., 1960, 1964; Webb, 1966a). The activated double bond of NEM reacts rapidly, selectively and irreversibly with protein SH groups at pH 5 - 7 to form a stable addition product (Fig. 6.3). Above pH 7, absolute specificity is progressively lost and some reactions with amino groups and with histidine residues may occur. NEM undergoes alkaline hydrolysis above pH 7 to N-ethylmaleamate, though it is fairly stable in acid conditions. NEM is a highly penetrant species due to its small size and uncharged nature, and thus readily traverses membranes so that, in principle, all parts of the cell are accessible to NEM action (Klingenberg et al., 1974; Gaudemer & Latruffe, 1975). Many enzymes possess sulphydryl groups, and are therefore potentially susceptible to sulphydryl reagents, except in cases where 'buried' sulphydryl groups preclude successful modification. NEM has been shown to inhibit a wide range of cytoplasmic and also to inhibit organelle enzymes following penetration through intracellular membranes, e.g., rat liver mitochondrial hydroxybutyrate dehydrogenase (Gaudemer & Latruffe, 1975).

NEM has been used extensively in the study of membrane-bound transport systems (reviewed by Walker-Smith, 1984) see Section 6.4.4. Of particular relevance to these studies are the reports of inhibition
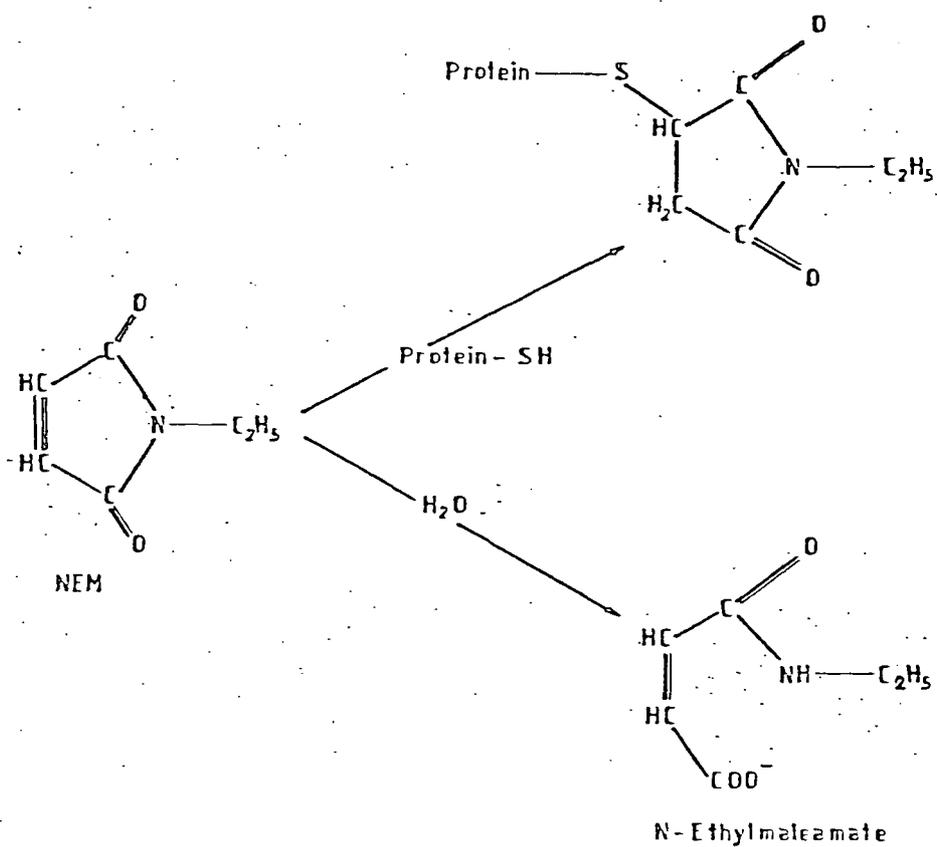
Figure 6.3   Reaction of N-ethylmaleimide with Sulphydryl Groups

NEM reacts with protein-bound thiols, to produce a stable addition product, although a second competing reaction can occur, which involves a hydrolytic ring-opening to form N-ethylmaleamate.

by NEM of amino acid transport in _Sacc. cerevisiae_ (Ramos et al.,

1980) and of peptide transport in _Candida albicans_ (Logan et al.,

1979) and in barley embryos (Walker-Smith & Payne, 1983a,b).

6.3.1.2 Effect of Preincubation of Cells with N-ethylmaleimide

Cells were preincubated with NEM (1 - 5 mM) for up to 10 min

before harvesting by centrifugation (Section 6.2.1), and assaying for

transport of Ala-Ala (0.2 mM) (Fig. 6.4). Preincubation with 5 mM NEM

resulted in complete inhibition of transport within 2 min, whereas with

1 mM NEM a 10 min preincubation resulted in about 40% inhibition

To determine whether the inhibition seen with Ala-Ala might be

specific for dipeptides, the effect of 2 min preincubation with 5 mM

NEM on the uptake of a variety of di- and oligopeptides and some amino

acids was also tested (Table 6.1)

6.3.1.3 Monitoring the Effect of N-Ethylmaleimide Addition During
Substrate Uptake

Candida albicans B2630 was grown in Pep-Medium overnight,

harvested and resuspended in PCG incubation buffer pH 4.5 (Section

4.2.2). The effect of NEM (0.1 - 5 mM) on the uptake of peptide and

amino acid substrates (all 0.2 mM) was examined using the standard

manual fluorescamine assay (Section 2.5.2), NEM having being shown not

to interfere with the fluorescence assay (Section 6.2.3). The effects

of NEM on the uptake of Ala-Ala, Ala-Ala-Ala and Gln are shown (Figs.

6.5 - 6.7).

6.3.1.4 Effect of N-Ethylmaleimide on the Uptake of Gly-[U^{14}C]Phe

Candida albicans B2630 was grown in Pep-Medium overnight,

harvested by membrane filtration and resuspended in PCG incubation

buffer, pH 4.5 (4 ml, 1.3 - 1.55 (mg dry wt.)ml^{-1}). Cells were
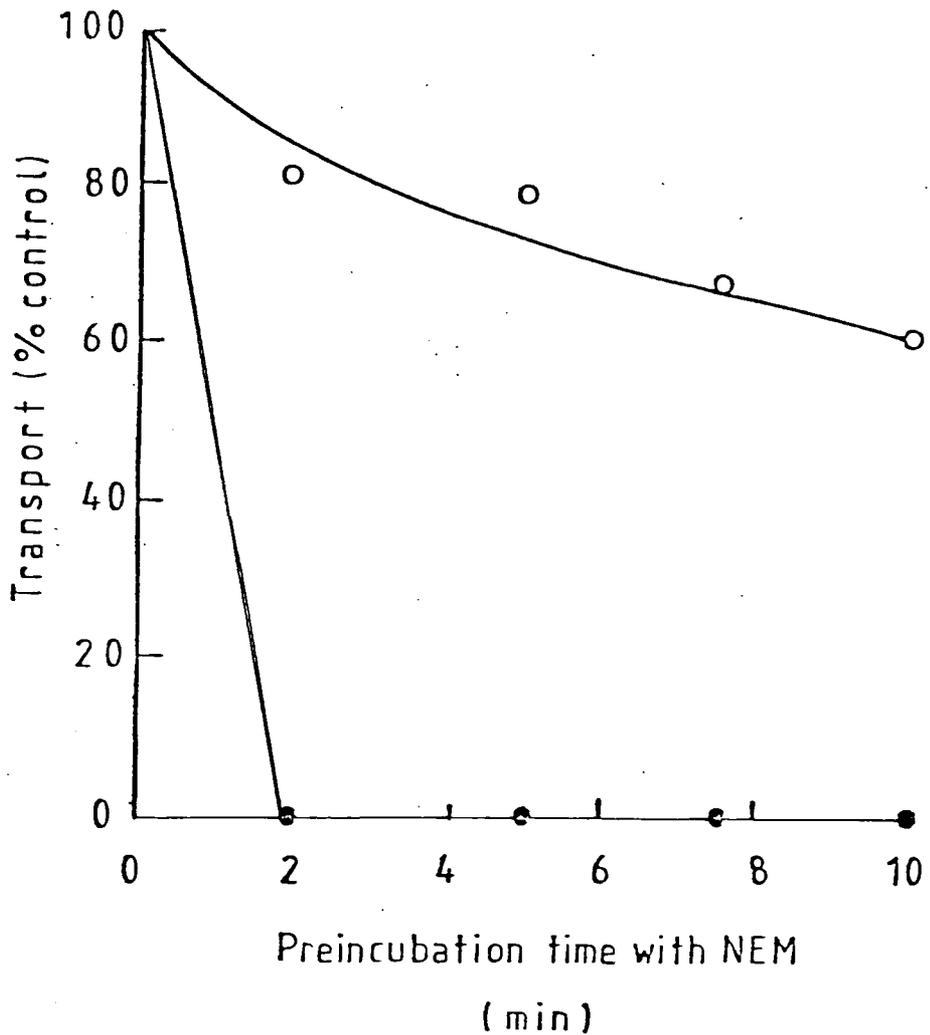
Figure 6.4   Inhibition of Ala-Ala Transport Following Preincubation

with NEM

Uptake of Ala-Ala (0.2 mM) by Candida albicans B2630 after
preincubation with NEM (1 mM (O) and 5 mM (●)) for the indicated
times.   Rates are expressed as a percentage of untreated controls.
Each value is the average of at least 2 separate determinations.

Substrate	% Inhibition
Ala-Ala	100
Ala-Ala (Pro-Medium grown)	100
Ala-Ala-Ala	100
Ala-Ala-Ala-Ala	100
Gln	100
Leu	100

TABLE 6.1   Effect of Preincubation (2 min) with NEM (5 mM) on

Transport Activity

Candida albicans B2630 was grown in Pep-Medium overnight (except as indicated) harvested and resuspended in PCG incubation buffer pH 4.5 (Section 4.2.2). Cells were preincubated for 5 min (28°), NEM (5 mM) was added and incubation continued for a further 2 min before reharvesting by centrifugation (Section 6.2.1). Transport of substrate (0.2 mM, except for Pro-Medium grown, 0.1 mM) was assayed using the standard, manual fluorescamine assay (Section 2.5.2). Results are the percentage inhibition relative to an untreated control.

Figure 6.5 Effect of NEM Addition During Ala-Ala Uptake

The effect of NEM (0.1 mM) on the Uptake of Ala-Ala (0.2 mM) by Candida

albicans B2630 (1.21 (mg. dry wt.)ml^{-1}).

Arrow indicates point of addition of NEM

(●) Control   (O) NEM treated

Figure 6.6   Effect of NEM Addition During Ala-Ala-Ala Uptake

A   The effect of NEM (1 mM) on the uptake of Ala-Ala-Ala (0.2 mM) by

      Candida albicans B2630 (0.78 (mg. dry wt)ml^{-1})

B   The effect of NEM (0.1 mM) on the uptake of Ala·Ala-Ala (0.2 mM)

      by Candida albicans B2630 (0.62 (mg. dry wt.)ml^{-1}).

Arrow indicates point of addition of NEM

(○) Control   (○) NEM treated
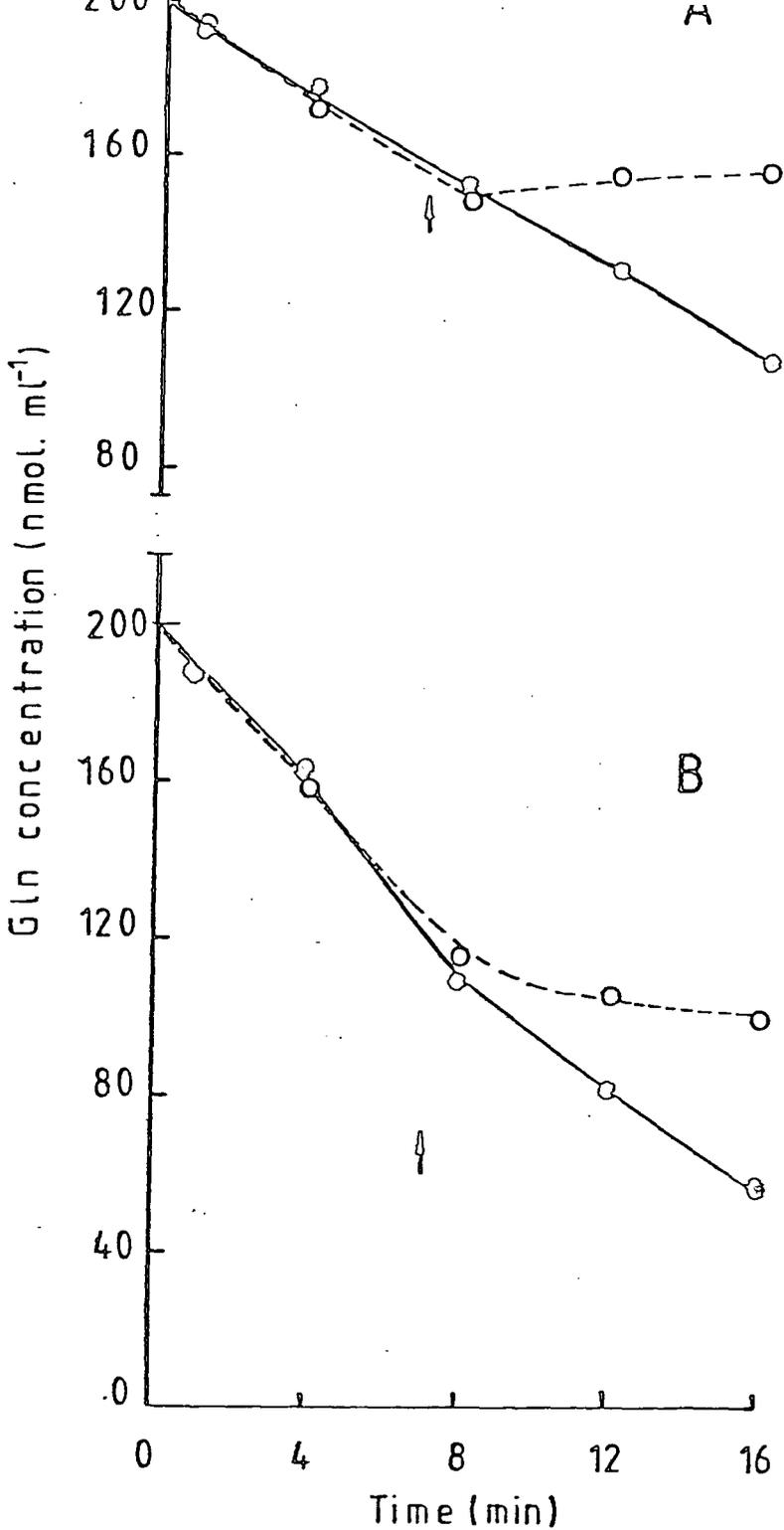
Figure 6.7 Effect of NEM Addition During Gln Uptake

A   The effect of NEM (1 mM) on the uptake of Gln (0.2 mM) by Candida
     albicans B2630 (1.16 (mg. dry wt.)ml^{-1}).

