Aspects of peptide transport in saccharomyces cerevisiae and streptococcus spp.

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Aspects of Peptide Transport in *Saccharomyces cerevisiae* and *Streptococcus* spp.

by

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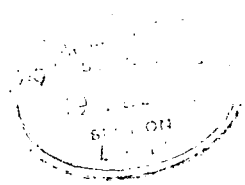
A thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy in the University of Durham

October 1980



Abstract

Two novel fluorescence methods for the assay of peptide transport in microorganisms are described. These methods are compared with conventional radiotracer assays, and certain advantages of the fluorescence techniques are highlighted.

Peptide uptake in the yeast *Saccharomyces cerevisiae* is investigated. It is shown that peptide and amino acid uptake are distinct, intact peptide accumulation is demonstrated, and the presence of a single main peptide permease, which handles a range of di- and tripeptides, is demonstrated. A mutant deficient in this permease is isolated on the basis of resistance to the dipeptide-mimetic antibiotic bacilysin.

Peptide uptake in *Streptococcus faecalis* is also shown to be distinct from amino acid transport, and intact peptide accumulation is again demonstrated. A strain of *Strep. faecalis* (*faecium*) is shown to possess two peptide permeases, a high-rate system for di- and tripeptides, and a low-rate oligopeptide system. Other strains of *Strep. faecalis* are shown to differ in their peptide transport specificity. Mutant strains deficient in peptide permeases or intracellular peptidases are isolated on the basis of resistance to peptide mimetic antibiotics.

The coupling of metabolic energy to peptide transport is investigated in *Sacc. cerevisiae* and *Strep. faecalis*. In both cases a proton-motive-force is implicated. Some preliminary investigations of the energy consumption of peptide transport in *Strep. faecalis*, implying the same stoichiometry for tri-, tetra- and pentaalanine uptake, are reported.

Acknowledgments

I should like to thank Prof. D. Boulter for the use of facilities in the Department of Botany, and the Science Research Council and Roche Products Ltd. for financial support.

I should like to acknowledge the considerable contributions of Dr. C.F. Higgins and Mr. G. Bell to the development of the fluorescence assays used in this work. I am also grateful to members of the Botany Department, University of Durham, especially Dr. C.F. Higgins and Mr. R.A. Alves, for many stimulating discussions.

Thanks are due to my industrial supervisors, Dr. W.J. Lloyd and Dr. P.S. Ringrose of Roche Products Ltd., Welwyn Garden City, for their guidance and support, and to the members of staff, in particular Mrs. D. Barber and Mrs. A. Broadhurst, who were so helpful during the periods I spent at Roche Products Ltd.

I should also like to thank Mrs. E. Ellis for her efficient typing, Mr. A. Jamieson for photographic work, and Mr. A. Reid, Mr. C. McCarty and Miss H. Morton for assistance in drawing diagrams.

Finally, I am deeply grateful to my supervisor, Dr. J.W. Payne, for his help, encouragement and guidance during the course of this study, and during the preparation of the manuscript.

Memorandum

Parts of the work presented in this thesis also appear in the following publications:

1) Nisbet, T.M., Payne, J.W. (1979a) Peptide transport in *Saccharomyces cerevisiae*: Characteristics of transport system shared by di- and tripeptides. J. Gen. Microbiol. 115, 127-133.

2) Nisbet, T.M., Payne, J.W. (1979b) Specificity of peptide uptake in *Saccharomyces cerevisiae*, and isolation of a bacilysin-resistant, peptide-transport-deficient mutant. FEMS Lett. 6, 193-196.

3) Nisbet, T.M., Payne, J.W. (1980) Characterization of two peptide-transport systems in *Streptococcus faecalis*. Trans. Biochem. Soc. 8, 705-706

4) Payne, J.W., Nisbet, T.M. (1980a) Peptide Transport in Microorganisms. Trans. Biochem. Soc. 8, 683-685

5) Payne, J.W., Nisbet, T.M. (1980b) Limitations to the use of radioactively labelled substrates for studying peptide transport in microorganisms. FEBS Lett. 119, 73-76

6) Payne, J.W., Nisbet, T.M. (1980c) Continuous monitoring of substrate uptake using fluorescamine: Application in studies of energy coupling to peptide transport in *Saccharomyces cerevisiae* and *Streptococcus faecalis*. J. Bacteriol. (submitted for publication)

CONTENTS

Figures

Tables

CHAPTER 1

INTRODUCTION

Peptides have long been recognised as important nutrients for microorganisms, but it is only comparatively recently that the details of their utilization have been determined. Work in the period after the Second World War demonstrated that peptides are nutritionally useful as sources of amino acids, and thus must undergo uptake by the cell and hydrolysis to be utilized. Transport systems for intact peptides have since been demonstrated in a wide range of micro-organisms (Section 1-1), mammalian organs (Matthews, 1975) and germinating barley embryos (Higgins and Payne, 1977).

Although peptide transport has been demonstrated in many micro-organisms, the uptake systems have been well characterized in only very few, in particular Escherichia coli. In the present work, the peptide transport systems of the yeast, Saccharomyces cerevisiae, and the Gram-positive bacterium Streptococcus faecalis have been characterised, and some information about the energy coupling of the transport has been obtained. Apart from the use of the work in allowing comparisons to be made between peptide transport systems in different organisms, such information is necessary if the rational design of peptide mimetic antibiotics (Section 1-1-9), active against specific organisms, is to be possible.

In this introductory chapter, a survey of the literature in the following areas is given:

Section 1-1 Peptide transport in microorganisms. The characteristics of transport in those organisms studied to date.

Section 1-2 Nitrogen metabolism in Sacc. cerevisiae. Peptide and amino acid uptake and metabolism.

Section 1-3 Nitrogen metabolism in Strep. faecalis. As for 1-2.

Section 1-4 Energetics of transport in microorganisms. The modes of energy coupling and action of metabolic inhibitors, with particular reference to the two organisms studied here.

1-1 Peptide Transport in Microorganisms

1-1-1 Introduction

As long ago as the latter part of the 19th century, media containing small peptides were used for the cultivation of bacteria, and today many standard media use preparations of peptide-containing 'peptones' (see for example the Difco Manual, 9th Edition, 1953). Although the importance of peptides in the nutrition of microorganisms has thus been long assumed, it was only with the availability of defined synthetic peptides in the 1940's that the mechanisms involved in the utilization of peptides could be elucidated. It has since become apparent that peptides act as amino acid sources for many organisms, and that their utilization thus 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 these organisms (e.g. gut bacteria) live in environments where small peptides are readily available, so that the possession of peptide transport systems is clearly of advantage.

Since synthetic peptides became readily available and in particular over the last dozen years, there has been a large body of research into peptide transport in microorganisms, in much of which E.coli has been used as a test organism. The volume of literature makes an exhaustive survey impracticable in the space available here, and so this review concentrates on those areas most relevant to the

results presented in this thesis, and in particular on more recent reports. For further information on some of the topics not covered in detail here, the reader is referred to the many reviews published in the last decade (Barak and Gilvarg, 1975a; Matthews and Payne, 1975a, 1979a,b; Payne, 1972a, 1975, 1976, 1977; Payne and Gilvarg, 1971, 1978; Sussman and Gilvarg, 1971). Summaries of the most recent work are given in reviews on bacteria (Payne, 1980a), yeasts (Becker and Naider, 1980), and other fungi (Wolfinbarger, 1980).

The properties of peptide transport systems in yeasts and Streptococci are not covered in detail in this section, these being dealt with specifically in Sections 1-2-2 and 1-3-2, respectively.

1-1-2 Mechanisms of Peptide Uptake and Utilization

1-1-2-1 Models for Peptide Uptake

The utilization of a peptide as an amino acid source in a prokaryote may occur in one of three ways: uptake of intact peptide, followed by cleavage; cleavage of peptide during translocation across the cytoplasmic membrane; cleavage extracellularly and uptake as free amino acids (Fig. 1-1). In eukaryotes, the situation is more complex, as the possibility arises for intracellular cleavage to occur not only in the cytoplasm, but in some intracellular organelle(s) such as the vacuole (Fig. 1-1d). The implications of these mechanisms and the evidence for and against their operation in a range of organisms are given below.