B . The effect of NEM (0.1 mM) on the uptake of Gln (0.2 mM) by
     Candida albicans B2630 (1.68 (mg. dry wt.) ml^{-1}).

Arrow indicates point of addition of NEM.

(o) Control   (O) NEM treated

preincubated for 10 min (28°C) before addition of Gly-[U^{14}C]Phe (final

concentration 0.2 mM, 0.01 uCi ml^{-1}). Samples (0.5 ml) were taken

periodically (1 - 18 min) and assayed for radioactivity as described

previously (Section 2.6.2). After 10 min, NEM (1 mM) (20 ul of 100 mM

stock solution) was pulsed into one sample (2 ml) of cell suspension.

This treatment rapidly inhibited accumulation of Gly-[U^{14}C]Phe (Fig.

6.8). The apparent loss of accumulated Gly-[U^{14}C]Phe after addition of

NEM may be due to either an experimental artifact or a time-dependent

exodus of radioactively-labelled material following the addition of

NEM.

6.3.2 Effect of p-Chloromercuribenzenesulphonic Acid on Transport Activity

6.3.2.1 Introduction - Chemical Properties

Organic mercurial compounds react rapidly and specifically with

sulphydryl groups at pH 5, and have been used extensively to gain

insight into the role of thiol groups in various proteins. Organic

mercurials react with sulphydryl groups to form mercaptides (Equation

1), the reaction being readily reversible by the addition of excess low

molecular weight thiol to regenerate the original SH group (Equation

2).

(1) $R_1 - SH + Hg - R_2 \rightleftharpoons R_1 - S - Hg - R_2$

(2) $R_1 - S - Hg - R_2 + R_3 \rightleftharpoons R_1 - SH + R_3 - S - Hg - R_2$

The commonly used mercurials are derivatives of the phenylmercuric

ion, and include p-mercuribenzoate (PMB), p-chloromercuribenzoate

(PCMB), p-mercuribenzenesulphonate (PMBS) and p-

chloromercuribenzenesulphonate (PCMBS), most of which are available as

the sodium salt and are consequently freely soluble. The unsubstituted
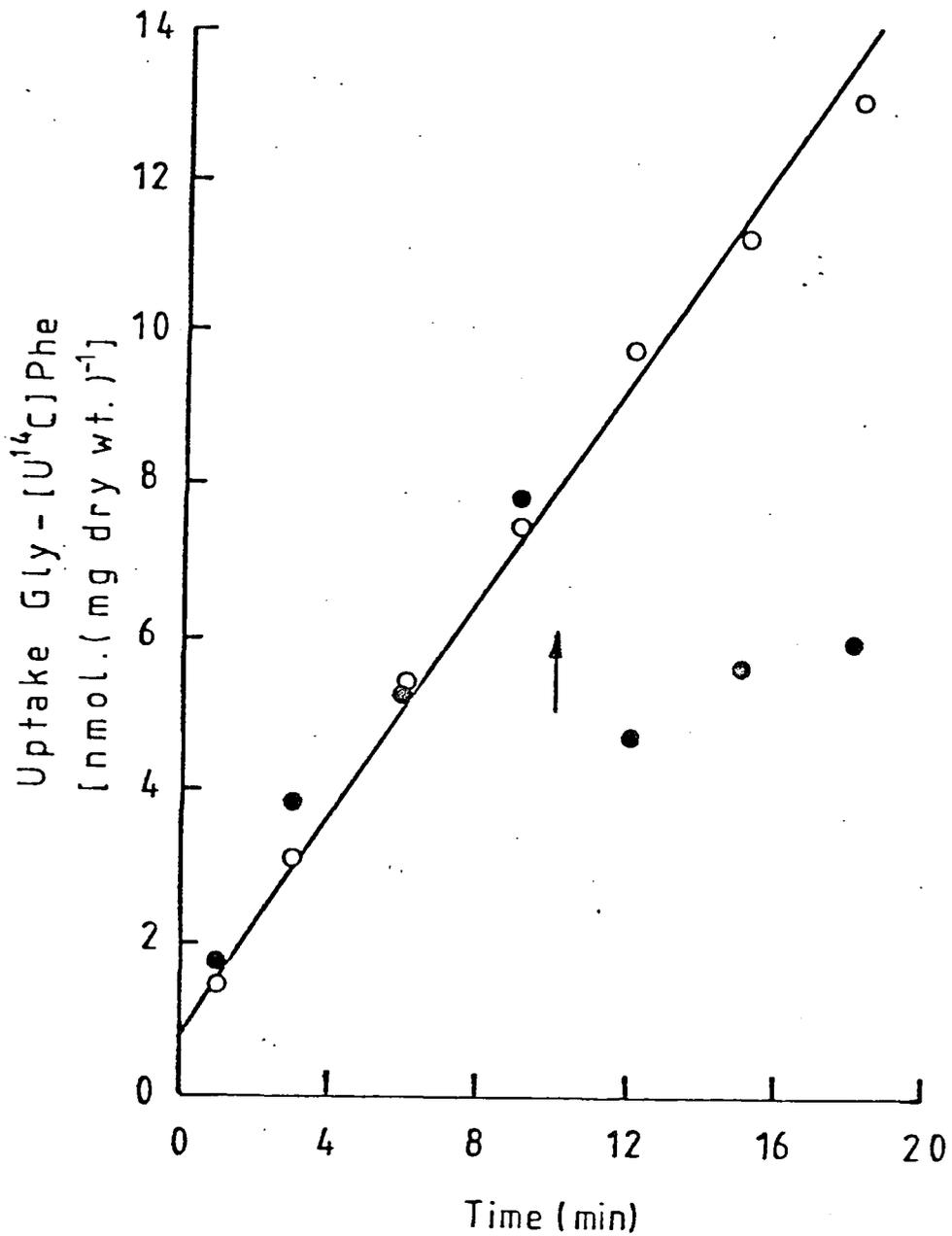
Figure 6.8 Effect of Addition of NEM During Uptake of Gly-[U^{14}C]Phe

Effect of addition of NEM (1 mM) (indicated by arrow) on uptake of Gly-[U^{14}C]Phe (0.2 mM, 0.01 uCi ml^{-1}) by <u>Candida albicans</u> B2630, relative to an untreated control. Both experiments were performed using aliquots from the same batch of cells.

(O) Control   (●) NEM treated

phenylmercuric ion is lipid soluble enabling it to traverse membranes, but the addition of charged carboxyl or sulphonate groups reduces the penetrability of these derivatives (Webb, 1966b). Thus, PCMBS is considered to be virtually impermeable, though limited access to the interior of certain cells, e.g. in the erythrocyte, may possibly be afforded by anion channels (Rothstein, 1970). Thus, the non-penetrative properties of PCMBS contrast markedly to those of NEM, and the two reagents may be used in conjunction to gain information on sulphydryl groups located on the inside and/or outside of a membrane (Giaquinta, 1976; Delrot et al., 1980; Despeghel & Delrot, 1983). Other related mercurial organics have been employed in both enzyme and transport studies, including p-hydroxymercuribenzoate (PHMB) and p-hydroxymercuribenzenesulphonate (PHMBS) (Plagemann & Wohlheuter, 1984; Ahmad et al., 1984).

PCMBS has been used extensively in enzyme studies to investigate the role of thiol groups (Galanta & Hatefi, 1978) and in the study of membrane-bound transport proteins (reviewed by Walker-Smith, 1984).

PCMBS has been shown specifically to inhibit peptide transport in barley embryos, peptide substrates being able to protect against PCMBS inactivation (Walker-Smith & Payne, 1983a,b). The reducing agent, dithiothreitol reversed the inactivation caused by PCMBS leading to the conclusion that this transport system contains a redox-sensitive protein(s).

6.3.2.2 Effect of Preincubation of Cells with p-Chloromercuribenzene-
          sulphonic Acid

Candida albicans B2630 was grown in Pep-Medium overnight, harvested by membrane filtration, and resuspended in PCG incubation buffer pH 4.5 (Section 4.2.2). Cells were preincubated with PCMBS (5 mM) for 5 min

before reharvesting by centrifugation (Section 6.2.1) and assaying for Ala-Ala (0.2 mM) transport by the standard manual fluorescamine procedure (Section 2.5.2).

Preincubation with PCMBS (5 mM) resulted in about 40% inhibition of Ala-Ala transport, relative to an untreated control performed with the same initial batch of cells (data not shown).

6.3.2.3 Monitoring the Effect of p-chloromercuribenzenesulphonic Acid Addition During Substrate Uptake

Candida albicans B2630 was grown in Pep-Medium overnight, harvested and resuspended in PCG incubation buffer pH 4.5 (Section 4.2.2). The effect of PCMBS (0.1 - 2 mM) on the uptake of peptide and amino acid substrates (all 0.2 mM) was examined using the standard manual fluorescamine assay (Section 2.5.2), PCMBS having been shown not to interfere with the fluorescence assay (Section 6.2.3). The effects of PCMBS on the uptake of Ala-Ala, Ala-Ala-Ala and Gln are shown (Figs. 6.9 - 6.11).

6.3.3 Effect of Phenylarsine Oxide on Transport Activity

6.3 3.1 Introduction - Chemical Properties

Studies were undertaken with phenylarsine oxide (PAO), which complexes selectively with vicinal dithiols, to determine its effects upon peptide transport in Candida albicans.

Arsenical compounds have been known to be toxic to biological systems for many years and their biochemistry has recently been reviewed (Knowles & Benson, 1983). Trivalent arsenicals (all derivatives of arsine, AsH_3) react rapidly and specifically with thiol groups via a substitution mechanism which is not fully understood
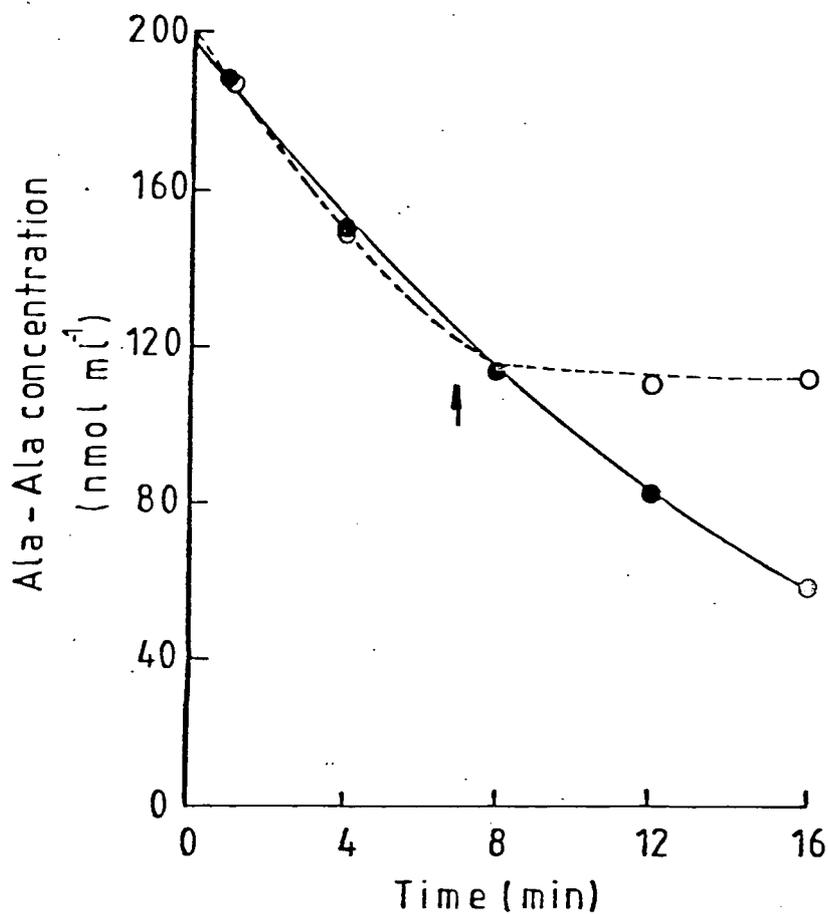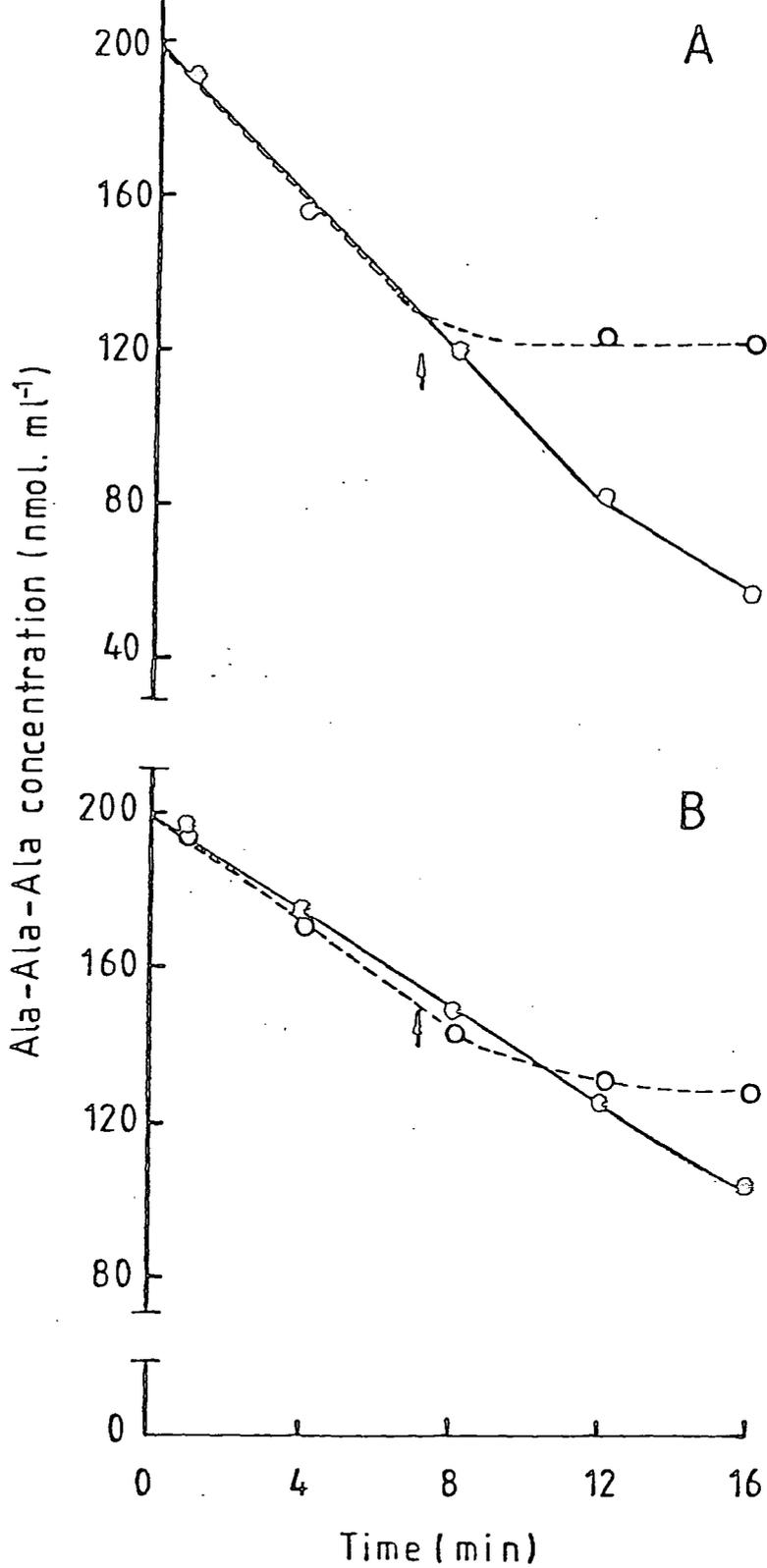
Figure 6.9 Effect of PCMBS Addition During Ala-Ala Uptake