1-1-2-2 In Many Organisms Peptide Hydrolysis is not Extracellular

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

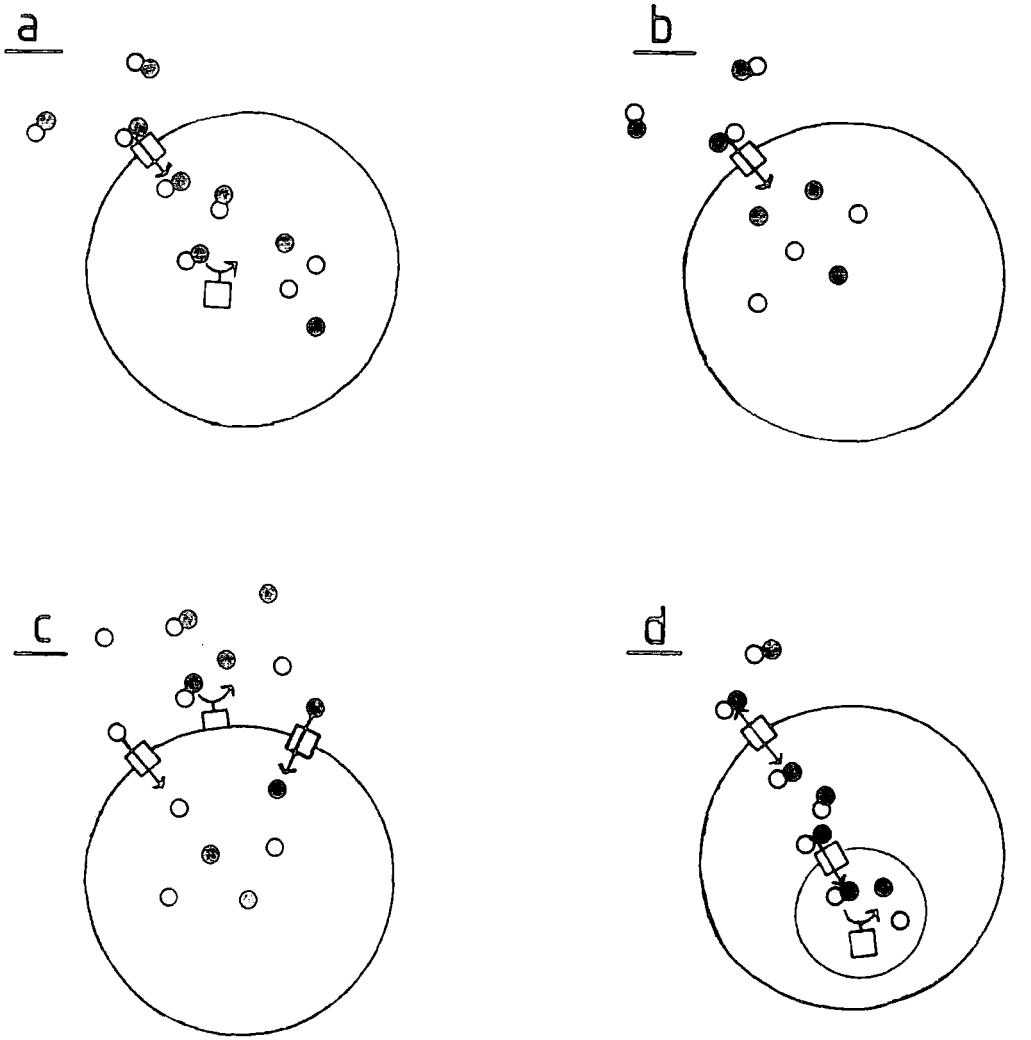


Fig 1-1 Models for Peptide Uptake

a) Uptake of intact peptide, b) cleavage of peptide during uptake, c) extracellular cleavage and uptake of free amino acids, d) cleavage in an organelle of a eukaryote . (OO dipeptide, ⊕ O amino acids, permease, peptidase)

a) Lack of inhibition of peptide uptake by free amino acids.

b) Uptake in peptide-linked form of amino acid residues impermeant in the free form.

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

d) Uptake of amino acids in peptide-permease-deficient strains.

Lack of competition by free amino acids for the uptake of labelled peptides has been shown in E.coli (Cowell, 1974; De Felice et al., 1973; Smith et al., 1970). Strep.faecalis (Brock and Wooley, 1964), Leuconostoc mesenteroides (Mayshak et al., 1966; Yoder et al., 1965), Lactobacillus casei (Leach and Snell, 1960), Pseudomonas spp. (Cascieri and Mallette, 1976a), Bacteroides ruminicola (Pittman et al., 1967), Staphylococcus aureus (Perry and Abraham, 1979), Strep. lactis, Strep. cremoris (Law, 1978), Sacc. cerevisiae (Becker and Naider, 1977). Candida albicans (Logan et al., 1979), and Neurospora crassa (Wolfinbarger and Marzluf, 1975a). Lack of competition has also been shown by direct fluorescence assays of transport in E.coli (Payne and Bell, 1977a) and Salmonella typhimurium (J.W. Payne, personal communication), and by less direct growth assays in many organisms (reviewed in Payne and Gilvarg, 1978).

The uptake of impermeant amino acid derivatives in peptide form is of great value in the development of novel antibacterial agents (Section 1-1-9) and has been demonstrated in E.coli (Fickel and Gilvarg, 1973), Salmonella typhimurium (Ames et al., 1973), and a range of other organisms (Atherton et al., 1979a).

Mutants that are deficient in amino acid transport but that can still take up the peptide-linked amino acid have been reported for E.coli (De Felice et al., 1973; Guardiola and Iaccarino, 1971;

Guardiola et al., 1974a,b) Salmonella typhimurium (Kustu and Ames, 1973), and Lactobacillus casei (Peters et al., 1953).

Mutants that are deficient in peptide utilization but that retain unimpaired utilization of amino acids have been isolated from many organisms (Payne and Gilvarg, 1978). However, the isolation of such mutants does not, in itself, demonstrate the separation of peptide and amino acid transport, for, in principle, they could be defective in intra- or extracellular peptidase activity. Mutants which have been shown to be deficient in peptide transport per se have, however, been isolated from E.coli (Payne and Gilvarg, 1978; Alves and Payne, 1980), Salmonella typhimurium (Ames et al., 1973), Staph. aureus (Perry and Abraham, 1979), Sacc. cerevisiae (Marder et al., 1979), and Neurospora crassa (Wolfinbarger and Marzluf, 1975a).

The evidence presented in this section shows that peptide transport is distinct from amino acid uptake in a number of micro-organisms, and so extracellular peptidase action does not play a significant role in peptide utilization in these species.

1-1-2-3 The Site of Hydrolysis Following Peptide Uptake

In those prokaryotes where peptide uptake has been shown to be distinct from amino acid uptake, cleavage could occur either cytoplasmically (Fig. 1-1a) and/or during translocation (Fig 1-1b). The demonstration of intact peptide accumulation would rule out the possibility of cleavage being an obligate feature of uptake, but the generally high levels of intracellular peptidase relative to transport in those organisms (e.g. E.coli) extensively studied makes the detection of such accumulation difficult. However, certain peptides are

resistant to cleavage but still retain adequate affinity for transport, allowing intact accumulation to be detected; this has been observed for glycyl-sarcosine (Payne, 1972b) and triornithine (Payne, 1968; Gilvarg and Levin, 1972) in E.coli. Recent studies on the uptake of peptides by mutants of Salmonella typhimurium deficient in particular cytoplasmic peptidases (Payne and Nisbet, 1980a; J.W. Payne, personal communication), showed that intact accumulation of many peptides occurred. Thus, cytoplasmic cleavage is implicated in the utilization of peptides in these strains.

It should be stressed, however, that 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 and Becker, 1978) although this does not seem to be the case in Ps. maltophilia and Ps. putida (Cascieri and Mallette, 1976a,b).

In eukaryotic organisms, cleavage may be in compartments other than the cytoplasm (Fig 1-1d). Consideration of the possible site(s) of peptidase activity following peptide uptake in Sacc. cerevisiae is made in Section 1-2-3-3.

1-1-2-4 Extracellular Cleavage

Although many microorganisms secrete proteases, there are relatively few reports of secretion of enzymes capable of cleaving small peptides. Such peptidases have been reported for Aeromonas proteolytica (Wilkes et al., 1973), Bacillus licheniformis (Hall et al., 1966), Bacillus subtilis (Ray and Wagner, 1972), Strep. cremoris and Strep. lactis (Law, 1977), and peptidases associated with the external surface of the cell have been found in Strep.lactis

(Law, 1977), Strep. cremoris (Exterkate, 1975, 1976), and Sacc. cerevisiae (Matile et al., 1971) (peptidases that have been shown to play a specific role in cross-linking of cell wall components are not considered here).

It is interesting to note that, whereas Strep. cremoris and Strep. lactis both display extracellular dipeptidase activity, they also possess transport systems for dipeptides (Law, 1978). It is to be presumed, therefore, that a proportion of peptide is cleaved extracellularly and taken up as the free amino acids in these organisms. In Neurospora crassa, (Wolfinbarger, 1980) tripeptides are transported as such, but utilization of dipeptides is subject to inhibition by amino acids; presumably extracellular dipeptidase activity is involved.

1-1-3 Peptide Uptake and Amino Acid Exodus

In a natural environment such as the gut, microorganisms are presented with a wide range of peptide substrates. In contrast, in a typical assay of peptide uptake in vitro, a large amount of one or a few amino acids is introduced into the cell after uptake and cleavage of a particular peptide. Studies on E.coli have shown that uptake of peptides under such conditions is accompanied by rapid exodus of the constituent and metabolically related amino acids (Payne and Bell, 1977a; Payne and Bell, 1979); similar rapid exodus has also been noted for Salmonella typhimurium (J.W. Payne, personal communication) and for Staph. aureus (Perry and Abraham, 1979). Presumably it occurs via amino acid permeases, although attempts to verify this in E.coli have so far been inconclusive (J.W. Payne, personal communication).

Although it is not known how widespread the exodus phenomenon is in bacteria, it seems a logical method of regulating the amino acid pools during peptide utilization (Payne, 1975); to inhibit peptide uptake in a response to an excess of one pool amino acid would not be an efficient alternative. In the few eukaryotic microorganisms studied to date, no amino acid exodus has been detected. This is discussed in relation to peptide transport in Sacc. cerevisiae in Section 3-5-3-3.

1-1-4 Kinetic Parameters of Peptide Transport

In studies of kinetic parameters of peptide transport, it is difficult to exclude the effects of cleavage on the apparent affinity for, and velocity of transport. The best published values are those derived from data from the rapid uptake of radiolabelled peptides, though even here cleavage and amino acid exodus may influence the results (Section 2-6).

The following apparent values have been derived from the application of Michaelis-Menten Kinetics to data from the uptake of labelled peptides in E.coli: Gly-Gly, K_m 1 x 10^{-5} M, V_{max} 10 nmol min^{-1} mg dry wt^{-1} (De Felice et al., 1973), K_m 3 x 10^{-6} M, V_{max} 5 nmol min^{-1} mg dry wt^{-1} (Cowell, 1974); Gly-Gly-Gly, K_m 1 x 10^{-6} M, V_{max} 5 nmol min mg dry wt^{-1} (De Felice et al., 1973). When data were fitted to a model for two transport systems, the following values were obtained: Ala-Ala, K_m 6 x 10^{-8} M and 5 x 10^{-5} M, V_{max} 14 and 33 nmol min^{-1} mg $protein^{-1}$; Ala-Ala-Ala, K_m 7 x 10^{-8} M and 1 x 10^{-5} M, V_{max} 5 and 29 nmol min^{-1} mg $protein^{-1}$ (Ringrose and Lloyd, 1979).

In Staph. aureus, Gly-Phe had a K_m of 3 x 10^{-4} M and a V_{max} of 56 nmol min^{-1} mg dry wt^{-1} (Perry and Abraham, 1979), in Neurospora

crassa Gly-Leu-Tyr had a K_m of 3.4×10^{-5} M and a V_{max} of 2 nmol min^{-1} mg dry wt^{-1} (Wolfinbarger and Marzluf, 1975a) and the transport of Met-Met-Met had a K_m of 8×10^{-5} M in Sacc. cerevisiae (Becker and Naider, 1977) and 3×10^{-5} M in Candida albicans (Logan et al., 1979). Kinetic parameters for uptake independent of peptidase activity were obtained for Gly-Pro in a peptidase-deficient strain of Salmonella typhimurium (Yang et al., 1977). These were a K_m of 5.3×10^{-7} M and a V_{max} of 1.4 nmol min^{-1} mg dry wt^{-1}.

There are, not surprisingly, wide differences in kinetic parameters of peptide uptake between organisms. However, even in E.coli, the observed affinities for transport vary considerably. Although these affinities may be for different permeases (Section 1-1-5) the wide range means that studies of competition for peptide transport must be carefully designed if they are to be meaningful (see Sections 1-1-5 and 2-1).

1-1-5 The Presence of one or more Peptide Permeases

In many of the organisms studied to date, there is more than one peptide permease. Evidence for this comes from studies of competition between peptides and from the isolation of transport deficient mutants.

In considering the literature evidence for the existence of separate systems, the limitations of competition studies, particularly using indirect methods (Section 2-1), must be borne in mind. As the affinities for transport of different peptides may vary widely (Section 1-1-4), large inhibitor: substrate ratios may need to be used to establish whether or not mutual competition for uptake is occurring; literature evidence which relies on competition studies

alone is thus rarely rigorous. For example, the report (Law, 1978) that dipeptides inhibited tripeptide utilization in Strep. lactis and Strep. cremoris but that tripeptides did not inhibit dipeptide uptake is equally consistent with the presence of one peptide transport system with a higher affinity for dipeptides as it is with the author's conclusion that two transport systems are present. It is also of importance to consider whether the transport system is saturated; studies performed at concentrations well below saturation will show negligible competition between substrates sharing the same permease.

The isolation of transport-deficient mutants yields much firmer evidence for the existence of one or more permeases, though here adequate transport studies must also be performed to allow unambiguous conclusions. In E.coli, mutants isolated as resistant to triornithine lose the ability to utilize many oligopeptides but not dipeptides (Payne, 1968), leading to the suggestion that E.coli possesses separate di- and oligopeptide permeases. Later reports (Barak and Gilvarg, 1975; Naider and Becker, 1975) suggested the presence of a further oligopeptide permease of restricted specificity. However, the use of sensitive fluorescence assays of transport in mutants lacking the di- or oligopeptide permease, or both (Alves and Payne, 1980), showed a residual transport activity of broad specificity, which is presumably identical to the 'specialised' systems previously reported. It therefore seems that there are at least three peptide permeases in E.coli.

Mutants defective in oligopeptide uptake but unaltered in dipeptide uptake have also been isolated from Salmonella typhimurium (Ames et al., 1973; Jackson et al., 1976) and the presence of at least two permeases has been confirmed by competition studies (Yang et al., 1977). In Sacc. cerevisiae, in contrast, it appears that there is only one major permease (Section 1-2-2-3). There are also reports of separate di- and oligopeptide permeases in Pseudomonas spp. (Cascieri and Mallette, 1976a), Streptococcus spp. (Law, 1978; Rice et al., 1978), Lactobacillus casei (Dunn et al., 1957), and Leuconostoc mesenteroides (Shelton and Nutter, 1964), although the evidence is not as conclusive as in the above cases.

1-1-6 Size Limit for Peptide Transport

An upper size limit for peptide transport could be a property of the permease itself, or occur as a result of a diffusion barrier in the cell wall or outer membrane. In E.coli, Payne and Gilvarg (1968a) showed that the upper size limit for peptide transport depended not simply on the number of amino acid residues, but rather on the Stokes' radius of the peptide, and suggested that the exclusion, which occurred at about the pentapeptide size, was more likely a feature of the cell wall or outer membrane rather than the permease. Subsequent work has also shown an exclusion limit of about 600 daltons for saccharides and polyethylene glycols by the outer membrane of Salmonella typhimurium (Nakae and Nikaido, 1975; Decad and Nikaido, 1976); such a limit would explain the results with peptides in E.coli (Payne and Gilvarg, 1968a; Smith et al., 1970). In contrast, the size limit for peptide utilization in Neurospora crassa (Wolfinbarger and Marzluf, 1975b) seems to be a

function of the permease *per se*. Reports of an upper size limit for peptide transport in *Pseudomonas putida* (Cascieri and Mallette, 1976a), *Strep. lactis* (Rice *et al.*, 1978), and *Sacc. cerevisiae* (Section 1-2-2-4) do not distinguish between the two possibilities.

1-1-7 Substrate Specificity of Peptide Uptake

1-1-7-1 General

Considering only the twenty natural L-amino acids, there are 400 possible dipeptides, 8000 tripeptides and 160,000 tetrapeptides. As the available evidence (Section 1-1-5) suggests that most organisms possess only a few peptide transport systems, these must clearly be able to handle a wide range of amino acid residues in order to be useful. The specificity of the uptake system for peptides must therefore depend on other features of the substrate, such as the N-terminal amino group, C-terminal carboxyl group and the peptide bond itself. These and other structural specificities are discussed below.

1-1-7-2 Side Chain Specificity

Although there have not to date been any systematic studies of the effect of different amino acid residues on the velocity of, or affinity for, transport of peptides, those systems studied will transport many different peptide sequences. Thus, competition for uptake has been shown for a range of dipeptides in *E.coli* (Payne and Gilvarg, 1978), *Salmonella typhimurium* (Yang *et al.*, 1977), *Pseudomonas putida* (Cascieri and Mallette, 1976a), *Leuconostoc mesenteroides* (Yoder *et al.*, 1965), *Strep. lactis* and *Strep. cremoris* (Law, 1978), and *Staph. aureus* (Perry and Abraham, 1979), and for oligopeptides in *E.coli* (Payne and Gilvarg, 1978), *Salmonella*

typhimurium (Jackson et al., 1976), Pseudomonas putida (Cascieri and Mallette, 1976a), Strep. lactis (Rice et al., 1978), Saccharomyces cerevisiae (Becker and Naider, 1977), Candida albicans (Logan et al., 1979), and Neurospora crassa (Wolfinbarger and Marzluf, 1975a). The reported specialised oligopeptide permease(s) in E.coli (Barak and Gilvarg, 1975; Naider and Becker, 1975) is likely to be identical to the residual, broad specificity uptake found later (Alves and Payne, 1980), the initial characterisation of it as having narrow specificity probably arising from the insensitive assays and limited substrates used.

1-1-7-3 Stereospecificity

The lack of systematic studies makes it difficult to draw firm conclusions about the uptake of peptides containing D-amino acids, and the lack of peptidase activity against many such substrates makes some studies of peptide utilization hard to interpret. However, all peptide transport systems studied to date display a marked preference for L-residues.

In E.coli, various dipeptides containing D-residues do not inhibit uptake of Leu-Gly or Gly-Gly (Levine and Simmonds 1962 ; Kessel and Lubin, 1963), and the uptake of several L-D, D-L and D-D peptides is indetectable when assayed directly (Payne, 1980). The presence of a D-residue at the C-terminal of an oligopeptide is, in contrast, tolerated: Payne (1980) demonstrated extensive uptake of L-Ala-L-Ala-D-Ala, L-Val-L-Val-D-Val, and Gly-Gly-D-Leu, but not of LDL- or DDD-Ala-Ala-Ala, D-Val-D-Val-D-Val or D-Leu-Gly-Gly.

Other organisms have not been studied to the same extent as E.coli, the most detailed studies being those on yeast (Section 1-2-2-5).

In other organisms there are some reports of uptake of peptides containing D-residues (e.g. Kihara et al., 1961), but, in general, uptake is much less than the corresponding all-L-peptide (see Payne and Gilvarg, 1978).

1-1-7-4 N-terminal α-Amino Group

The transport of N-substituted peptides is influenced by the nature of the added group, and a systematic study of the uptake of different substituted peptides is needed in order to draw firm conclusions about the N-terminal requirement. In E.coli, substitutions that preserve the positive charge of the group allow peptide utilization, but those that neutralise the charge do not (Payne, 1971, 1974). Such detailed studies have not been carried out in other organisms. However,substituted peptides in which the positive charge is not preserved do not seem to be transport substrates in Pseudomonas spp. (Cascieri and Mallette, 1976a; Miller and Becker, 1978), Salmonella typhimurium (Jackson et al., 1976), Streptococcus spp. (Law, 1977, 1978), or Neurospora crassa (Wolfinbarger and Marzluf, 1975a), but in Strep. faecalis (Section 1-3-2-5) and yeasts (Section 1-2-2-5) there is some evidence for the transport of such substrates.

1-1-7-5 C-terminal α-Carboxyl Group

In general, peptide transport systems that handle oligopeptides recognise substrates with modified C-termini, although the unmodified parent peptide is invariably a better substrate. Utilization of C-terminal substituted peptides has been shown in E.coli (Payne, 1973; Becker and Naider, 1974; Fickel and Gilvarg, 1973), Salmonella typhimurium (Ames et al., 1973), Pseudomonas putida (Cascieri and Mallette, 1976a), a range of lactic acid bacteria (Shankman et al., 1960)

and in Sacc. cerevisiae (Section 1-2-2-5). Antibacterial phosphonopeptides, which carry a C-terminal phosphonic acid group, are active against many species (Atherton et al., 1979a; Allen et al., 1979). In contrast, in Candida albicans C-terminal methyl esters are not utilized (Lichliter et al., 1976).

There is some evidence that the dipeptide permease in E.coli has a more rigorous C-terminal requirement than the oligopeptide permeases. Payne and Gilvarg (1968b) found that lysyl cadaverine (essentially Lys-Lys lacking the α-carboxyl group) acted as a lysine source for an auxotroph of E.coli, but that a mutant lacking an oligopeptide permease did not utilize the peptide. However, the phosphonodipeptide alafosfalin was found to enter oligopeptide-permease-deficient E.coli cells (Ringrose and Lloyd, 1979). The presence of a third peptide-permease in E.coli (Section 1-1-5) and the insensitivity of the growth assay used in the former study, do not yet allow firm conclusions to be drawn about the C-terminal requirement of the dipeptide permease.