A   The effect of PCMBS (1 mM) on the uptake of Ala-Ala (0.2 mM) by
      Candida albicans B2630 (1.12 (mg.dry wt.)ml^{-1})

B   The effect of PCMBS (0.1 mM) on the uptake of Ala-Ala (0.2 mM) by
      Candida albicans B2630 (1.21 (mg.dry wt.)ml^{-1}).

Arrow indicates point of addition of PCMBS

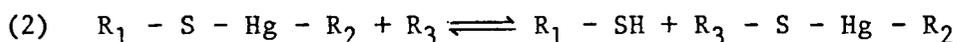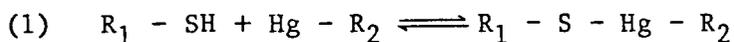(●) Control   (○) PCMBS added after 7 min.

Figure 6.10   Effect of PCMBS Addition During Ala-Ala-Ala Uptake

A   The effect of PCMBS (1 mM) on the uptake of Ala-Ala-Ala (0.2 mM)

by <u>Candida</u> <u>albicans</u> B2630 (0.7 (mg.dry wt)ml^{-1}).

B   The effect of PCMBS (0.1 mM) on the uptake of Ala-Ala-Ala (0.2

mM) by <u>Candida</u> <u>albicans</u> B2630 (0.62 (mg.dry wt.)ml^{-1}).

Arrow indicates point of addition of PCMBS

(●) Control   (○) PCMBS added after 7 min.

Figure 6.11 Effect of PCMBS Addition During Gln Uptake

A The effect of PCMBS (1 mM) on the uptake of Gln (0.2 mM) by

Candida albicans B2630 (1.38 (mg.dry wt.)ml^{-1})

B The effect of PCMBS (0.1 mM) on the uptake of Gln (0.2 mM) by

Candida albicans B2630 (1.68 (mg.dry wt.)ml^{-1})

Arrow indicates point of addition of PCMBS

(o) Control   (o) PCMBS added after 7 min.

(Webb, 1966c). Trivalent arsenicals fall into two categories :-

1) monosubstituted alkyldihaloarsines (RAsX$_2$) and alkyl arsenoxides (RAsO).

2) dialkylhaloarsines (R$_1$R$_2$AsX).

Monosubstituted compounds react reversibly with vicinal, or adjacent dithiols on a protein to form a stable thioarsinite ring structure, reaction with monothiols produces an unstable product (Fig. 6.12). Dialkylhaloarsines give stable derivatives with single cysteine residues only (Webb, 1966c). The decomposition of cyclic thioarsinite, to regenerate the original sulphydryl groups is readily brought about by the addition of excess vicinal dithiol e.g., dimercaptopropanol, dithiothreitol or dithioalkanes which compete for reaction with the inhibitor (Walker-Smith, 1984). Thus, many enzymes have been classified as dithiol-dependent on the basis of their inhibition by alkyl arsenoxides (Webb, 1966c; Stevenson et al., 1978). Recently, these compounds have been employed in the characterization of the role of vicinal dithiols in transport processes. However, it is possible that enzymic inactivation may be brought about by the arsenical compound combining with two distally located SH groups in a particularly flexible protein. The ability of low molecular weight dithiols to regenerate the original sulphydryl groups would indicate that inhibition is not achieved through this sort of coformational change in the protein.

The trivalent arsenical, phenylarsine oxide (PAO) was employed in these studies to examine the possible role of vicinal dithiols in peptide transport. PAO is only slightly soluble in water, but because of its hydrophobic, uncharged nature it is considered to be lipid soluble and thus to have some degree of permeability (Webb, 1966c).
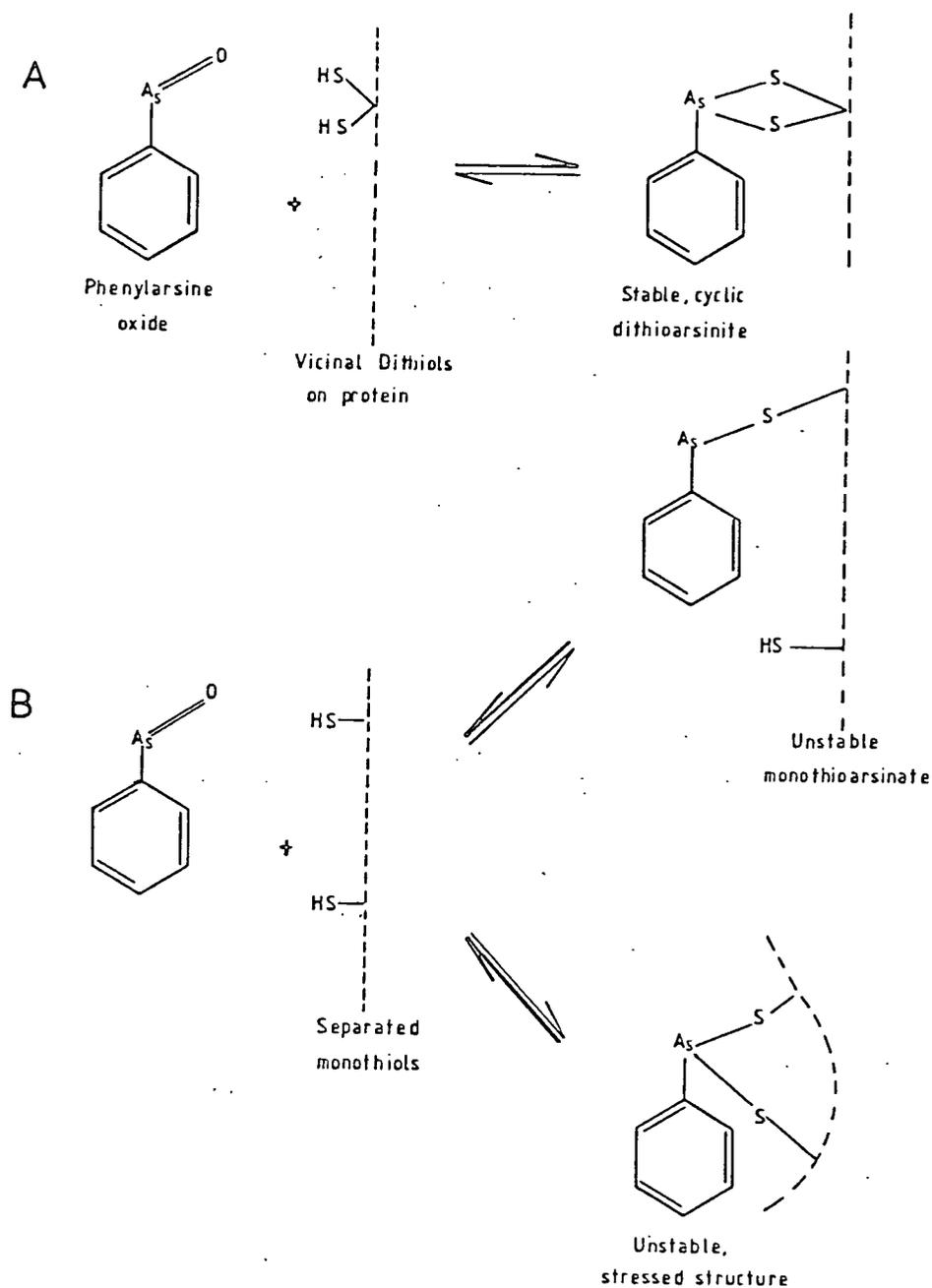
Figure 6.12 Reaction of Phenylarsine Oxide with Thiols and Vicinal
            Dithiols

A   PAO can react with vicinal dithiols to form a stable cyclic
     dithioarsinite.

B   Reaction with a single thiol produces an unstable
     monothioarsinite, reaction with two separated monothiols would
     produce a distorted product which is only likely to be a
     transient species.

Evidence has been reported for the important role of vicinal dithiols in proline transport by E.coli (Robillard & Konings, 1981) and in D-glucose transport by the renal outer cortical brush border (Turner & George, 1983). Peptide uptake by barley embryos is inhibited by PAO, and this inhibition is reversed by the addition of dithiothreitol. Thus, it was concluded that this peptide transport system contains a redox sensitive, dithiol-dependent protein (Walker-Smith & Payne, 1983a,b).

6.3.3.2 Monitoring the Effect of Phenylarsineoxide Oxide Addition During Substrate Uptake

Phenylarsine oxide (PAO) is only slightly soluble in aqueous solution; approximately 0.5 mM representing a saturated solution in phosphate-citrate buffer pH 3.8 (50 mM) phosphate) at 20°C (Walker-Smith, 1984). However, it is possible to obtain a higher concentration by first dissolving PAO in ethanol (upto 200 mM) and adding the required volume to PCG incubation buffer, pH 4.5 preincubated at 28°C. In this way it was possible to obtain concentrations upto about 2 mM, the higher solubility being attributed to a combination of increased pH and temperature, and possibly a reduced buffer concentration. By first dissolving PAO in ethanol it was also possible to use this protein modification reagent for inhibitor-pulse studies (Section 6.2.2). The concentration of ethanol in incubations never exceeded 1%, this concentration having previously being shown not to affect peptide transport (Section 4.6.2.1).

Candida albicans B2630 was grown in Pep-Medium overnight, harvested and resuspended in PCG incubation buffer pH 4.5 (Section 4.2.2). The effect of PAO (0.1 - 2 mM) on the uptake of peptide and

amino acid substrates (all 0.2 mM) was examined by using the standard manual fluorescamine assay (Section 2.5.2), PAO having been shown not to interfere with the fluorescence assay (Section 6.2.3). The effects of PAO on the uptake of Ala-Ala, Ala-Ala-Ala and Gln are shown (Figs. 6.13 - 6.15).

6.3.4 Discussion

Several thiol-specific reagents have been used in these studies in an attempt to inhibit the activity of transport proteins located in the plasma membrane of Candida albicans B2630. Cells were generally grown in Pep-Medium overnight, conditions in which peptide transport activity is greatest (Section 4.4), as high transport rates (of the order 10 nmol.min^{-1} (mg.dry wt)$^{-1}$) more easily allow quantification of low levels of inhibition. The transport activities of the dipeptide and oligopeptide transport systems together with the glutamine transport system were examined in these studies.