1-1-7-6 The Peptide Bond

Those systems studied to date seem to have a requirement for an α-peptide linkage, although very few substrates have been studied. Thus, β-Asp-Gly, β-Asp-Ala, γ-Glu-Gly, Ala-ε-Lys and γ-Glu-ε-Lys did not compete for the uptake of the corresponding α-linked peptides in E.coli (Payne, 1972a) although β-Ala-His was utilized (Payne, 1973; see also Kirsh et al., 1978). β-Ala-His did not inhibit Gly-Met utilization in Pseudomonas putida (Cascieri and Mallette, 1976a) and β-Ala-Leu did not compete with Gly-Pro uptake in Salmonella typhimurium (Yang et al., 1977), Strep. lactis or Strep. cremoris

(Law, 1978). However the uptake of β-, γ- or ϵ-linked peptides has not to date been studied using direct uptake assays.

Although on the available evidence, a requirement for the α-peptide bond seems quite strict, peptides containing imino residues (X - Pro, etc) and those in which the peptide bond nitrogen is methylated (e.g. glycyl-sarcosine) are transported by E.coli (Payne, 1972b). The resistance of the latter bonds to peptidase action makes sarcosine-containing peptides suitable for the demonstration of intact peptide accumulation by cells.

1-1-8 Regulation of Peptide Transport

There have been very few studies to date on the regulation of peptide transport. In those organisms studied, peptide transport appears to be constitutive (Payne, 1980b), and regulation of amino acid pool levels during peptide uptake by bacteria appears to be by amino acid exodus (Section 1-1-3). However, lower rates of peptide uptake in E.coli were observed when several amino acids were present in in the medium,although the mechanism of this effect was not established (Payne and Bell, 1977b). In Sacc. cerevisiae, peptide uptake is coregulated with certain amino acid permeases and catabolic enzymes (Section 1-2-4-4).

1-1-9 Peptide Mimetic Antibiotics

Antibiotics generally act by the inhibition of a specific enzyme reaction essential to the metabolism of the target organism. However, many of these enzymes are intracellular, and potential inhibitors are often unable to reach them as they are excluded by the plasmalemma and are not recognised by transport systems. In contrast to many permeases, peptide transport systems have a broad

specificity, particularly with respect to side-chains and the C-terminus (Section 1-1-7), and will transport peptides containing normally impermeant amino acid derivatives (Ames et al., 1973; Fickel and Gilvarg, 1973; Ringrose, 1980). Thus, if a peptide containing a toxic amino acid derivative is taken up and cleaved by a cell, the peptide permease can be exploited as a 'warhead delivery system' for a normally impermeant moiety. The application of this 'smugglin' concept (Matthews and Payne, 1975b) has led to the development of some novel synthetic antimicrobial agents, and some natural antibiotics have since been shown to share this exploitation of peptide permeases.

A comprehensive survey of peptides as antimicrobial agents is given by Ringrose (1980), and only a brief summary is given here. Of the synthetic peptide antibiotics developed over the last few years, the phosphonopeptides (Section 4-4), in which the C-terminal carboxyl group is replaced by phosphonic acid, are perhaps the most promising. Substitutions of the carboxyl group by tetrazole (Ringrose, 1980) or sulphonate groups (Ringrose, 1980) did not yield comparably active compounds. Many peptides containing amino acids with derivatised side chains have also been assayed for antimicrobial activity (Ringrose, 1980).

Many antibiotics are secreted by microorganisms, particularly by Bacillus and Streptomyces spp., and some of these have been demonstrated to exploit peptide permeases in their target organisms. These natural antibiotics include bacilysin (Section 3-4), Fumarylcarboxyamide-L-2,3-diaminopropionyl-L-alanine (Lindenbein) (Ringrose, 1980) phosphinothricyl-alanyl-alanine (Bayer et al.,

1972), and alazopeptin (Patterson et al., 1966) which carries two molecules of 6-diazo-5-oxo-L-norleucine linked to an N-terminal alanine moiety.

Some toxic, linear peptides have been shown to act as the intact molecule. Thus the growth inhibitory effects of glycyl-leucine (Simmonds, 1970) and triornithine (Payne, 1968) against E.coli are attributable to the intact peptide.

In the present studies, toxic peptides are used as biochemical tools for the selection of resistant mutants. For a peptide that acts as a 'delivery' system for a toxic amino acid derivative, such mutants may be:
a) deficient in peptide uptake; b) deficient in peptidase activity; c) possess an altered 'target' for the toxic moiety.

In the majority of studies to date, spontaneously resistant mutants to toxic peptides have been found to be transport-defective (Payne, 1968; Ames et al., 1973; Perry and Abraham, 1979; J.W. Payne, personal communication), although Miller and Schwartz (1978) isolated peptidase-deficient mutants of E.coli K-12 on the basis of resistance to valyl-peptides, and resistant mutants of E.coli altered in the 'target site' of the toxic moiety have been obtained (J.W. Payne, personal communication).

Presumably, if a peptide containing a toxic amino acid enters the cell via a single permease and is cleaved by more than one peptidase, resistant mutants are much more likely to be defective in transport rather than peptidase activity. Conversely, if the peptide enters via two or more permeases, and is cleaved by a single peptidase, peptidase-deficient mutants are likely to be selected.

1-2 Nitrogen Metabolism in Yeasts

1-2-1 Introduction

The yeasts are a group of fungi in which the predominant form is unicellular, and in which asexual reproduction is usually by budding. However, many species display a mycelial or pseudo-mycelial form and there are examples of division by binary fusion.

Perhaps the best known, and certainly the most intensively studied yeasts, are those of the genus Saccharomyces, involved in beer, wine and bread making. However, other yeasts live in a wide range of environments, including soil, the surface of plants, lakes, and seas. Some species (e.g. Candida albicans and Cryptococcus neoformans) are human pathogens. Yeasts can grow on a wide range of nitrogen sources. Wickerham (1946) showed that, in the presence of vitamins and trace elements, many species of yeasts grew on ammonium sulphate as a sole nitrogen source. Amino acids (Section 1-2-4-1), small peptides (Section 1-2-2-1), urea, and purine and pyrimidine bases have also been demonstrated as nitrogen sources in some yeasts (Suomalainen and Oura, 1971). Sacc. cerevisiae was chosen as an organism for study in the present work for a number of reasons: First, whereas the literature on peptide transport in bacteria is extensive, that on eukaryotic microorganisms is more limited (Section 1-1). As Sacc. cerevisiae has been intensively studied in other respects, it was chosen as a 'model' eukaryote. Second, in its commercial use, Sacc. cerevisiae is grown in conditions where the ability to transport small peptides would be an advantage, the predominant nitrogen nutrients in, for example, brewers' malt wort being amino acids and small peptides (Rainbow,

1970). It is reasonable, therefore, to expect Sacc. cerevisiae to possess a transport system for peptides. Third, it is to be hoped that the characteristics of peptide transport in Sacc. cerevisiae are generally similar to those in other yeast species. Thus, a knowledge of peptide transport in Saccharomyces may lay the foundations for the design of specific peptide-mimetic antibiotics (Section 1-1-9) for use against pathogenic yeasts such as Candida albicans.

1-2-2 Peptide Transport in Saccharomyces cerevisiae

1-2-2-1 Introduction

There are numerous examples in the early fermentation literature of the ability of yeasts to use peptides as a source of nitrogen (Suomalainen and Oura, 1971). Nielson (1943) studied the assimilation of nitrogen by Sacc. cerevisiae from a variety of amino acid and peptide sources, though his study did not exclude the possibility of extracellular hydrolysis of peptides. The first systematic studies of peptide transport were those performed by Becker, Naider, and their co-workers in the 1970's.

1-2-2-2 Distinction of Amino Acid and Peptide Transport

Evidence for the separate uptake of amino acids and peptides in Sacc. cerevisiae comes from the results of competition studies. Thus, through competition for a shared permease, phenylalanine can prevent the growth of a leucine auxotroph on free leucine, but growth is not inhibited when Leu-Leu is used as a leucine source (Marder et al., 1977). Similarly, the uptake of Met-Met- [^{14}C]Met, although subject to inhibition by a range of peptides, is relatively insensitive to inhibition by free methionine (Becker and Naider, 1977).

Marder et al., (1978) isolated a mutant of a leucine auxotroph

that grows normally on free leucine, but not on Leu-Leu or Leu-Leu-Leu as a leucine source. Since the mutant possesses peptidase activity against the peptides, this suggests that the transport of the peptides has been lost, whereas transport of free leucine is retained. All the available evidence, therefore, indicates that amino acid and peptide transport are mediated by separate systems.

1-2-2-3 Evidence for a Single Peptide Transport System

When the present work was undertaken, information on the number of peptide transport systems in Sacc. cerevisiae was poor. Becker and Naider (1977) had reported that Met-Met was a poor inhibitor of Met-Met-[^{14}C] Met uptake, leading them to speculate that separate di- and oligopeptide systems might exist. However, it is clear that a system handling a wide range of peptide substrates can also possess a wide range of affinities for those substrates, and the observation that a single dipeptide is a poor inhibitor of Met-Met- [^{14}C] Met uptake is not good evidence for the presence of two transport systems (see Section 1-1-5).

Studies using a Lys/Leu auxotroph of Sacc. cerevisiae (Marder et al., 1977) provided circumstantial evidence for there being a single system for di- and oligopeptides. Thus 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. It appears likely that these lags arose from competition at the transport step. However, the explanation given for the lags observed (Marder et al., 1977), namely that there was no growth until the concentration of the

competing peptide had been significantly depleted, does not seem likely. It can be calculated that, with the small amount of yeast initially present in the incubations, it would take over 10,000 hours for all the competing peptide to be used if the rate of uptake was typical of those found for other peptides (Becker and Naider, 1977; Section 3-5). It seems more likely that the lags result from the need for a minimum internal pool of the essential amino acids before growth can occur.