Cells treated with both NEM and PCMBS showed a marked concentration dependence, depending on whether the cells were preincubated with inhibitor or that the inhibitor was added during substrate uptake. Preincubation with 5 mM NEM for 2 min resulted in complete inhibition of the dipeptide transport system (and the transport systems for oligopeptides, glutamine and leucine), whereas preincubation with 1 mM NEM for 10 min resulted in only 40% inhibition. Preincubation with 5 mM PCMBS for 5 min resulted in 40% inhibition of the dipeptide transport system. This difference is presumably due to differences in permeability of the two inhibitors, PCMBS being virtually impermeable (Section 6.3.2.1), and thus is considered to be inaccessible to intracellular target sites. Addition of these
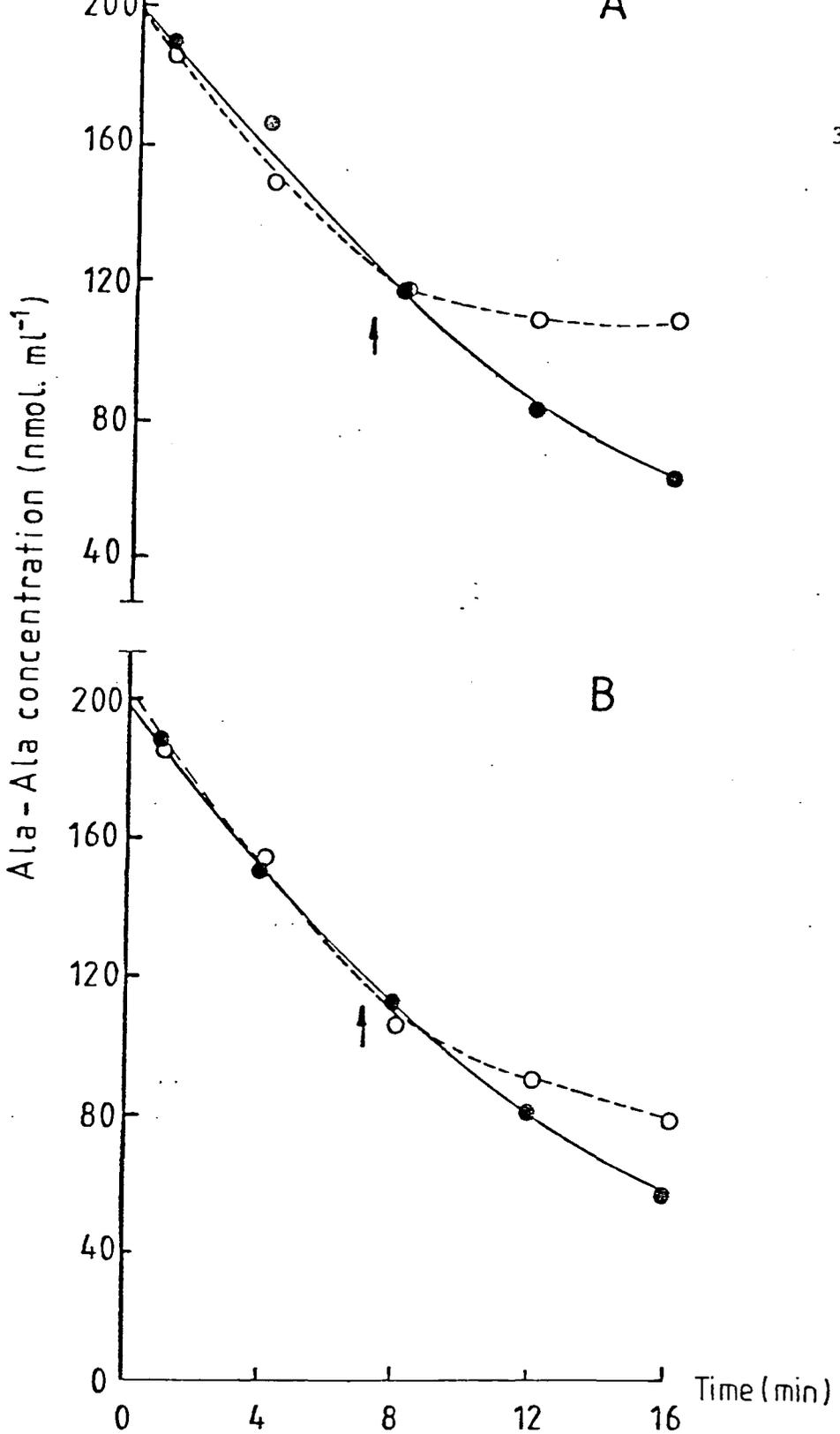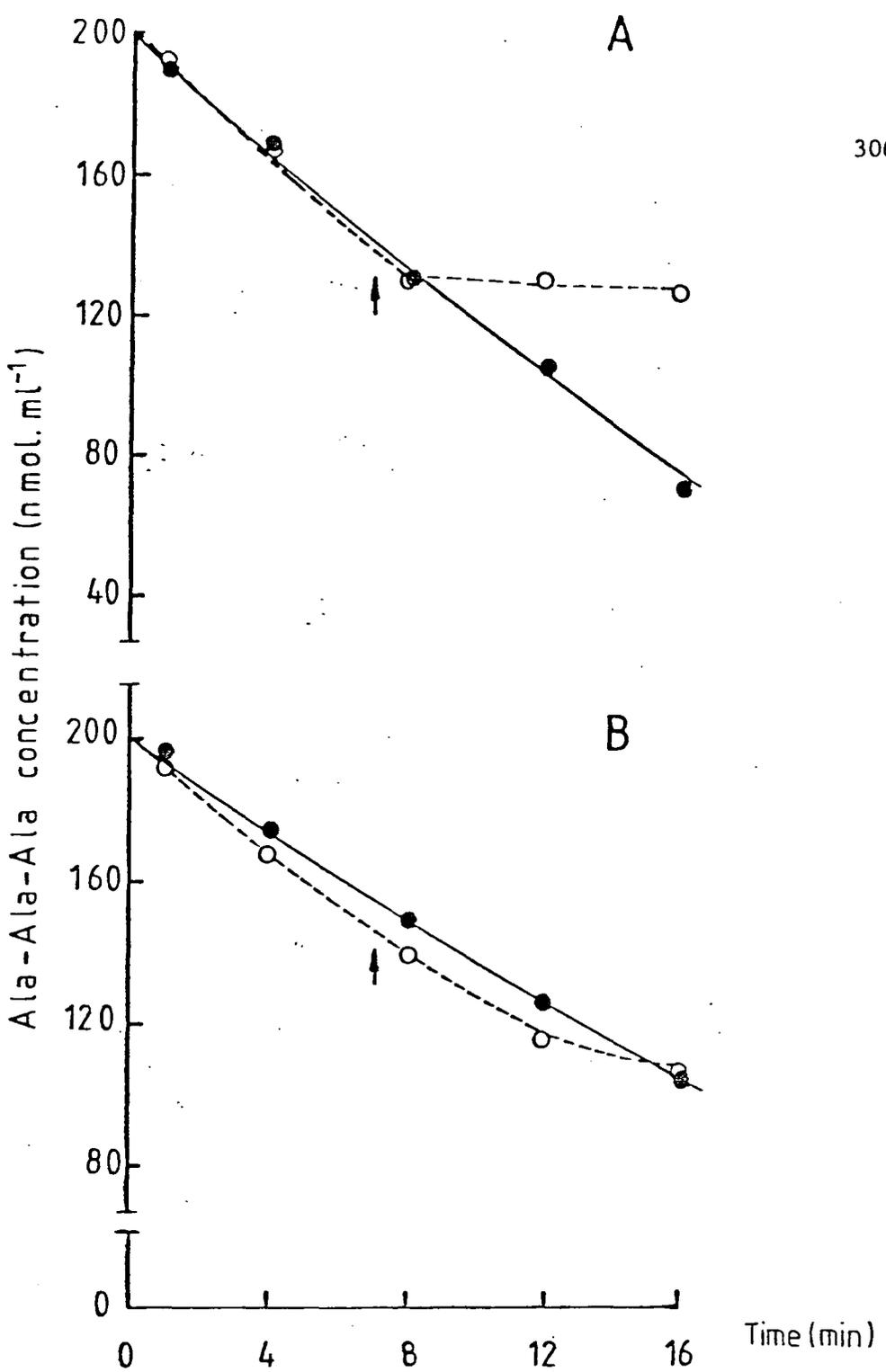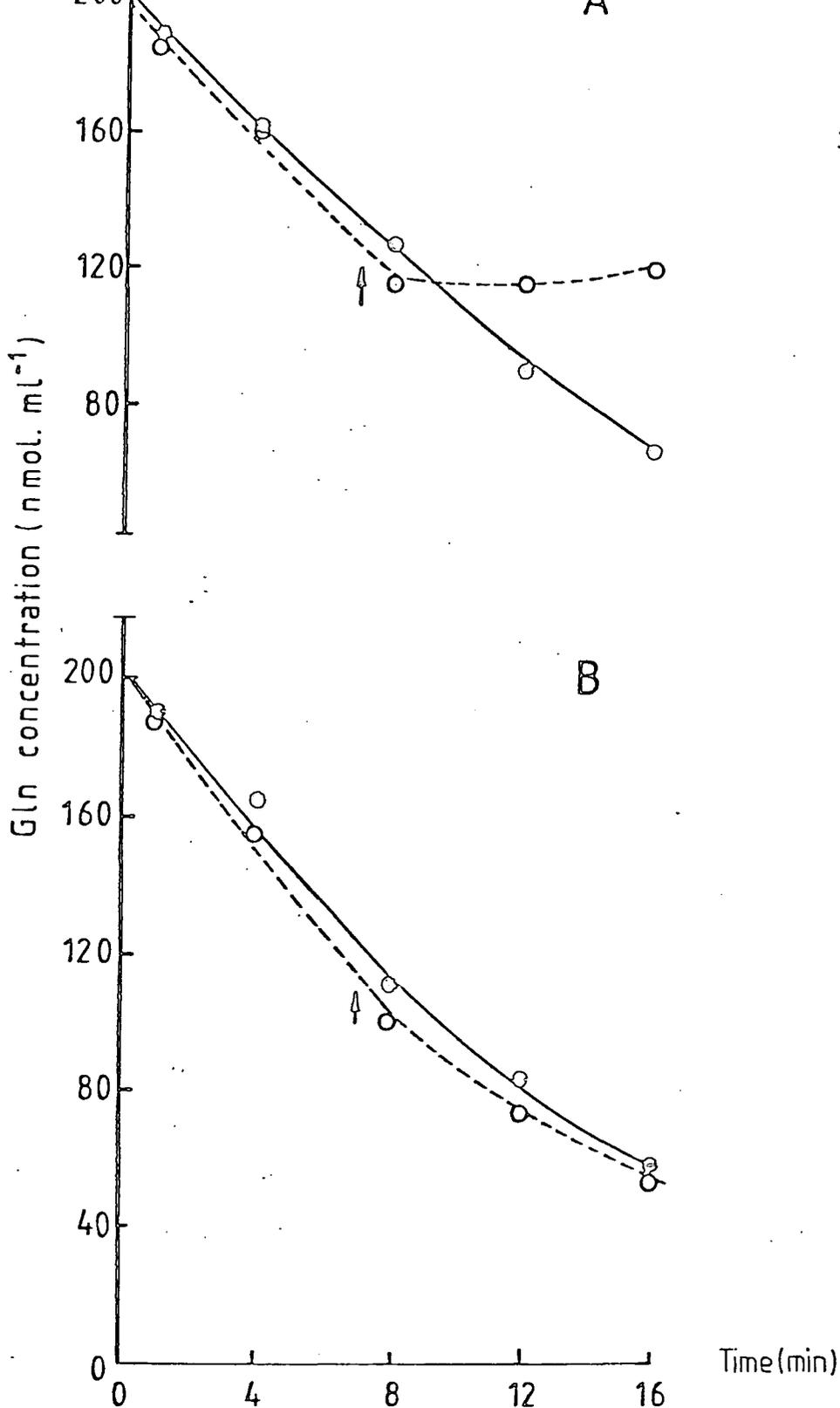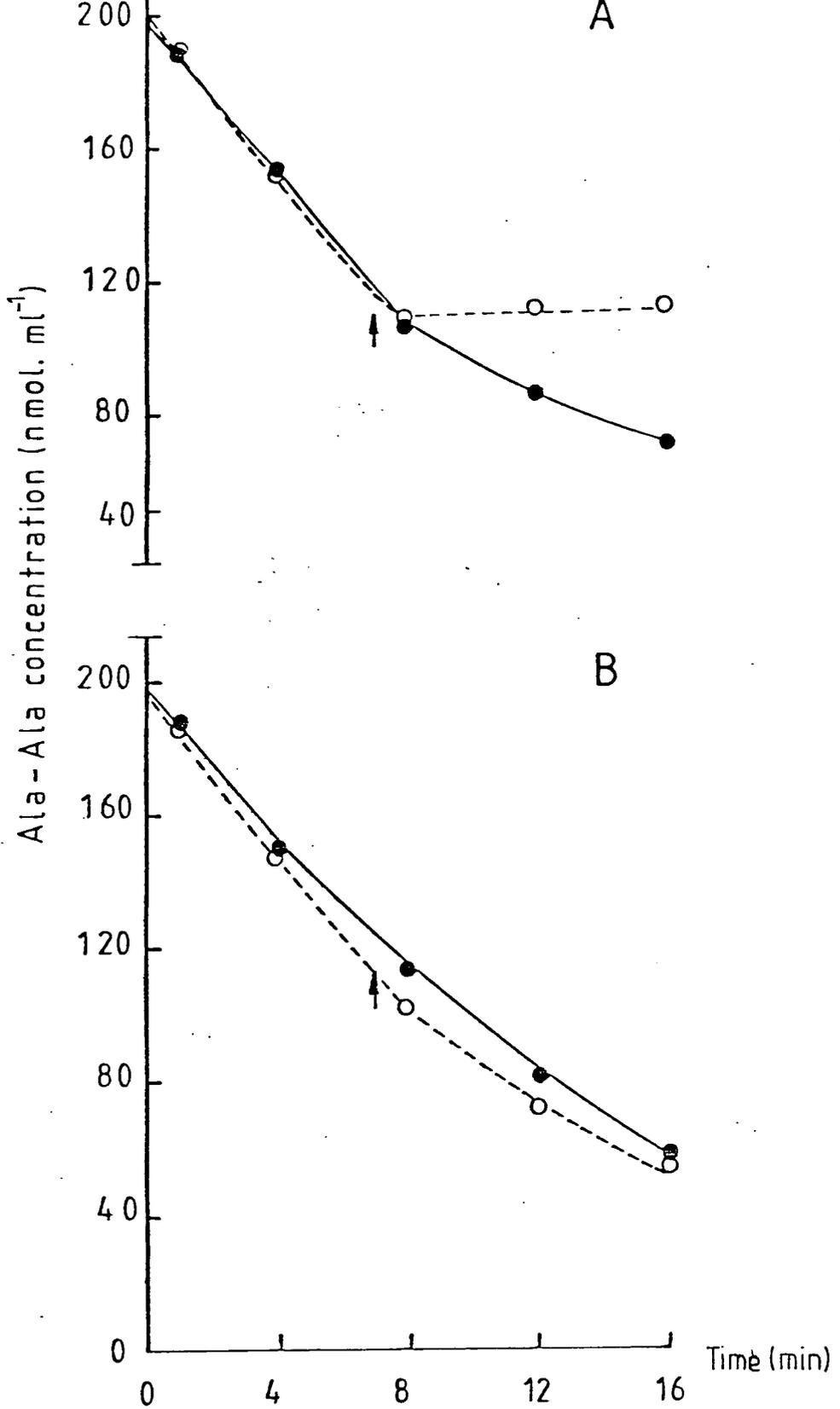
Figure 6.13   Effect of PAO Addition During Ala-Ala Uptake

A   The effect of PAO (1 mM) on the uptake of Ala-Ala (0.2mM) by

Candida albicans B2630 (1.42 (mg dry wt)ml^{-1})

B   The effect of PAO (0.1 mM) on the uptake of Ala-Ala (0.2 mM) by

Candida albicans B2630 (1.21 (mg dry wt)ml^{-1}).

Arrow indicates point of addition of PAO.