During the course of the present work, Marder et al., (1978) isolated a mutant of a Leu/Lys auxotroph of Sacc. cerevisiae that is resistant to the toxic peptide L-ethionyl-L-alanine. This mutant has lost the ability of the parent to utilize Leu-Leu and Leu-Leu-Leu as leucine sources, and is unable to accumulate label from [^{14}C] Gly-Leu. The isolation of this mutant is good evidence for a common transport system for di- and tripeptides.

1-2-2-4 Size Limit for Peptide Transport

The available evidence suggests that size limit for peptide transport may vary in different strains. Strain G 1333 utilized $(Met)_5$ and $(Met)_5$ methyl ester as methionine sources (Naider et al., 1974), Strain XJBI-1C did not use $(Met)_5$ and grew poorly on tetrapeptides (Becker and Naider, 1980) and strain Z1 - 2D did not grow on leucine-containing tetrapeptides (Marder et al., 1977).

The size limit for peptide transport could arise as a result of the specificity of the permease or from a 'sieving' effect of the cell wall. Experiments on cell wall porosity in Sacc. cerevisiae give an apparent cut-off point at a molecular weight of about 700 (Scherrer et al., 1974). This could account for the exclusion of

substrates larger than $(Met)_5$ (Naider et al., 1974) but not for the exclusion of tetrapeptides (Marder et al., 1977).

It is interesting to note that the yeast mating factor α is an oligopeptide of 12- or 13- amino acids (Stotzler et al., 1976; Tanaka et al., 1977). Either the receptor for this factor is located on the periphery of the cell or the apparent porosity of the cell wall is larger than suggested by Scherrer et al., (1974).

If uptake of peptides into yeast spheroplasts were to be studied this would presumably resolve the question of whether the size limit is a property of the cell wall or the permease itself.

1-2-2-5 Specificity of Uptake. Influence of Constituent Amino Acids, Stereochemistry, and N- and C-terminal Modifications

The work of Becker, Naider, and co-workers has shown that peptides containing a variety of L-amino acids can be transported by Sacc. cerevisiae. However, the majority of the work was performed using growth tests, and it is not possible to draw definite conclusions about the affinity or rate of uptake of peptides of different sequences by this indirect approach.

Nielsen (1943) described nitrogen assimilation by Sacc. cerevisiae from D-Leu-Gly-Gly and to a low extent from D-Leu-Gly, although assimilation from the respective L-stereoisomers was much greater. Assimilation from Gly-D-Leu was negligible whereas Gly-L-Leu was a good substrate. Studies on a Leu/Lys auxotroph (Marder et al., 1977) showed that neither L-Leu-D-Leu nor D-Leu-L-Leu would act as a leucine source, and although this could result from an inability to cleave these peptides it was also shown that both these peptides and L-Ala-L-Ala-D-Ala and D-Ala-D-Ala-D-Ala were poor inhibitors of uptake

of other peptides. Both L-Met-L-Met-D-Met and L-Met-D-Met-L-Met were somewhat inhibitory of Met-Met- [^{14}C] Met uptake in another strain (Becker and Naider, 1977). Because only two stereoisomers of Met-Met-Met methyl ester (L-L-L and L-L-D) acted as methionine sources in strain G 1333 (Becker and Naider, 1980), the authors concluded that the other stereoisomers were not transported. However, it may be that they are transported, but at a rate insufficient to promote growth. In all these growth tests, however, the extent to which cleavage may be the rate limiting step in growth is not clear. In summary, the generally indirect evidence suggests that the presence of D-residues in a peptide reduces its rate of transport and its competitive ability markedly, and although there is some evidence that D-residues can be 'tolerated' more at the C- than at the N-terminus, the influence of the position of a D-residue in the peptide sequence is not clear.

The effect of derivitization of the peptide N-terminal amino group on transport is also unclear. Sarcosyl-glycine and N-methyl-Leu-Gly were not assimilated, whereas benzoyl-Gly-Gly was (Nielsen, 1943). Dunn and Dittmer (1951) showed that benzyloxy-carbonyl-derivatives of peptides were utilized less efficiently than their 'parent' peptides. In more recent studies, some peptides with either acetyl- or tert-butyloxycarbonyl groups on the N-terminus were utilized in one strain (Naider et al., 1974), N-acetyl-Met-Met-Met was transported in another (Becker and Naider, 1977), but N-acetyl peptides were not utilized in a third (Marder et al., 1977). Although the intervention of peptidases and N-acylases in the growth responses is a complicating factor, this evidence may be interpreted to indicate that substitution of the N-terminal-α-amino group of a

peptide lowers its affinity for, and/or its rate of,transport but that there is not an absolute requirement for the group to be unsubstituted.

Esterification of the C-terminal-α-carboxyl groups of a number of peptides did not affect their utilization in Sacc. cerevisiae (Naider et al., 1974; Marder et al., 1977) indicating that a free carboxyl-terminus is not necessary for transport.

1-2-2-6 Peptide Transport in Candida albicans

The only other yeast in which peptide transport has been reported is Candida albicans. Peptide transport is distinct from amino acid transport (Logan et al., 1979) but the number of systems present is not clear. Methionine-containing peptides up to the size of pentamethione and incorporating a range of other amino acids supported growth of a Met/Lys auxotroph (Lichliter et al., 1976). N-acylation of peptides did not prevent their utilization, but C-terminal methyl esters were not utilized (Lichliter et al., 1976). This apparent requirement for an underivatized C-terminus is paralleled by the affinity for transport of peptides containing D-residues: whereas L-Met-L-Met-D-Met has virtually no effect on L-Met-L-Met-L-Met uptake, D-Met-L-Met-L-Met is a good inhibitor (Logan et al., 1979).

It seems that the characteristics of peptide transport are broadly similar in Candida albicans and Sacc. cerevisiae. The rates of peptide uptake are of the same order in both species, and both display quicker uptake when grown on a poor nitrogen source (Logan et al., 1979, Becker and Naider, 1977). The effects of N- and C-terminal derivatization seem, however, to be different in the two organisms.

1-2-3 Peptidases in Saccharomyces cerevisiae

1-2-3-1 Introduction

A wide range of functions for the proteases of Sacc. cerevisiae has been reported. These functions include: the interconversion of biologically active and inactive protein; protein maturation; protein turnover during growth, starvation and sporulation; and the utilization of small peptides as amino acid and nitrogen sources. The discussion here is limited to the last role. For a recent review of yeast proteolysis in general, see Wolf and Holzer (1980).

1-2-3-2 The Peptidases and their Specificities

The intracellular peptidases that have been characterised to date in Sacc. cerevisiae are: two endopeptidases, proteases A and B; two carboxypeptidases, Y and S; and at least four aminopeptidases (Wolf and Holzer, 1980).

No cleavage of small peptides by protease A has been detected (Hata et al., 1967). Protease B failed to hydrolyse a range of benzyloxycarbonyl dipeptides (Lenney and Dalbec, 1967), although it did cleave some amino acid p-nitrophenyl esters (Ulane and Cabib, 1976).

Carboxypeptidase Y has the ability to remove most amino acid residues, including proline, from the C-termini of peptides. Studies on a range of N-terminal blocked dipeptides (Hayashi, 1976) have shown that it has the following specificity: an absolute requirement for an L- amino acid at the C-terminus; slow release of C-terminal histidine, arginine and lysine; good cleavage of peptides with aromatic or aliphatic side chain in the penultimate

or terminal position, whereas glycine in these positions results in poor cleavage. Despite the cleavage of N-terminal blocked dipeptides, activity against free dipeptides is negligible (Hayashi, 1976). Carboxypeptidase S was detected in mutants lacking carboxypeptidase Y activity, and seems to have a narrower substrate specificity (Wolf and Weiser, 1977). Activities of both carboxypeptidases increase several-fold when cells are grown on N-Cbz-Gly-Leu as a sole nitrogen source (Wolf and Ehmann, 1978; Wolf et al., 1979). A mutant auxotrophic for leucine, and lacking both carboxypeptidases was unable to grow on N-Cbz-Gly-Leu as a leucine source (Wolf et al., 1979). At least one carboxypeptidase is thus implicated in the utilization of small peptides.

The number and specificity of aminopeptidases is less clear. Matile and co-workers (Matile and Wiemken, 1967; Matile et al., 1971) reported four aminopeptidases, as did Rose et al., (1979). Masuda et al., (1975) isolated two aminopeptidases, both of which cleaved dipeptides. Another enzyme active against a number of peptides with free N-termini, but not those containing N-terminal lysine (Wolf and Holzer, 1980) has been isolated (Metz et al., 1974; Metz and Rohm, 1976). Rohm and co-workers also isolated a second aminopeptidase (Wolf and Holzer, 1980) and a specific dipeptidase (Rohm, 1974) which they claimed were identical with the aminopeptidases of Masuda et al. Besides the presence of four aminopeptidases, Rose et al., (1979) found a specific dipeptidase. The activity of the different enzymes towards a number of free peptides was assayed. The dipeptidase was found not to cleave X-Pro peptides or those containing D- amino acids, but cleaved all

other dipeptides tested.

1-2-3-3 Subcellular Localisation

There is a large volume of evidence that proteases A & B and carboxypeptidase Y are located in the vacuole of Sacc. cerevisiae (Matile and Wiemken, 1967; Lenney et al., 1974; Matern et al., 1974), although Cabib et al., (1973) were unable to locate protease A in the vacuolar fraction. Carboxypeptidase S seems to be associated with a membrane fraction in lysed cells (Wolf and Holzer, 1980). Of the aminopeptidases, one is localised in the vacuole (Matile et al., 1971), whereas the dipeptidase and one of the aminopeptidase activities isolated by Frey and Rohm are localised in the cytosol (Wolf and Holzer, 1980).

There is some evidence for the presence of extracellular peptidase activity. One of the aminopeptidase activities detected by Matile et al., (1971) was lost upon production of spheroplasts, indicating that it may be associated with the cell wall or the outside of the plasmalemma. The presence of an extracellular aminopeptidase was confirmed by Frey and Rohm (1979). Recent studies on the action of the yeast α-mating factor on type a cells (Stotzler et al., 1977) have provided evidence for an extracellular peptidase capable of cleaving Trp from Trp-Leu-Gln-Leu. However, this conclusion is based on the ability of a Trp/Leu auxotroph to grow on the peptide as a Trp source, but not as a Leu source, even though the peptide is totally cleaved by a cell extract. However, this conclusion is not rigorous, for if the leucine requirement of the cells is considerably higher than the tryptophan requirement, these results could be explained by slow transport and solely intracellular cleavage of the

peptide. In another study, Tanaka and Kita (1977) showed that cells of mating type α will cleave α-factor between the 6th and 7th amino acid residues.

Despite these reports of extracellular peptidase activity, no extracellular hydrolysis of peptides used in transport studies has been detected either in whole cells or in supernatant solutions after osmotic shock (Becker et al., 1973). Furthermore, the lack of competition for uptake between peptides and amino acids is strong circumstantial evidence that extracellular cleavage does not play a role in small peptide uptake. It seems likely that any extracellular peptidase activity is highly specific, and may be related to mating factor responses.

It is clear that there are peptidases capable of cleaving small peptides in both the cytoplasm and the vacuole, and so their location does not indicate the likely site of hydrolysis following peptide transport.

1-2-4 Amino acid Transport and Metabolism in Saccharomyces cerevisiae

1-2-4-1 Introduction

Amino acids have long been known to act as nitrogen sources for yeast. Thorne (1941) found that most amino acids, except histidine, glycine, cysteine and lysine, supported the growth of brewers' yeast. More recent work on Sacc. cerevisiae (Watson, 1976) confirms these conclusions, glycine being a very poor nitrogen source, and histidine, cysteine and lysine not supporting growth.

Studies of the uptake and subsequent metabolism of amino acids are clearly relevant to work on peptide transport, and hence a brief survey of these topics is included.

1-2-4-2 Amino acid Permeases

Early studies on the transport of amino acids into Sacc. cerevisiae indicated that the permease(s) present had a broad specificity (Surdin et al., 1965). Work by Grenson and her co-workers on Sacc. cerevisiae strain Σ1278b (used in the present study) has shown the presence of one general amino acid permease, and several more specific systems. The general amino acid permease transports a wide range of L-amino acids (but not proline) (Grenson et al., 1970), and also D-amino acids (Rytka, 1975). The activity of the general permease is very low when cells are grown in the presence of ammonia. This feature, together with the isolation of transport-deficient mutants, has allowed the study of other specific systems. These include systems for L-arginine (Grenson et al., 1966), L-lysine (Grenson, 1966), two systems for L-methionine (Gits and Grenson, 1967), two for L-histidine (Crabeel and Grenson, 1970) and two for L-glutamic acid (Joiris and Grenson, 1969; Darte and Grenson, 1975). A proline permease has been characterised in Sacc. chevalieri (Magana-Schwencke and Schwencke, 1969). Table 1-1 summarises the characteristics of these permeases.

1-2-4-3 Metabolism of Amino Acids

When different amino acids are supplied as nitrogen sources, the pool levels of all amino acids vary considerably (Watson, 1976). This arises partly from the influence of the supplied amino acid on closely related amino acid biosynthetic pathways, but also reflects the efficiency with which the α-amino nitrogen can be utilized for general synthesis. Transamination reactions are important in the utilization of many amino acids as nitrogen sources. The first step in the utilization of tyrosine was shown to be a transamination with α-

Table 1-1 Properties of the Amino Acid Permeases of *Sacc. cerevisiae*

Permease	Substrate	Affinity	Rate (nmol min^{-1} $mg\ protein^{-1}$ from 0.1 mM solution)
gap	neutral and basic a-a's eg		
	Arg	7.6×10^{-6}	
	Lys	3.1×10^{-6}	
	Trp	1×10^{-5}	
	His		79
	Ser		98
	Ala		58
	Met		54
	D-amino acids		
arg P_1	Arg	10^{-5}	20
	Lys	2×10^{-4}	6
lys P_1	Lys	2.5×10^{-5}	6
met P_1	Met	1.2×10^{-5}	13
met P_2	Met	7.7×10^{-4}	2
	Thr		
	Ser		
his P_1	His	10^{-5}	
his P_2	His	10^{-3}	
	Tyr		
Dicarboxylic amino acids		$\approx 10^{-5}$	
Proline (*Sacc. chevalieri*)		2.5×10^{-5}	

Data are derived from the references in Section 1-2-4-2

ketoglutarate (Sentheshanmuganathan and Elsden, 1958) and a cell free extract of Sacc. cerevisiae was shown to catalyse the transfer of amino groups from Asp, Leu, Nle, Ile, Val, Nva, Met, Phe, Tyr, and Trp to α- ketoglutarate forming glutamic acid and a keto-acid (Sentheshanmuganathan, 1960). The results of Woodward and Cirillo (1977) essentially confirm these findings. They found significant transaminase activity against Asp, Glu, Leu, Ile, Val, Met, Phe, and Tyr with α-ketoglutarate as acceptor, and against Ala with pyruvate as acceptor.

The metabolism of arginine, ornithine, citrulline and proline is closely linked with that of glutamic acid (Jauniaux et al., 1978). Thus it can be seen that those amino acids that act as nitrogen sources can be metabolised so that their α-amino nitrogen is in the form of glutamic acid, a substance central in the nitrogen metabolism of yeast. When an amino acid is provided as a sole nitrogen source, large amounts of α-keto-acid may be formed by transamination. This α-keto-acid is decarboxylated to form the corresponding aldehyde, which is then usually reduced to form a fusel alcohol (although the aldehyde formed after transamination and decarboxylation of glutamic acid is oxidised to form succinic acid) (Rainbow, 1970). Fusel alcohols have long been known as products of yeast growth on amino acid sources, and rapid exodus of such compounds has been demonstrated during amino acid transport (Woodward and Cirillo, 1977). The decarboxylation reaction and subsequent exodus of the fusel alcohol clearly has relevance to the use of radiolabelled substrates for the study of amino acid and peptide transport (see Section 2-7).

1-2-4-4 Regulation of Amino Acid Transport and Metabolism

Amino acids accumulated by Sacc. cerevisiae are not freely exchangable with those in the medium, in marked contrast to some bacterial amino acid permeases (e.g. Quay et al., 1977; Section 1-3-4-4).

Thus, cells preloaded with labelled histidine do not lose label when incubated in a medium containing unlabelled histidine (Grenson, 1973). In cells preloaded with unlabelled histidine (Grenson, 1973), valine, arginine, lysine, or α-amino isobutyric acid (Woodward and Cirillo, 1977) uptake of the corresponding labelled amino acid is severely inhibited. This process of transinhibition could lead to the apparent inhibition of amino acid uptake by peptides, amino acids released by intracellular hydrolysis transinhibiting uptake of free amino acids.

When Sacc. cerevisiae is grown on a poor nitrogen source such as proline, all the amino acid transport systems are fully active. When cells are grown on ammonia, however, the activity of the general amino acid permease is severely inhibited (Grenson et al., 1970). This ammonia inhibition has also been shown for peptide transport (Becker and Naider, 1977) transaminases (Woodward and Cirillo, 1977), the synthesis of a number of catabolic enzymes (Dubois et al., 1973) and, in Sacc. chevalieri, for the proline permease (Schwencke and Magana-Schwencke, 1969). It seems clear that the role of the ammonia inhibition is to avoid energetically expensive processes involved in the use of amino acids as nitrogen sources, when a better source is freely available.

It has been shown that the ammonia inhibition of the general

amino acid permease (Grenson and Hou, 1972) and of the synthesis of arginase, urea amidolyase and allantoinase (Dubois et al., 1973) is released in mutants lacking a functional NADP-glutamate dehydrogenase (NADP-GDH). It seems likely that the NADP-GDH in Sacc. cerevisiae acts in a similar regulatory fashion to that proposed for the enzyme in Aspergillus nidulans (Pateman and 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.

1-2-4-5 The Role of the Vacuole in Amino Acid Metabolism

Wiemken and Durr (1974) showed that about 60% of the total amino acid pool of Sacc. cerevisiae was localised in the vacuolar fraction. Basic amino acids were present in a higher proportion in the vacuole than in the cytoplasm, and did not exchange freely between the two pools. Other amino acids were more evenly distributed, and exchange was freer. There was relatively little glutamate in the vacuolar pool. High concentrations of allantoin have also been reported as sequestered in a compartment other than the cytoplasm (Zacharski and Cooper, 1978).

Boller et al., (1975) demonstrated the presence of a specific arginine permease in isolated vacuoles. This permease allowed the vacuoles to accumulate arginine, which is clearly compatible with the high vacuolar arginine pools found previously (Wiemken and Durr, 1974).

The available evidence indicates that the vacuole acts as a

store of nitrogen metabolites. Basic amino acids are suitable storage compounds, as their metabolism is closely linked to that of glutamate (Jauniaux et al., 1978).

1-2-5 The γ-Glutamyl Cycle - Its Possible Involvement in Transport of Peptides and Amino Acids

The γ-glutamyl cycle is a metabolic pathway that degrades and synthesises glutathione (γ-glutamyl-cysteinyl-glycine). There is a great deal of evidence that the cycle operates in a range of mammalian tissues, and it has been proposed that one of the enzymes of the cycle, the membrane-bound γ-glutamyl transpeptidase, may mediate the translocation of amino acids across the cell membrane. (For a review of the γ-glutamyl cycle in mammalian systems, see Meister and Tate, 1976).

γ-Glutamyl transpeptidase catalyses the conversion of glutathione and an amino acid to the γ-glutamyl amino acid and cysteinyl-glycine. It was first isolated from E.coli by Samuels, (1953) and has since been found in a range of microorganisms. However, only in Sacc. cerevisiae have all the enzymes of the cycle been found (Mooz and Wigglesworth, 1976). Transpeptidase extracts were shown to have increased activity in the presence of Met, Ala, Glu, Pro, Cys, Glu , Gly and Gly-Gly (Mooz and Wigglesworth, 1976).