(●) Control   (○) PAO treated
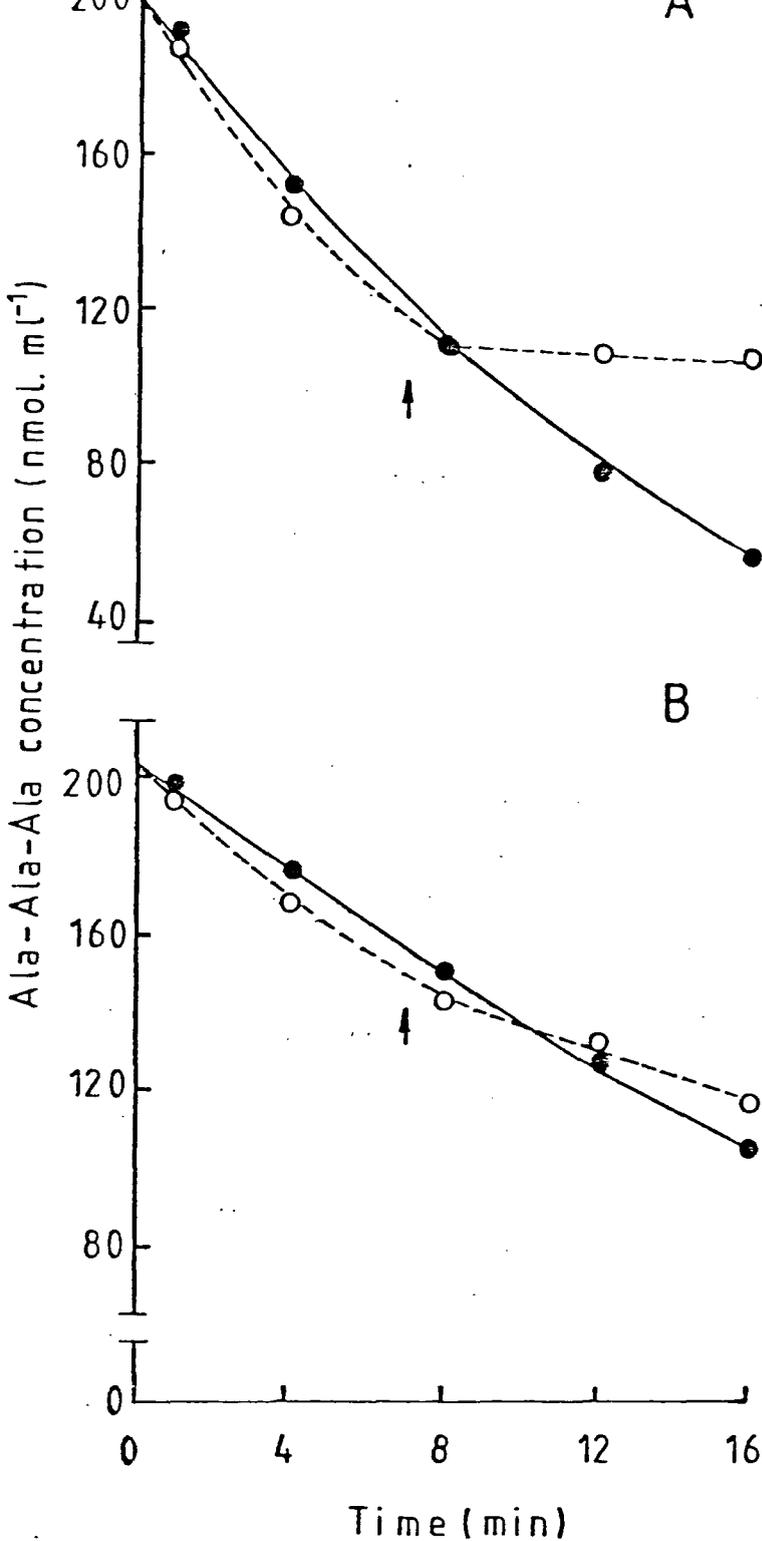
Figure 6.14 Effect of PAO Addition During Ala-Ala-Ala Uptake

A   The effect of PAO (1 mM) on the uptake of Ala-Ala-Ala (0.2 mM) by
      Candida albicans B2630 (0.9 (mg.dry wt.)ml^{-1})

B   The effect of PAO (0 1 mM) on the uptake of Ala-Ala Ala (0.2 mM)
      by Candida albicans B2630 (0.62 (mg.dry wt.)ml^{-1}).

Arrow indicates point of addition of PAO.

(●) Control   (○) PAO added after 7 min.
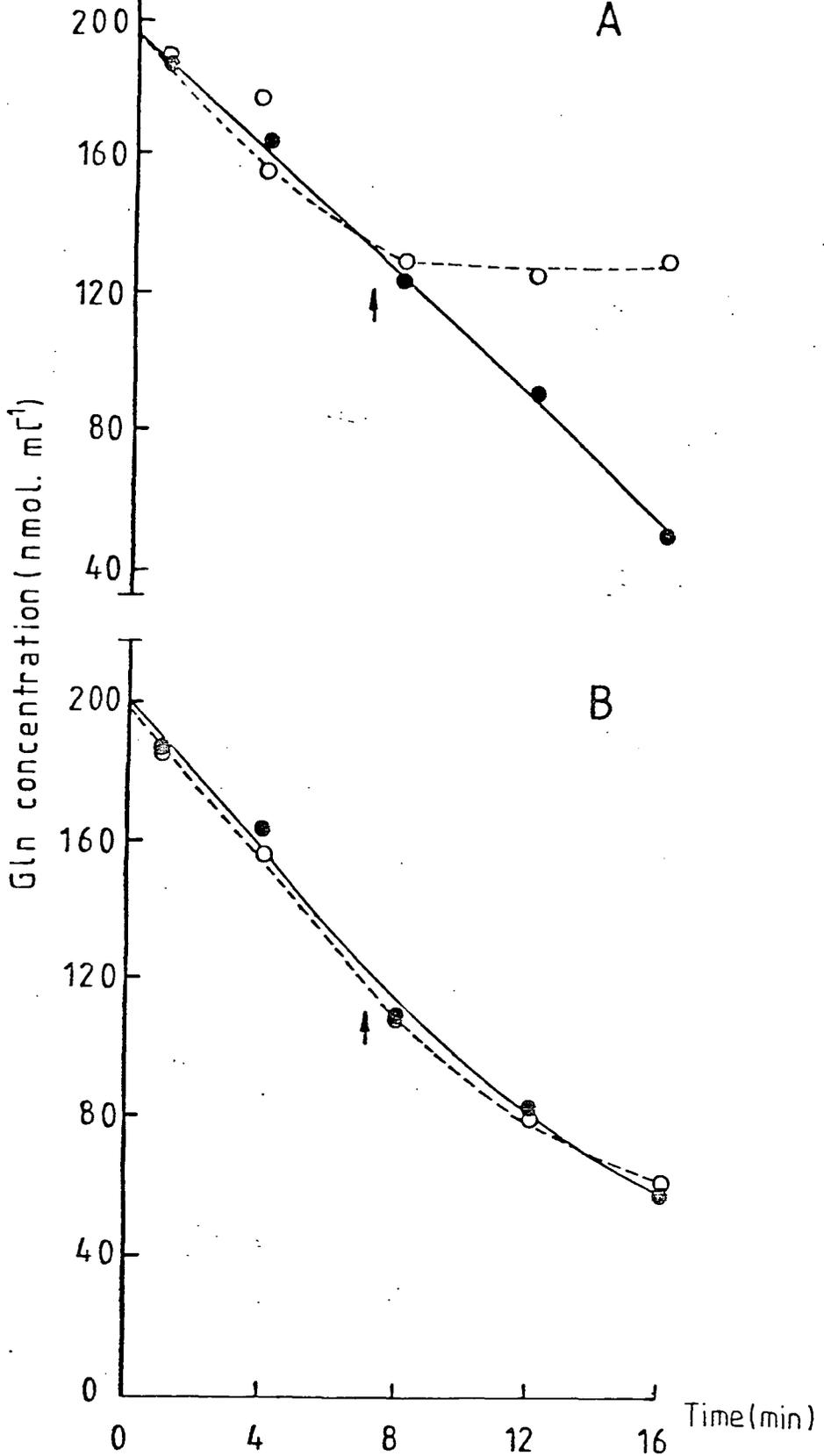
Figure 6.15 Effect of PAO Addition During Gln Uptake

A   The effect of PAO (1 mM) on the uptake of Gln (0.2 mM) by Candida
        albicans B2630 (1·4 (mg.dry wt)ml^{-1}).

B   The effect of PAO (0.1 mM) on the uptake of Gln (0.2 mM) by
        Candida albicans B2630 (1.68 (mg.dry wt.)ml^{-1}).

Arrow indicates point of addition of PAO

(●) Control   (○) PAO added after 7 min

inhibitors during substrate uptake gave interesting results. Addition

of PCMBS and PAO (at 1 mM) resulted in immediate inhibition of Ala-Ala,

Ala-Ala-Ala and Gln uptake, whereas addition of these inhibitors at 0.1

mM, resulted in only minimal inhibition. NEM inhibition of

oligopeptide (Ala-Ala-Ala) and glutamine transport showed a similar

concentration dependence, whereas addition of 0.1 mM during Ala-Ala

uptake resulted in immediate and complete inhibition of the activity of

the dipeptide transport activity. Thus, some degree of selectivity was

detected. Addition of these inhibitors (1 mM) also resulted in the

release of fluorescamine-positive material. Addition of NEM (1 mM) was

also shown to inhibit the uptake of Gly-$[U^{14}C]$Phe, when added during

uptake of this substrate. This result indicates that any observed

inhibition was not a consequence of the assay method employed.

Thus, addition of these thiol-specific inhibitors produced very

similar results, though they have different sites of activity; PAO is

specific for vicinal dithiols located both intra- and extracellularly

(as this reagent is highly permeable); PCMBS would be expected only to

be accessible for externally-located thiol groups and NEM to be

accessible to both intra- and extracellularly located thiols. The lack

of selectivity, and similar kinetics of inhibition, suggests that

inhibition of transport may be a generalised effect, possibly on a

common component essential for translocation (e.g. a component involved

in the energisation of transport) or a disruption of the integrity of

the plasma membrane, resulting in inhibition of transport.

The rapid inhibition of transport activity observed in the

presence of substrate could possibily be due to increased access of the

inhibitors for the permeases thiol groups when the carrier is in

operation, or that addition of inhibitors during uptake may facilitate access to a secondary component essential for transport.

Enhanced inhibition of proline transport in E.coli by NEM, was also observed in the presence of transport substrate (proline) (Janick et al., 1977). This was attributed to the exposure of a critical sulphydryl group during a protein conformational change as a result of substrate translocation.

Thiol groups have long been implicated in mediated transport in many systems (reviewed by Walker-Smith, 1984). The neutral, basic and general amino acid transport systems of Neurospora crassa have been shown to be inhibited by thiol reagents (Nelson et al., 1975), though the plasma membrane ATPase of this organism is also sensitive to cysteine modification (Brooker & Slayman, 1983; Serrano, 1983). Thus interference with the energisation of amino acid transport may be partially responsible for some of the observed inhibition. The leucine transport system in Sacc. cerevisiae has been shown to be dependent on thiol groups (Ramos et al., 1980), these thiols were proposed to be located on the inner surface of the plasma membrane, as they are sensitive to NEM but not to PCMB (Ramos et al., 1983). Peptide transport in Candida albicans has been shown to be completely inhibited by preincubation with 10 mM NEM (Logan et al., 1979). In E.coli the transport systems for ornithine, phenylalanine and proline have been shown to have essential sulphydryl groups (Janick et al., 1977), in the proline transport system these groups exist as paired, redox-sensitive dithiols (Poolman et al., 1983). However, the transport systems for glutamate, glycine, tyrosine, leucine and lysine, were shown not to be inhibited by modification of cysteine residues (Kaback & Patel, 1978).

Osmotic shock-sensitive transport systems, (i.e. those systems involving a perisplasmic binding protein, a membrane protein component and dependent on phosphate-bond energy) are generally less susceptible to sulphydryl modification relative to osmotic shock-resistant systems (Berger & Heppel 1974; Janick et al., 1977), though the reasons for this distinction are unclear.

Sulphydryl groups have been shown to possess an essential role in certain solute transport systems, through a redox-sensitive dithiol-disulphide interchange mechanism (Robilliard & Konings, 1982; Walker-Smith, 1984). Several transport systems have been shown to be dependent on this interchange for activity including those systems for phosphoenolpyruvate-dependent hexose uptake (Robilliard & Konings, 1981), lactose uptake (Konings & Robilliard, 1982) and proline uptake (Poolman et al., 1983), all in E.coli; and the scutellar peptide transport system in germinating barley embryos (Walker Smith & Payne, 1983a, b).

6.4   Further Chemical Modification Studies

6.4.1   Effect of N-Ethyl-5-Phenylisoxazolium-3'-Sulphonate (Woodwards Reagent K) on Transport Activity

6.4.1.1   Introduction - Chemical Properties

Selective derivatization of carboxyl groups may be achieved by treatment with isoxazolium salts such as Woodwards Reagent K (WRK) Isoxazolium salts were originally developed as activators for carboxylic acids in peptide synthesis (Woodward & Olofson, 1966; Woodward et al, 1966), though their application has been extended to protein modification studies. WRK reacts specifically with carboxyl

groups to form an enol ester derivative over a wide pH range

(Bodlaender et al., 1969). Due to its aromatic nature, WRK may be

expected to be slightly soluble in the lipid bilayer, despite the

presence of a negatively charged sulphonate group on the molecule

(Walker-Smith, 1984).

Treatment with WRK has indicated that carboxyl groups are

essential for the activity of several enzymes, including bovine

carboxy-peptidase (Petra, 1971; Petra & Neurath, 1971), chloroplastic

ATPase (Arana & Vallejos, 1980), ferredoxin-NADP$^+$ oxidoreductase

(Carrillo et al., 1981) and murine β-glucuronidase (Homandberg, 1982)

WRK was shown not to affect the uptake of peptides, amino acids, or

glucose by barley embryos, though this may reflect the lack of

accessability of the reagent to these uptake systems (Walker-Smith &

Payne, 1983a; Walker-Smith, 1984).

6.4.1.2 Effect of Preincubation of Cells with Woodwards Reagent K

Cells were preincubated with Woodwards Reagent K (WRK) (5 mM) for

up to 30 min before reharvesting by centrifugation (Section 6.2.1), and

assaying for peptide and amino acid transport (Fig. 6.16) by using the

standard, manual-fluorescamine assay (Section 2.5.2). Interestingly,

with this reagent, some selectivity of inhibition on the three

transport systems examined was observed, with the peptide systems

showing greater susceptability.

6.4.2 Effect of Phenylglyoxal on Transport Activity

6.4.2.1 Introduction - Chemical Properties

Arginine residues are often important for enzymes function as the

guanidyl group carries a positive charge at physiological pH values.

Specific rates have been assigned to certain arginine residues in
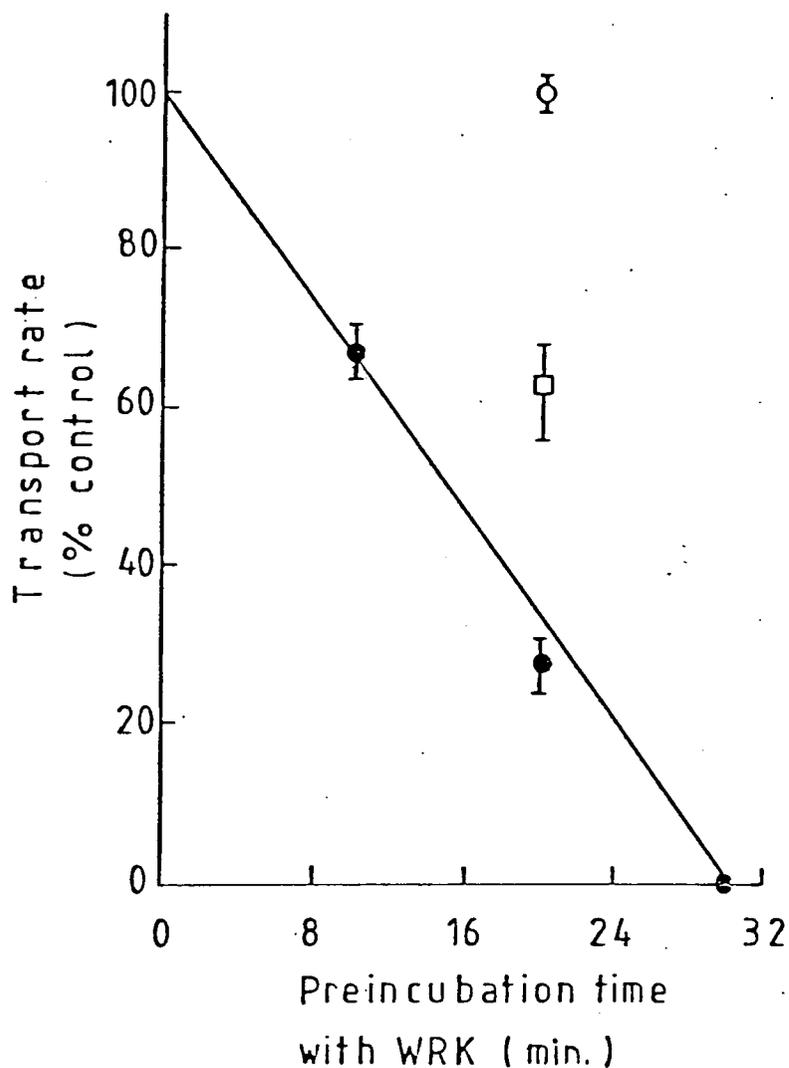
Figure 6.16   Effect of Woodwards Reagent K on Peptide and Amino Acid

Transport

Uptake of Ala-Ala (●) and Ala-Ala-Ala (□), (both 0.2 mM) and Gln

(○) (0.5 mM) by Candida albicans B2630 after preincubation with WRK

(5 mM) for the indicated times.   Rates are expressed as a percentage of

untreated controls.   Each value is the average of at least 2 separate

determinations.

proteins e.g. at the binding site for substrates or cofactors bearing a negative charge, and localization at the surface of an enzyme to enhance its solubility.

Several selective reagents are available for the covalent modification of the arginyl guanido group including 1,2,cyclohexanedione (Toi et al., 1967), butanedione (Riordan, 1973), camphorquinone-10-sulphonic acid (Pande et al., 1980) and phenylglyoxal (Takahaski, 1968). Derivatives of phenylglyoxal have been developed, e.g. p-hydroxyphenylglyoxal, which has a greater solubility in water (Yamasaki et al., 1980); and p-nitrophenylglyoxal, which allows a colorimetric determination of arginine residues to be performed (Yamasaki et al., 1981). Two molecules of phenylglyoxal (PG) react with one guanidyl group at pH 7 - 8 to give a complex ring structure, this reaction may be reversed at elevated temperatures. The derivatization is highly specific, though some side reactions with amino groups may occur (Takahaski, 1968)

Treatment with PG has indicated that arginyl groups are essential for the activity of several enzymes including alkaline phosphatase (Daemen & Riordan, 1974) and choline acetyltransferase (Mautner et al., 1971). Similarly, studies with PG have shown that arginine residues are essential for the sodium-dependent transport of phosphate, glucose and alanine across the renal brush-border membrane (Strevey et al., 1984), for sulphate exchange across erthrocyte membranes (mediated by the integral band 3 membrane protein) (Zaki, 1984), and for chloride exchange in erythrocytes (Wieth et al., 1982). The kinetics of PG inhibition of peptide transport in barley embryos implicated the role of arginine residues in peptide transport, though substrate protection

experiments offered no evidence for the location of the residue(s) at the binding or active site of the carrier protein (Walker-Smith & Payne, 1983a).

6.4.2.2 Effect of Preincubation of Cells with Phenylglyoxal

Candida albicans B2630 was grown in Pep-Medium overnight, harvested by membrane filtration (Section 4.2.2) and resuspended in PCG incubation buffer pH 7 (Section 6.2.1). Organisms (1.6 - 1.9 (mg dry wt) ml^{-1}) were preincubated with phenylglyoxal (PG) (5 mM) for 20 min before reharvesting by centrifugation, and assaying for peptide transport by the standard, manual fluorescamine procedure (Section 2.5.2).

Preincubation of cells with PG had no effect on the transport of Ala-Ala and Ala-Ala-Ala (both 0.2 mM) relative to untreated controls also preincubated at pH 7 (results not shown). The preincubation of cells at pH 7 for 40 min had no adverse effects on transport of Ala-Ala, relative to cells preincubated for the same period at pH 4.5 before reharvesting by centrifugation (Section 6.2.1).

6.4.3 Effect of Diethylpyrocarbonate on Transport Activity

6.4.3.1 Introduction - Chemical Properties

Selective derivatization of histidine residues may be achieved by photo-oxidation after sensitizing the residue with the dye Rose Bengal (Westhead, 1965) or by treatment with diethylpyrocarbonate (ethoxyformic anhydride).

Because of its greater selectivity, diethylpyrocarbonate (DEPC) was employed in this study to evaluate the role of histidine residues in peptide and amino acid uptake by Candida albicans. The use of DEPC in protein chemistry has been reviewed (Miles, 1977). DEPC will modify

the unprotonated form of a range of nucleophilic amino acid side-chains; thus, at alkaline pH, sulphydryl, tyrosyl, guanidyl and amino moieties are easily derivatized (Burch & Ticku, 1981). However, at pH 6 - 7, the reagent is specific for the imidazole ring of histidine, forming an N-ethoxyformyl-histidine complex in a reaction which can be followed spectrophotometrically (Roosemont, 1978). The N-ethoxyformyl-histidine complex is of limited stability (Melchior & Fahrney, 1970) and its breakdown to regenerate the amino acid is rapidly achieved by the addition of hydroxylamine (Padan et al., 1979). Excess DEPC is rapidly hydrolysed to carbon dioxide with a half-life of 24 min at 25°C (Walker-Smith, 1984).

Treatment with DEPC has indicated that histidine residues are essential for the activity of many enzymes, including succinyl-CoA synthetase from E.coli (Collier & Nishimura, 1979), fungal amylase (Kita et al., 1982), and dopamine β-hydroxylase (Sams & Matthews, 1984). Similarly, studies with DEPC have shown that histidine residues are essential for the biological activity of Botulinum neurotoxins A and B (Dasgupta & Rasmussen, 1984) and for the cryoprecipitation of monoclonal human cryoglobulin M (Kosarev et al., 1984).

DEPC has seen limited application to transport studies; it was shown to inhibit the uptake of lactose and proline by E.coli vesicles (Padan et al., 1979), and to inhibit the lactose-induced proton influx (Patel et al., 1982). Inactivation of peptide and amino acid uptake in barley embryos by DEPC was neither rapid nor complete, and substrate screening experiments gave conflicting results (Walker-Smith, 1984). Both D-, and L-peptide stereoisomers were shown to confer protection on both peptide and amino acid uptake, but D- and L-amino acids did not

substrate screen peptide uptake from DEPC inhibition (this effect was not due to the progressive inactivation of DEPC by peptides). The lack of detailed knowledge of the complicated chemistry of this reagent precluded a satisfactory explanation of these anomalous results.

6.4.3.2 Effect of Preincubation of Cells with Diethylpyrocarbonate

Candida albicans B2630 was grown in Pep-Medium overnight, harvested by membrane filtration (Section 4.2.2), and resuspended in phosphate-citrate incubation buffer pH 6.5 (10 mM phosphate) to 1.7 (mg dry wt) ml.$^{-1}$ (2 x 5 ml) and preincubated for 5 min at 28°C. DEPC was diluted with ethanol (50% v/v) and 14.5 ul of this were added to the cell suspension (5 ml) with vigorous stirring to give a 10 mM solution. DEPC was always prepared just prior to use, because its susceptibility to aqueous hydrolysis gives it a relatively short half-life (Section 6.4.3.1). Organisms were reharvested by centrifugation and resuspended in PCG incubation buffer pH 4.5 (Section 6.2.1) for assay of peptide and amino acid transport according to the standard, manual fluorescamine procedure (Section 2.5.2).

Preincubation of cells with DEPC (10 mM) resulted in 100% inhibition of transport of Ala-Ala, Ala-Ala-Ala, and Gln (all 0.2 mM) transport (Table 6.2).

6.4.4 Discussion

Several other chemical modification agents were used in these studies in an attempt to find a selective inhibitor of peptide transport : specifically, carboxyl groups (WRK), arginine residues (PG) and histidine residues (DEPC).

Preincubation with WRK was shown to inhibit selectively peptide transport, a 20 min. preincubation with WRK (5 mM) resulted in a 73%

$$\underline{\text{Transport Rate}} \text{ nmol min.}^{-1}(\text{mg dry wt.})^{-1}$$

Substrate	Untreated Control	Cells Preincubated with DEPC
Ala-Ala	3.6	ND
Ala-Ala-Ala	7.8	ND
Gln	10	ND

TABLE 6.2 Effect of Preincubation (5 min) with DEPC (10 mM)

on Transport Activity

Uptake of peptide and amino acid (all 0.2 mM) by Candida albicans B2630
after preincubation (5 min) with DEPC (10 mM).

ND - not detectable.

inhibition of Ala-Ala-transport (via the dipeptide transport system), a 37% inhibition of Ala-Ala-Ala transport (via the oligopeptide transport system), but had no effect on Gln transport. Such selectivity, implies that these results may be due to a direct inhibition of transport system components, and not due to secondary effects on, for example, plasma membrane-bound ATPases. This observed selectivity offers possibilities for further studies, and may possibly enable the selective labelling of the dipeptide transport system using radioactively-labelled WRK, with labelling of the oligopeptide transport system being minimised by substrate screening with tripeptides. Comparison of membrane protein profiles from WRK-labelled cells with those from the dipeptide transport deficient mutant, PA8035 (Section 5) could aid resolution of the components of the dipeptide transport system in a similar manner to that reported for the gap system in Sacc. cerevisiae (Woodward & Kornberg, 1980, 1981)

The lack of inhibition of the transport activities of the dipeptide, oligopeptide and Gln transport systems, by preincubation with PG, suggests that arginine residues are not essential for the activities of these systems. However, the possibility that essential arginine residues associated with these systems, are not accessible to PG by the preincubation treatment employed in these studies, cannot be excluded.

Conversely, preincubation with DEPC resulted in complete inhibition of the activity of all three systems, suggesting that histidine residues are essential for the activity of all three systems. However, the possibility of inhibition through secondary effects, (e.g. inhibition of ATPase, or disruption of membrane integrity) cannot be excluded.

EFFECTS OF OSMOTIC SHOCK ON TRANSPORT IN YEAST

6.5 Introduction

The effects of osmotic shock treatment on peptide and amino acid transport, together with an examination of those proteins released by this treatment, are presented in the following sections. The mannitol-based osmotic shock medium described by Schwencke et al., (1971), was used throughout these studies, sucrose being ineffective as a shock inducer in yeast (Schwencke et al., 1971).

Osmotic shock treatment has been shown to result in the selective release of hydrolytic enzymes and periplasmic binding proteins, located in a region between the cell wall and the cytoplasmic membrane (Heppel, 1967; Berger et al., 1974). Substrate specific binding proteins associated with bacterial amino acid transport systems have been reviewed (Oxender & Quay, 1975; Anraku, 1980). The majority of osmotic shock studies reported in the literature have been concerned with bacterial systems, studies with yeast being hampered by technical difficulties (Haskovec & Kotyk, 1969; Schwencke et al, 1971; Horak & Kotyk, 1973; Opekarova et al., 1975). The osmotic shock procedure of Schwencke et al (1971) was reported to produce a reasonable release of extracellular enzymes, without a parallel liberation of intracellular enzymes, whilst maintaining a good viability of the shocked cells. Application of this procedure to the study of transport in Candida albicans B2630 in conjunction with corresponding transport-deficient mutants, was hoped to provide further insight into the mechanism of transport in this organism.

6.6 Methods

6.6.1 Organisms and Growth Conditions

Candida albicans B2630 and its corresponding dipeptide transport-deficient mutant PA8035 (Chapter 5), and Saccharomyces cerevisiae Σ 1278b and its corresponding peptide transport-deficient mutant Σ 1278b gpp were used throughout these studies, and have been described previously (Section 2.8.2.1).

Organisms were grown overnight at 28°C in liquid media (50 ml) containing L-proline (4 mg ml.$^{-1}$), Bacto-Peptone (2% w/v) or ammonium sulphate (0.5% w/v) as the sole nitrogen source (referred to as Pro-, Pep- and NH_4^+- Medium respectively, see Section 4.2.1). All overnight cultures were innoculated from stock cultures, grown to mid-exponential phase in Pro-Medium and maintained at 4°C.

All materials used in these studies were from Sigma (London) and BDH (Poole, Dorset), unless otherwise stated.

6.6.2 Osmotic Shock Procedure

Cells were osmotically shocked according to the method of Schwenke et al., (1971). Exponentially-growing yeast cells were first suspended in a concentrated solution of mannitol, EDTA 2-mercaptoethanol and Tris, before harvesting by centrifugation and quickly resuspending in a solution of $MgCl_2$ of low osmotic strength. Sucrose has been previously shown to be ineffective as a shock inducer in yeast (Schwenke et al., 1971) and was not used in this study.