The evidence for the involvement of the γ-glutamyl cycle in amino acid or peptide transport in yeast is not good (Meister, 1980). There is a report (Osuji, 1979) of increased glutathione turnover during amino acid uptake in Candida utilis, but other workers were unable to reproduce Osuji's results (Robins and Davies, 1980) and concluded that the γ-glutamyl cycle could account for only a few

per cent of the amino acid transport observed. The transpeptidase level in Sacc. cerevisiae was shown to be unaltered in a mutant lacking the general amino acid permease (Penninckx et al., 1980) although an apf mutant (which has low activities of all amino acid permeases) had a low transpeptidase activity. As the transpeptidase was also subject to ammonia repression, (Penninckx et al., 1980) it seems likely that the transpeptidase is controlled by the same regulatory system as the amino acid permeases (Section 1-2-4-4). Despite this co-regulation, the available evidence indicates that the γ-glutamyl cycle does not play a significant role in amino acid uptake. In particular, the results of Eddy and co-workers (Section 1-4-3-2) on the energy coupling of amino acid transport are hard to reconcile with the action of γ-glutamyl transpeptidase.

1-3 Nitrogen Metabolism in Streptococcus faecalis

1-3-1 Introduction

The family Streptococcaceae contains the genera Streptococcus, Leuconostoc, Pediococcus, Aerococcus and Gemella (Deibel and Seeley, 1974). These Gram-positive cocci are generally fermentative and facultatively anaerobic. The genus Streptococcus contains species that grow in diverse environments: Strep. pyogenes and Strep. pneumoniae are respiratory tract pathogens, Strep. sanguis is found in dental plaque, Strep. lactis and Strep. cremoris are used as cheese starters, and Strep. faecalis is a gut bacterium.

Strep. faecalis has variously been assigned to the 'group D streptococci', 'enterococci' and 'faecal streptococci'; the relationships between these terms are shown in Fig. 1-2. Group D is one of the serological groups of Lancefield (1933), who

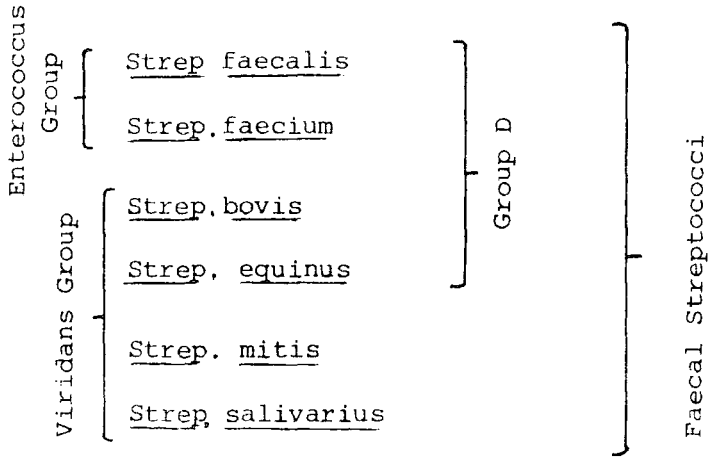


Fig 1-2 Definition of the terms "enterococci", "group D streptococci", and "faecal streptococci" (adapted from Hartman et al., 1966).

classified the streptococci according to their cell wall antigens. However, later classification has been based on other factors and so the 'Lancefield Group D' streptococci include members of the enterococcus and viridans taxonomic groups. These two groups are often termed the 'faecal streptococci' because of their usual environment.

Strep. faecalis was chosen for study here for several reasons: its peptide transport systems have not previously been characterised; the energetics of transport of other substrates have been extensively studied by Harold and co-workers (Section 1-4-3-3) making it a suitable organism for studies on the energy coupling of peptide transport; it is a causative agent of bacterial endocarditis and urinary tract infections, and is refractive to many commonly used antibiotics, making it an organism worthy of study with a view to developing effective 'smugglin' antibiotics (Section 1-1-9).

It is clear (Table 1-2) that Strep. faecalis and Strep. faecium are closely related; in fact Strep. faecium was recognised as a synonym of Strep. faecalis in the 6th Edition of Bergey's Manual of Determinative Bacteriology (Breed et al., 1948). However, there is much evidence (reviewed in Hartman et al., 1966) that the two species have separate characteristics, and they are recognised as such in the eighth edition of Bergey's Manual (Deibel and Seeley, 1974). Some strains, including one of those used here, ATCC 9790, were originally isolated as Strep. faecalis and are now considered to be Strep. faecium. Strain ATCC 9790, however, will use arginine as an energy source (Bakker and Harold, 1980), one of the features characteristic of Strep. faecalis but not of Strep. faecium (Diebel

and Seeley, 1974). This strain will thus be referred to as Strep. faecalis (faecium) after Asghar et al., (1973).

In this section, the relevant literature on Strep. faecalis and Strep. faecium is surveyed. Where relevant, references to work on other streptococci are included, but in these cases, no attempt at an exhaustive survey has been made.

1-3-2 Peptide Transport in Strep. faecalis

1-3-2-1 Introduction

The earliest indication that peptide transport occurs in streptococci came from the search for the apparent peptide growth factor 'strepogenin'. Whilst most of the work in this field was carried out using Lactobacillus casei there were early reports that such a factor stimulated the growth of Strep. lactis (Smith, 1943) and a haemolytic streptococcus (Sprince and Wooley, 1944). It later became clear that 'strepogenin' did not exist as such, but that the effects observed reflected the superiority of peptides as amino acid sources in these organisms (for a review of the work on 'strepogenin' see Matthews and Payne, 1975a). The knowledge that fastidious streptococci grow well on media containing peptone is also indicative of peptide transport.

Despite these early indications that peptide transport is present in streptococci, there have been few studies of peptide transport as such, and most of these have only yielded inconclusive data. However, the available information is summarised below.

1-3-2-2 Distinction of Amino Acid and Peptide Transport

The report of Kihara et al., (1952) that under some incubation

conditions Leu-Tyr and Gly-Tyr are better tyrosine sources than the free amino acid was the first indication that amino acid and peptide transport may be separate in Strep. faecalis. Subsequently, (Kihara et al., 1961) the same was shown for D-Ala-L-Ala and D-Ala-Gly as D-alanine sources. The absence of inhibition of [^{14}C] Gly-Gly uptake by free alanine or glycine in strains of Strep. faecium and Strep. faecalis (Brock and Wooley, 1964) confirmed that separate transport systems exist.

1-3-2-3 The Number of Peptide Transport Systems

There is no literature evidence indicating how many peptide transport systems are present in Strep. faecalis.

1-3-2-4 Size Limit for Peptide Transport

Literature information on the size limit for peptide transport is ambiguous, as in most cases the possibility of extracellular cleavage was not excluded. Krehl and Fruton (1948) reported utilization of di-, tri- and tetrapeptides containing leucine, and Shankman et al., (1960) demonstrated utilization of di-, tri- and tetraleucine. The latter authors argued that, as N-phthalyl derivatives are less efficient leucine sources than the unsubstituted peptides, extracellular cleavage is not important; if extracellular cleavage were rate limiting, however, and N -phthalyl derivatives were more slowly cleaved than their parent peptides, the observed effect would also result. Studies on the antibacterial effect of phosphonopeptides (Atherton et al., 1979a) against Strep. faecalis showed activity of di- to hexapeptides, with a peak at the tripeptide.

1-3-2-5 Specificity of Uptake. Influence of Constituent Amino Acids, Stereospecificity and N- and C-terminal Modifications

Although no systematic studies have been performed, peptides containing a range of different amino acids have been shown to be transported or utilized by Strep. faecalis. These include: Leu, Gly and Tyr (Krehl and Fruton, 1948; Kihara et al., 1952); Leu and Val (Shankman et al., 1960); Ala and Gly (Kihara et al., 1961); Gly (Brock and Wooley, 1964); Ala, Met, Phe, Val, Lys and Pro (Atherton et al., 1979a).

In studies into the stereochemical requirements for the utilization of homo-valine peptides (Shankman et al., 1960), the L-D dipeptide was shown to be essentially inactive, whereas the tripeptides studied ranked LLL > LLD > DLL. The activity of Ala-Ala-aminoethylphosphonic acid antibiotics (Atherton et al., 1979a) similarly showed the ranking LLL > LLD > DLL, although in both these studies the possibility that hydrolysis of the peptide may be the limiting factor was not investigated.. This feature is illustrated by the report (Kihara et al., 1961) that both D-Ala-L-Ala and L-Ala-D-Ala were taken up by Strep. faecalis, but only the former was cleaved.

Information about the effect of N- and C-terminal substitutions on uptake of peptides is sparse. N-phthalyl derivatives of di- to tetrapeptides were utilised slowly by Strep. faecalis, as were C-terminal methyl esters, but peptides blocked at both termini were essentially inactive (Shankman et al., 1960).

1-3-2-6 Peptide Transport in Other Streptococci

Most other studies of peptide transport in streptococci have

been carried out using those group N organisms used in the dairy industry. Law et al., (1976) demonstrated that some commercial cheese starters grew better on media supplemented with soy peptones (average 3-4 amino acids) than with papain digested skimmed milk (6-7 residues). Studies of peptide uptake are complicated by the secretion of dipeptidases by Strep. lactis and Strep. cremoris (Law, 1977); however, uptake of Gly-[^{14}C]Leu was shown to be insensitive to inhibition by those amino acids and the few tripeptides tested, but sensitive to dipeptides (Law, 1978), providing evidence for the transport of dipeptides as such. The ability to transport Gly-[^{14}C] Leu varied markedly from strain to strain. A systematic study of the uptake of several [^{14}C] leucine containing peptides by Strep. lactis (Rice et al., 1978) indicated that di- to penta-peptides are transported, but the authors' conclusion that separate uptake systems for di- and oligopeptides exist is not the only possible interpretation of the data. Whereas it seems likely that more than one system is present, mutual competition between di- and oligopeptides was detected, and the long incubation times (15 min) used for labelled peptide uptake mean that amino acid exodus (Section 1-1-3) is a possible complicating factor.

Apart from the above investigations on group N streptococci, studies on the action of phosphonopeptides have shown that peptide transport occurs in a range of other members of the genus (W.J. Lloyd, personal communication).