A mannitol-based medium of high osmotic pressure was used throughout this study. A saturated mannitol solution (13 g in 50 ml) in Tris-HCl (0.1 M Tris), pH 8, containing EDTA (0 - 5 mM) and 2-mercaptoethanol (1.0 mM) was stirred overnight, before filtering to

remove the insoluble precipitate (a 1.0 M mannitol solution in water represents a saturated solution).

Cells were grown in Pro-, or Pep-Medium overnight to mid-exponential phase (typically 0.5 (mg dry wt.)ml^{-1}), and harvested by membrane filtration (Section 4.2.2), cells being washed on the filter with phoshate-citrate buffer, pH 4.5. Cells were resuspended in the Tris-EDTA-mercaptoethanol-mannitol osmotic medium (referred to as TEMM) to approximately 0.5 (mg dry wt.)ml^{-1} (20 ml), and the suspension incubated at 28°C for 10 min at 28°C in a 'gently' shaking water bath. Cells were reharvested by centrifugation (2000xg, 5 min) using a MSE Bench Centrifuge. The pellet was resuspended in a low osmotic pressure solution (20 ml) containing ice-cold MgCl$_2$ (0.5 mM), and stirred continually in an ice bucket for 10 min. The suspension was centrifuged (2000xg., 5 min), the supernatant extract (19 ml) was removed, recentrifuged (2000xg, 15 min) and the supernatant solution (18 ml) carefully removed and immediately stored at -20°C or dialyzed (see below). This process of recentrifugation was shown to remove all viable cells from the supernatant fluid.

This supernatant solution (low osmotic pressure solution containing protein(s) released by the osmotic shock treatment hereafter called shock medium) was dialyzed to remove MgCl$_2$, the presence of which may be expected to affect the running of electrophoresis gels. Dialysis tubing (0.32 mm thick, 10 mm flat width, Gallenkamp) was placed in boiling water (approx. 150 ml, containing approximately 0.1g ammonium carbonate) for 5 min, before washing in distilled water (room temperature). The tubing containing the shock medium (18 ml) was sealed and placed in a large conical flask containing distilled water (5 l.) and dialyzed overnight at 4°C, the

water being continually stirred using a large magnetic stirrer bar. The water was replaced and the dialysis repeated for a further 6 h. The shock medium was then dried in vacuo, and stored at $-20^{\circ}C$.

Osmotically shocked cells were immediately taken for transport assays according to the standard method described (Section 4.2.2), the centrifuged pellet being resuspended in phosphate-citrate-glucose incubation buffer.

6.6.3 Polyacrylamide Gel Electrophoresis

Dissociating polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS, 0.1% v/v) was performed on 15% (w/v) acrylamide slabs (with a 3% (w/v) stacking gel) according to the method of Laemmli (1970) with a discontinuous buffer system (Tris-HCl, pH 6.8, stacking; Tris-Hcl, pH 8.8, separating). Gel preparation is extensively discussed by Ames (1974).

Separating and stacking gels were prepared from a stock acrylamide solution (30% w/v), containing bis-acrylamide (0.08% w/v). The final concentrations in the separating gel were : Tris-HCl, 0.375 M (pH 8.8), SDS 0.1% w/v., and in the stacking gel, Tris-HCl 0.125 M (pH 6.8), SDS 0.1% w/v. The gels were polymerized chemically by the addition of tetramethylethylenediamine (TEMED, 0.033% v/v) and freshly prepared ammonium persulphate (1.5 ml of a 0.15% (w/v) solution per 60 ml of unpolymerized gel solution). The electrophoresis buffer (pH 8.3) contained Tris (0.025 M), glycine (0.2 M) and SDS (0.1% w/v). Bromophenol blue marker dye (0.1 ml) was added to the upper reservoir, containing approximately 500 ml of electrophoresis buffer.

Freeze-dried protein samples of dialyzed shock medium (Section 6.9.2) were dissolved in sample buffer (0.3 ml) containing Tris-HCl, pH 6.8 (0.2 M Tris), SDS (2% w/v) and sucrose (10% w/v), and samples (20 - 30 ul) loaded into the wells of the stacking gel using a Hamilton

syringe. Gels were electrophoresed at constant power (7 watts, 14 - 25 mAmps) for 4 - 5 h, until the bromophenol blue marker dye reached the bottom of the gel.

Protein bands were vizualised by the ultrasensitive staining method of Morrissey (1981), see next section.

6.6.4 Silver-Staining of Polyacrylamide Gels

Polypeptides in polyacrylamide gels were detected by the silver stain method of Morrissey (1981), which itself is a modification of the original method, Switzer et al., (1979).

Silver staining was performed as follows, gentle but thorough agitation being carried out throughout using a shaking water bath.

1) Gel was prefixed in methanol (50% w/v), acetic acid (10% w/v) for 30 min, followed by methanol (5% w/v), acetic acid (7% w/v) for 30 min.

2) Gel was fixed in glutaraldehyde (100 ml, 10% v/v), for 30 min.

3) The gel was rinsed in distilled water, either in a large volume overnight, followed by a fresh water rinse the next morning for 30 min, or with several changes of water for 2 h.

4) The gel was soaked in dithiothreitol (200 ml, 5 ug ml^{-1}) for 30 min.

5) The dithiothreitol solution was poured off, and without rinsing, silver nitrate (100 ml, 0.1% w/v) was added, and the gel ~~tested~~ left for 30 min.

6) The gel was rapidly rinsed once with distilled water (100 ml), and then twice rapidly with developer (50 ml) containing sodium carbonate (3% w/v) and formaldehyde (50 ul of 37% solution). The gel was then soaked in developer (100 ml) until the desired

amount of staining was achieved. Staining was terminated by the addition of citric acid (5 ml, 2.3 M), directly to the developer and agitating for 10 min.

7) The solution was then discarded and the gel washed several times in distilled water over a 30 min period.

8) Gels were then photographed, wrapped in cellophane and dried using a gel drier (Bio-Rad Slab Gel Drier).

Gels were fixed and stained in a polyethylene container, the same container being used throughout the procedure. Since silver is not deposited on the surfaces, no special cleaning is required. Particular attention must be paid to the volumes of the carbonate and citric acid solutions, which must be balanced to bring the pH to neutrality (Morrissey, 1981). If the pH remains too high, the reaction will not stop, and if the pH is too low, the gel will bleach. Gels were handled throughout with rinsed plastic gloves to avoid staining of fingerprints on the gel.

The chemistry behind the silver staining process is not clear (Tunon & Johansson, 1984), although it seems likely that silver ions or diamine complexes of silver are bound to negatively charged groups on the proteins or free aldehyde groups introduced by glutaraldehyde fixation. The silver ions are then reduced by formaldehyde. The different methods of silver staining have recently been reviewed (Allen, 1983; Dunn & Burghes, 1983).

6.7 Results

6.7.1 Effect of Osmotic Shock on Transport

Cells were grown in Pro-, or Pep-Medium overnight, osmotically shocked as described (Section 6.6.2), and the transport properties of

the shocked cells examined by the standard manual fluorescamine assay (Section 2.5.2). Osmotic shock was shown not to abolish uptake of Ala-Ala or Gln by these strains (Table 6.3).

The effect of osmotic shock on the transport properties of cells grown in NH_4^+-Medium was also examined (Table 6.4). Under these growth conditions, synthesis of both the peptide transport systems (Section 4.3.2), and of the general amino acid permease (gap system) in Saccharomyces, would be expected to be repressed by the nitrogen regulation effect. Thus, only amino acid transport was measured by cells grown under these conditions.

Osmotic shock treatment was shown to inhibit transport of both Gln and Leu by Sacc. cerevisiae Σ 1278b grown in NH_4^+-Medium, relative to an unshocked control taken from the same batch of cells. This is compatible with these specific amino acid transport systems for this yeast being osmotically sensitive and possibly involving periplasmic binding proteins. However, transport of Gln by Σ 1278b grown in Pro-Medium is not affected by osmotic shock treatment, presumably reflecting differences either between the gap and Gln-specific transport system(s) in this yeast, or the fact that growth in Pro-Medium reduces the sensitivity to osmotic shock treatment.

The fact that peptide transport in both Candida and Saccharomyces was not susceptible to osmotic shock treatment indicates the absence of a periplasmic binding protein for these systems; thus precluding further studies using osmotic shock to investigate the mechanism of peptide transport.

6.7.2 Resolution of Proteins Released by Osmotic Shock by Polyacrylamide Gel Electrophoresis

Freeze-dried protein samples of dialyzed shock medium (Section 6.6.2) were resolved by dissociating polyacrylamide gel electrophoresis (PAGE) as described (Section 6.6.3).

Uptake Rate (nmol.min.$^{-1}$(mg dry wt.)$^{-1}$)

Strain	Growth Medium	Ala-Ala	Gln
B2630	Pro-	1.4 (1.2)	10.6 (19.8)
B2630	Pep-	7 (10.9)	-
Σ 1278b	Pro-	0.6 (2.2)	9.4
Σ1278b gpp	Pro-	ND (ND)	10.6

TABLE 6.3 Effect of Osmotic Shock on Ala-Ala and Gln Transport

Candida albicans B2630 and Sacc. cerevisiae Σ 1278b and its corresponding peptide transport deficient mutant Σ 1278b gpp were grown in either Pro-, or Pep-Medium overnight, harvested by membrane filtration and osmotically shocked (Section 6.6.2). The transport properties of shocked cells were examined by the standard manual fluorescamine assay (Section 2.5.2).

Initial substrate concentrations were : Ala-Ala, 0.1 mM for cells grown in Pro-Medium, 0.5 mM for cells grown in Pep-Medium; Gln, 0.5 mM throughout. Figures in parentheses give typical uptake rates (where available) for untreated cells.

-, not determined.   N.D. not detectable

Uptake Rate (nmol.min.$^{-1}$(mg dry wt.)$^{-1}$)

Strain	Treatment	Arg	Gln	Leu
B2630	Untreated Control	5.3	11	-
	Osmotically Shocked	6.7	7.9	-
Σ 1278b	Untreated Control	9.8	5	4.5
	Osmotically Shocked	4.2	ND	ND

TABLE 6.4   Effect of Osmotic Shock on Amino Acid Transport

in Cells Grown in NH_4^+-Medium

Candida albicans B2630 and Sacc. cerevisiae Σ 1278b were grown in

NH_4^+-Medium overnight, harvested by membrane filtration (Section

4.2.2). A portion of the cells was taken for osmotic shock treatment

(Section 6.6.2), and the remainder for assay of amino acid transport by

the standard manual fluorescamine procedure (Section 2.5.2). Cells

were resuspended to 1.2 - 2.1 (mg dry wt.)ml.$^{-1}$ for transport studies.

Initial substrate concentration was 0.5 mM throughout.

-, not determined.   N.D. not detectable

Freeze-dried protein samples were dissolved in sample buffer (0.3 ml), and samples (20 - 30 ul) carefully loaded into the wells using a Hamilton syringe. Gels were electrophoresed as described (Section 6.6.3), protein bands being visualized using the ultrasensitive silver staining method (Section 6.6.4).

Protein standards of known molecular weight were included in each gel run. The distances of migration of the various protein standards relative to the distance of migration of the bromophenol blue band are a linear function of the logarithm of the molecular weight of the marker proteins. Thus, molecular weights of shock proteins may be determined by comparing their mobility in SDS gels with those marker proteins of known molecular weight. Marker proteins were dissolved in sample buffer (Section 6.6.2) to 2 ug ml^{-1}. Routinely, ovalbumin (43000) and lysozyme (14000), both at 20 ng per well (the optimum level for silver staining), were included for each gel run.

Protein bands were visualized by the silver staining procedure (Section 6.6.4). Initially staining using Coomassie Blue (0.025% w/v) was attempted, but proved not to be sufficiently sensitive to detect the low levels of protein released by osmotic shock treatment. Briefly, gels were stained overnight in a Coomassie Blue solution (1 l, 0.025% w/v) containing methanol (50%, v/v) and glacial acetic acid (7%, v/v). Gels were destained in the same solution minus Coomassie Blue dye. The limit of detection of the standard proteins was approximately 0.2 ug $track^{-1}$, bands being very faint at this concentration, detection thus being limited to ug quantities.

Samples of shock medium were obtained from Candida albicans B2630 and Sacc. cerevisiae Σ 1278b grown overnight in NH_4^+-, Pro- and Pep-Medium. The profile of proteins resolved by SDS-PAGE showed growth

medium dependent differences (Fig. 6.17). Of particular interest was the enhanced expression by <u>Candida albicans</u> B2630 of a polypeptide (polypeptide P, track 4, Fig. 6.17) by cells grown overnight in Pep-Medium. To examine whether this polypeptide was possibly involved in the increased rates of transport exhibited by cells grown in Pep-Medium (Section 4.3.2), a comparison was made of the shock profiles of strain B2630 and its corresponding dipeptide-transport-deficient mutant PA8035 grown in Pro-Medium overnight, and transferred to Pep-Medium for 120 min.

Enhanced expression of this polypeptide was not observed in a preparation from B2630 grown overnight in Pro-Medium, and transferred to Pep-Medium for 120 min (Track 3, Fig. 6.17), relative to the protein preparation obtained from B2630 grown in Pro-Medium overnight (Track 2, Fig. 6.17). No major differences were observed between the protein profiles of B2630 and PA8035 grown either in Pro-Medium (compare Tracks 2 and 6) or transferred to Pep-Medium for 120 min (compare Tracks 3 and 7).

The fact that the rates of peptide transport are known to have been increased during this period in Pep-Medium (relative to rates exhibited by cells grown in Pro-Medium), and that enhanced expression of polypeptide P is not observed, suggests that this polypeptide is not a component of the peptide transport system(s).

On the assumption that all polypeptides are stained equally, an approximate protein yield may be calculated by comparing the relative intensities of the marker proteins and the total polypeptide content in each separate track. A yield of approximately 50 ug protein (g dry wt)$^{-1}$ was calculated.

Figure 6.17   Electrophoresis of Shock Medium from Osmotically

Shocked Cells

Cells were grown in NH_4^+-, Pro-, or Pep-Medium as described (Section

6.6.1), harvested and osmotically shocked (Section 6.6.2).   Samples of

shock medium were collected, dialyzed (Section 6.6.2) and

electrophoresed as described (Section 6.6.3).   Protein bands were

vizualized by the silver staining method (Section 6.6.4).

Samples (30 ul track^{-1}) are in order.

(1 - 4) Candida albicans B2630, was grown overnight in NH_4^+-(1), Pro-

(2) and Pep-Medium (4), or overnight in Pro-Medium and transferred to

Pep-Medium for 120 min before osmotically shocking (3).

(6 - 7) Candida albicans PA8035, grown overnight in Pro-Medium (6), or

Pro- overnight and transferred to Pep-Medium for 120 min before

osmotically shocking (7).

(9 - 11) Sacc. cerevisiae Σ 1278b was grown overnight in NH_4^+-, (9);

Pro-, (10); and Pep-Medium, (11).

Tracks (5) and (8) contained the standards lysozyme (L,14200 mw) and

ovalbumin (0,43,000 mw) at 20 ng track^{-1}, Migrated distances are

indicated by - 0, and - L. Gel polarity (+/-) is indicated.
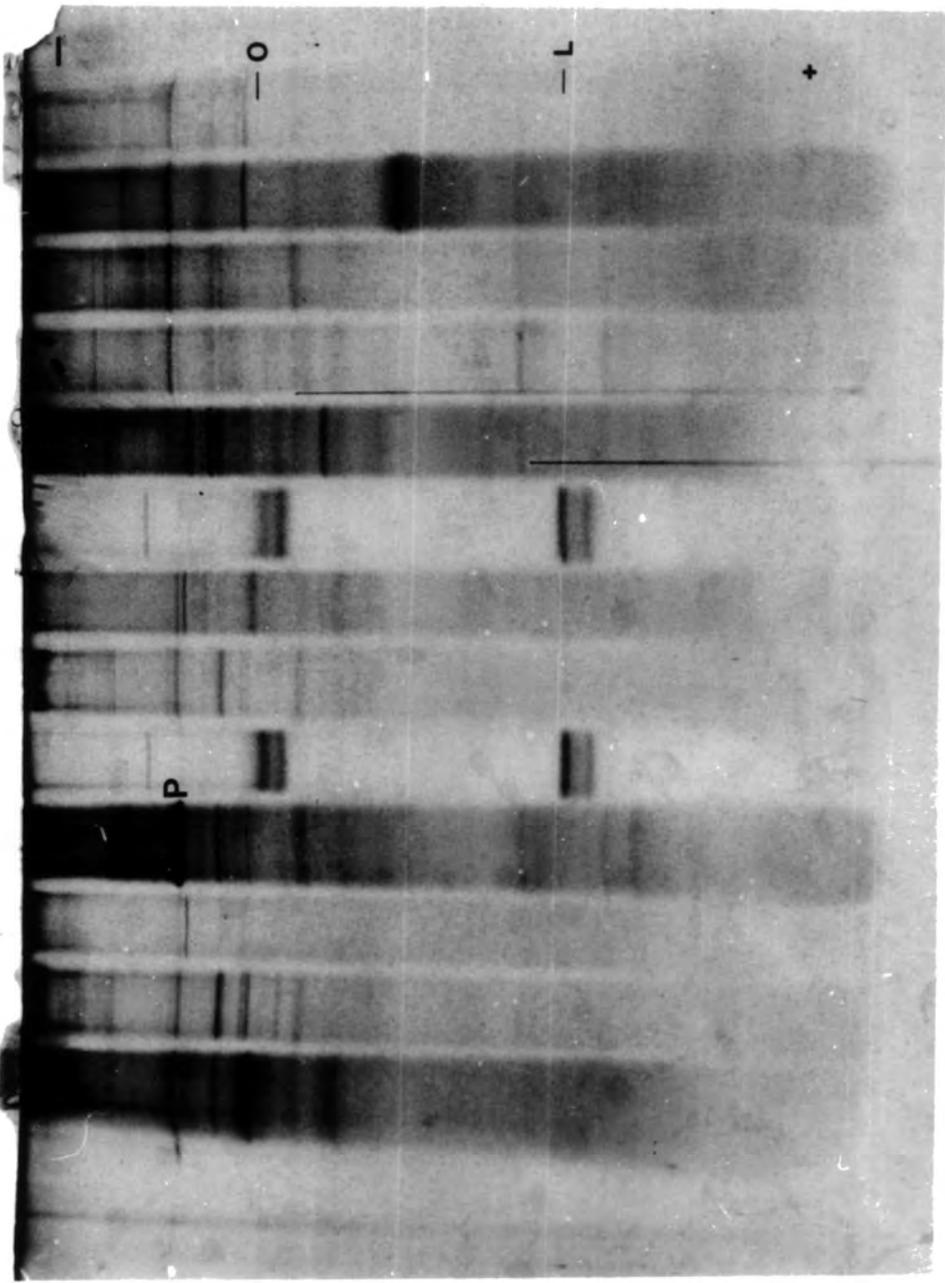
As the osmotic shock treatment was shown not to result in a loss of peptide transport (Section 6.7.1), further characterization of the proteins released by osmotic shock treatment was not attempted. However, the fact that the uptake of several amino acids is inhibited by osmotic shock for cells grown in NH_4^+-Medium, suggests the involvement of periplasmic binding proteins, and offers possibilities for further studies with these transport systems

6.8 Concluding Discussion

The osmotic shock protocol employed here was shown in controls with *Sacc. cerevisiae* to inhibit completely the transport of Gln and Leu, and give a 43% reduction in Arg transport, by cells grown overnight in NH_4^+-Medium (conditions under which expression of the general amino acid permease would be expected to be repressed). This result, coupled with the demonstration of the release of protein from *Candida albicans* by this treatment, encouraged us to use this protocol, on the assumption that *Candida albicans* is also susceptible to this treatment. The effectiveness of osmotic shock against ammonium-grown *Sacc. cerevisiae* implies that certain of the specific amino acid transport systems for this yeast are osmotically sensitive, and may involve periplasmic binding proteins. The fact that transport of Gln by *Sacc. cerevisiae* grown in Pro-Medium is not osmotically sensitive, presumably reflects differences between the *gap* and Gln-specific transport system(s) in this yeast, or possibly, that growth in Pro-Medium reduces the sensitivity to osmotic shock treatment.

The fact that transport of the amino acids in *Candida albicans* (grown in either Pro- or NH_4^+-Medium) was not completely inhibited by osmotic shock implies that these transport system(s) in this yeast may

not be sensitive to osmotic shock, or possibly that the treatment used was ineffective. However, the demonstration of protein release by this treatment tends to argue against this latter possibility

Transport of Ala-Ala by Candida albicans was insensitive to the osmotic shock treatment used here, which if substantiated implies the absence of a periplasmic binding protein for this transport system. This result dissuaded us from further studies using osmotic shock to investigate the mechanism of peptide transport in .this yeast.

Osmotic shock treatment of the yeast cells resulted in the release of a range of polypeptides in the shock medium. The profile of these polypeptides on a SDS-gel was dependent on the growth medium, presumably reflecting differences in the nature of periplasmic proteins produced in response to particular growth conditions.

Of particular interest was the enhanced expression of a polypeptide (polypeptide P, track 4, Fig. 6.17), by cells grown overnight in Pep-Medium. Initially, it was speculated that this polypeptide could possibly be involved in the enhanced uptake of peptide by cells grown in Pep-Medium. However, enhanced expression of this polypeptide was not observed in a preparation from B2630 grown overnight in Pro-Medium, and transferred to Pep-Medium for 120 min (track 3, Fig. 6.17), relative to B2630 grown overnight in Pro-Medium (track 2, Fig. 6.17). Taken together, these results indicate that this polypeptide is not a component of the peptide transport system(s). The identity of this polypeptide remains unclear, though it may be a peptidase or protease released in response to the presence of peptides in the growth medium (the nature and chain length of which is not specified). Release of proteases by Candida albicans, in response to the presence of proteins in the growth medium, has been reported (see Section 1.3.5)

As osmotic shock was shown not to inhibit peptide transport, further resolution and identification of the polypeptides released by shock treatment was not attempted. However, the methods described here do offer some potential for osmotic shock studies with this yeast.

CHAPTER 7

CONCLUDING DISCUSSION

CHAPTER 7   CONCLUDING DISCUSSION

This study has demonstrated the existence of at least two peptide permeases in Candida albicans, through the characterization of mutants isolated by a single-step procedure, on the basis of resistance to toxic peptides. The isolation of a bacilysin-resistant mutant that was shown to be defective in dipeptide transport whilst retaining typical wild-type rates of oligopeptide uptake, and was unable to utilise dipeptides as a sole nitrogen source, clearly indicates the existence of separate transport systems for di- and oligopeptides. These results were to some extent endorsed by other reports in the literature of mutants defective in dipeptide transport (Section 5.5), though these mutants were obtained from cells pretreated with mutagenic agents.

The availability of reasonable quantities of purified di- and tripeptide polyoxins and nikkomycins should facilitate further mutant studies. The isolation of a tripeptide-resistant mutant which is fully defective in oligopeptide transport is clearly desirable.

Strain B2630 used in these studies, was not very sensitive to m-fluorophenylalanyl-substituted peptides, but studies with a more sensitive strain should allow mutants to be isolated on the basis of resistance to the di- and tripeptide forms of these compounds.

To date, studies have been hampered by the limited availability of pure toxic peptides. It was not considered worthwhile attempting to synthesise individual polyoxins or nikkomycins, as they are very complex peptides, and their synthesis would be time-consuming and difficult, although methods of synthesis have been published.

The availability of a wider range of mutants resistant to toxic peptides should enable a detailed characterization of the specificities of the different peptide transport systems. The recent development of the automated fluorescamine assay coupled with the advanced computer software for kinetic analysis should permit a detailed kinetic characterization of these systems. Knowledge of the kinetic parameters for uptake will permit valid competition studies e.g., using radioactively-labelled substrates or the dansyl chloride assay. Such studies will enable a more detailed determination of the molecular determinants of transport, particularly with regard to side chain specificity, the results of which are essential for the rational design of novel peptide antibiotics and the use of peptides as drug delivery systems.

This study has characterized the peptide transport systems in Candida albicans in some detail and also characterized the ways in which the systems are regulated. Various levels of control occur, one involving ammonia repression (in a manner analagous to the Gap permease in Sacc. cerevisiae); and a novel mechanism by which transport activity varies depending on the presence of peptides in the growth medium. The addition of peptides (peptone), (even in the presence of ammonium) results in a rapid reversible-activation of peptide transport, a process which is independent of de novo synthesis of permease components. It was speculated that this Step Up of transport activity could be attributed to in situ activation of preformed permease components in the plasma membrane, and/or insertion of permease-containing vesicles into the plasma membrane in response to the presence of peptides. It would be of considerable interest to

characterise further the mechanism of Step Down of transport activity, these studies showed that this process was very rapid but did not determine whether this process was dependent on protein synthesis, e.g. inhibition of activity may possibly involve synthesis of a "repressor molecule". Of considerable interest with regard to the treatment of candidiosis would be the determination of the transport activity of cells taken directly from infected serum, to determine whether the presence of peptides in the serum results in cells having the high levels of peptide transport activity found in cells grown in defined peptone-based media. Thus, the advantageous nutritional response of inducing high levels of peptide transport activity in the presence of substrate, may well prove disadvantageous to the yeast when cells are exposed to toxic peptides. The presence of serum peptides means that potential toxic peptide drugs must be able to compete effectively with serum peptides for transport into the yeast cell, a good affinity (relative to serum peptides) will thus probably be a prerequisite for effective peptide drugs to combat systemic candidiosis.

Evidence suggests that the mycelial form of this dimorphic yeast, is probably the predominant species found at site infections (Shepèrd et al., 1985; Odds, 1979). It would thus be of considerable interest to examine the peptide transport activities of mycelial cells and their sensitivity to toxic peptides, to determine whether there are any important differences between the blastospore and mycelial forms of growth of Candida albicans. At the initiation of these studies, there was no defined medium which would have produced stable hyphae. However, recent work on the regulation of dimorphism has led to the development of a satisfactory medium for hyphal induction (Shepèrd et

<u>al</u>., 1985), which should permit future peptide transport studies, essential for any rational drug-development studies.

The high frequency of resistance to toxic peptides is of interest, particularly with regard to any future treatment of candidiosis. However, the use of a combination of toxic di- and oligopeptides, should provide some hope for effective treatment,

Related studies of peptide transport in <u>Candida albicans</u> demonstrated that peptide transport did not involve periplasmic binding proteins. However, the osmotic shock protocol employed here resulted in the release of a number of polypeptides, which may find application in future studies. The effects of various chemical modification agents on peptide transport was also studied, most of the reagents were non-specific with respect to their effect on the transport systems examined. However, Woodwards reagent K had some degree of specificity, which offers some potential for the selective labelling of the components of the dipeptide permease; isolation of the components of the peptide transport systems is of considerable interest. Studies involving peptide transport-deficient mutants, selective chemical modification agents and the recently developed peptide photoaffinity labels should all find use in future studies.

Thus, although there are undoubted unknowns and potential problems with the rational design of anticandidal peptide-drugs, this approach does have the potential for selective drug development. Therefore, synthetic antifungal peptides may eventually provide an alternative, selective therapy for the treatment of fungal infections, which are of increasing medical importance.

APPENDIX I

ABBREVIATIONS

NEM	N-ethylmaleimide
PCMBS	p-chloromercuribenzenesulphonic acid
PAO	phenylarsine oxide
WRK	N-ethyl-5-phenylisoxazolium-3'-sulphonate
	(Woodwards's Reagent K)
DEPC	diethylpyrocarbonate
PG	phenylglyoxal
CCCP	carbonylcyanide-m-chlorophenylhydrazone
DCCD	dicyclohexylcarbodiimide
DES	diethylstilbestrol
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
EDTA	ethylene diaminetetracetic acid
PAGE	polyacrylamide gel electrophoresis
TEMED	N,N,N',N'tetramethyl ethylene diamine
UDPNAG	uridine-diphosphate-N-acetylglucosamine
FPA	L-m-fluorophenylalanylalanine
FPAA	L-m-fluorophenylalanylalanylalanine

APPENDIX II

ISOLATED MUTANTS AND DESIGNATED STRAIN NUMBERS

All mutants were given a PA8... number, indicating the laboratory of origin (Payne), the number 8 indicating that the mutant is a Candida albicans.

PA8001 - PA8024	polyoxin-resistant, isolated on the basis of resistant to basic polyoxin fraction.
PA8026 - PA8029	nikkomycin-resistant; isolated on the basis of resistance to nikkomycin (mixture of X and Z).
PA8030 - PA8079	bacilysin-resistant.
PA8080 - PA8109	revertants of bacilysin-resistant mutant PA8035, isolated on the basis of enhanced utilisation of Ala-Ala.
PA8110 - PA8134	m-fluorophenylalanylalanylalanine-resistant.

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