1-3-3 Peptidases in Strep. faecalis

Although no peptidases from Strep. faecalis have been characterised, it is clear from the action of peptides as amino acid

sources (Section 1-3-2) that there is considerable peptidase activity present. The reports of Kihara *et al.*, (1952; 1961) that whole cells of *Strep. faecalis* display peptidase activity can be interpreted as most likely being due to the release of intracellular enzymes by autolysis (as post-exponential cells were used in the peptidase assay) and not to the presence of secreted extracellular peptidases.

In contrast, other streptococci do possess extracellular enzymes that cleave peptide bonds. Secretion of proteases has been reported for a Group A streptococcus (Liu and Elliot, 1971) and for *Strep. lactis* (Williamson *et al.*, 1964); there are cell-wall-bound proteases and peptidases in *Strep. cremoris* (Exterkate, 1975, 1976) and a surface-bound peptidase in *Strep. lactis* (Law, 1977). The production of extracellular proteases and peptidases by these organisms can be understood in relation to their environment. *Strep. cremoris* and *Strep. lactis* are used as cheese starters, and so grow in an environment where proteins and large oligopeptides are abundantly available as amino acid sources. In contrast, the gut environment of *Strep. faecalis* is not rich in such compounds. It therefore seems sensible that *Strep. faecalis* does not produce such extracellular proteases. However, in the absence of firm evidence, the possibility of extracellular cleavage is carefully considered in the present studies.

1-3-4 Amino Acid Transport and Metabolism in *Strep. faecalis*

1-3-4-1 Introduction

Strep. faecalis is unable to synthesise some 7 to 13 amino acids (Diebel and Seeley, 1974) and so needs an exogenous supply.

It has thus long been used as an organism for the bioassay of amino acids, e.g. Arg, His, Ile, Leu, Lys, Met, Thr, Trp and Val (Stokes et al., 1945). The utilization of exogenous amino acids is consistent with the presence of amino acid transport systems.

1-3-4-2 Amino Acid Transport Systems

There are several distinct amino acid transport systems in Strep. faecalis. A general system was first demonstrated by Brock and Moo-Penn (1962) who found that [^{14}C]- glycine uptake is inhibited by Ala, Ser, Thr, Val, and Cys. Asparagine transport is inhibited by (in decreasing order) Ala, Ser, Cys, Met, Gly, Trp and Thr, but not by Lys or Pro (Holden and Bunch, 1973). Ala, Ser, Gly and Thr undergo reciprocal exchange across the membrane, and a mutant defective in their uptake has been isolated (Asghar et al., 1973).

Apart from the general system, a specific uptake system for lysine (Friede et al., 1972) and high and low affinity systems for dicarboxylic amino acids (Reid et al., 1970; Utech et al., 1970) have been characterised.

1-3-4-3 Amino Acid Metabolism

As Strep. faecalis is unable to synthesise many amino acids, it is evident that a single amino acid cannot act as a nitrogen source. However, arginine and serine can act as energy sources (Diebel and Seeley, 1974). Apart from these two, most amino acids undergo very little if any metabolism subsequent to uptake. Gly and Thr were found to accumulate as such after amino acid uptake (Asghar et al., 1973) and His, Ile, Leu, Lys, Thr and Val were used for protein synthesis without significant degradation to other amino acids or breakdown for energy metabolism (Toennies and Shockman, 1953). Ala (Brock and

Moo-Penn, 1962; Asghar et al., 1973) and Glu (Reid et al., 1970) were found to be metabolised to some extent, though this may reflect incorporation into cell wall material (Toennies and Shockman, 1959). The general metabolic inactivity of amino acids in Strep. faecalis, and in particular the comparative lack of decarboxylation reactions, means that radiotracer assays of amino acid and peptide uptake are likely to be less suceptible to error than in some other organisms (see Sections 2-6, 2-7). There is a report, however, that Tyr undergoes decarboxylation under some conditions (Kihara et al., 1961).

1-3-4-4 Regulation of Amino Acid Uptake and Pool Levels

Amino acids such as Gly, Ala, Ser (Brock and Moo-Penn, 1962; Asghar et al., 1973) and Thr (Asghar et al., 1973) undergo reciprocal exchange across the membrane. It seems likely that this is true for all amino acids that use the general uptake system but it is not the case for Asp (Harold and Spitz, 1975), which presumably reflects the different mode of energy coupling for the uptake of this amino acid (Section 1-4-3-3).

Because of the exchange of amino acids between the medium and the cell, those which have been accumulated as a result of peptide uptake and hydrolysis may be exchanged for others in the medium; thus, Brock and Wooley (1964) found that Gly-Gly accelerated the uptake of [^{14}C] Gly in Strep. faecalis. In this way, amino acids whose cellular pool levels are low may be accumulated at the expense of exodus of another amino acid which is in excess as a result of peptide uptake; this may both maintain the pool levels of many amino acids and be energetically favourable.

1-4 The Coupling of Energy to Solute Transport in Microorganisms

1-4-1 Introduction - The Proton Motive Force

Those microorganisms studied to date maintain a potential, inside negative, across the cytoplasmic membrane. This potential is generated as a result of the pumping of ions across the membrane, the primary flux being of protons. Oxidative and photosynthetic electron transport chains, and proton-translocating ATP-ases are asymmetrically arranged across the membrane, and effectively pump protons from the interior to the exterior of the cell. The electrochemical gradient generated is termed the proton-motive force (p.m.f.), and is considered as the sum of the membrane potential and the pH gradient. (At high external pH values (>8), the pH gradient may be negligible, and so the p.m.f. becomes equivalent to the membrane potential).

1-4-2 Modes of Solute Uptake

Perhaps the simplest mode of solute uptake is diffusion through the lipid membrane. The low lipid solubilities of most polar nutrients, however, mean that such a mode of uptake would be nutritionally inadequate in most circumstances, and generally slower than experimentally observed rates; this mode can therefore be discounted under most normal and experimental circumstances. The majority of nutritional substrates enter and leave the cell via specific transport systems and their uptake can be classified into three types: facilitated diffusion; group translocation; active transport.

In facilitated diffusion the substrate moves down its electrochemical gradient. For a neutral substrate this is equivalent to its concentration gradient, but for charged substrates the membrane

potential also influences the point of equilibrium. Such transport requires no input of energy and, if the substrate is metabolised intracellularly, it is 'pulled' by the metabolism. Facilitated diffusion has been identified as the mode of uptake of glycerol in E.coli (Lin, 1970) and some sugars in Sacc. cerevisiae (Section 1-4-3-2).

Group translocations involve a chemical modification of the substrate during translocation. Examples are the phosphoenolpyruvate: hexose phosphotransferase systems of a range of bacteria, fatty acid transport in E.coli, and systems for the uptake of purines, pyrimidines, and nucleosides in E.coli (Hays, 1978).

The majority of solute uptake systems are active, that is, they involve the transport of a substrate against its electrochemical gradient. The energy required for this can be derived from a coupled exothermic chemical reaction , or from the linked transport of another solute down its electrochemical gradient. The modes of energy coupling to active transport are discussed in the next section.

1-4-3 Active Transport - Modes of Energy Coupling

1-4-3-1 Energy Coupling in E.coli

The most intensively studied organism in relation to energy coupling to transport is E.coli, and the discussion here will be limited to this organism. Energy coupling in Sacc. cerevisiae and Strep. faecalis is discussed in the succeeding sections.

E.coli produces a p.m.f. as a result of oxidative respiration, the electron transport chain in the membrane acting to extrude

protons. The energy thus stored can be converted to ATP by the membrane ATP-ase. Thus, under aerobic conditions, ATP from the metabolism of glucose can be of glycolytic or respiratory origin, and under anaerobic conditions glycolytically derived ATP can be used to maintain the p.m.f.

Active accumulation of solutes could clearly be powered directly by the cleavage of a 'high energy' compound such as ATP. The experiments of Berger (1973) implicated ATP, rather than the p.m.f., in the active transport of glutamine: in energy-starved cells of a membrane ATP-ase mutant of E.coli, which is unable to use p.m.f. for ATP production, glucose was much more effective than the electron-transport chain substrate, lactate, in stimulating glutamine uptake. Subsequently, other transport systems in E.coli, e.g. methionine, leucine and ribose, were shown to have a similar dependence on phosphate bond energy (Booth and Hamilton, 1980), although there is recent evidence that the energy donor is acetyl phosphate, rather than ATP (Hong et al., 1979).

Uptake systems linked to phosphate bond energy are characterised by being sensitive to arsenate, which drastically reduces ATP levels in E.coli (Klein and Boyer , 1972), and to osmotic-shock treatment. The sensitivity to osmotic shock can be ascribed to the requirement for periplasmic binding proteins for this class of transport system (Wilson and Smith, 1978).

The powering of active transport by the linked transport of another solute can operate by the movement of the second solute in the same direction (symport) or in the opposite direction (antiport). The most obvious candidate for symport is the proton,

as the proton-gradient is the primary gradient produced by respiration. Proton symport was first demonstrated in E.coli for lactose transport (West, 1970) and has since been demonstrated for many other substrates, including proline, glycine, gluconate and glucose-6-phosphate (Rosen and Kashket, 1978).

E.coli also maintains a gradient of sodium ions which is generated by a proton antiport mechanism. The transport of glutamate (MacDonald et al., 1977) and melibose (Tsuchiya et al., 1977) has been shown to be via sodium symport. For a discussion of the relative advantages of proton and sodium symport, see Booth and Hamilton (1980).

1-4-3-2 Energy Coupling in Sacc. cerevisiae

Yeast cells are known to maintain a marked pH gradient across the plasmalemma during energy metabolism. Studies on the distribution of the weak acid propionate (Ryan and Ryan, 1972; Seaston et al., 1976), and ^{31}P nuclear magnetic resonance studies (Salhany et al., 1975) have shown the intracellular pH to be near 6 in a medium of pH 4.5.

In many prokaryotic cells, such as E.coli, the proton pumps of the cytoplasmic membrane are associated with redox reactions. However, in eukaryotic cells the weight of evidence suggests that the proton pumps in the plasmalemma are generally ATP-ases (Raven and Smith, 1977). It seems that the proton gradient in yeast is maintained by an electrogenic ATP-ase (Serrano, 1980) similar to that found in Neurospora (Bowman and Slayman, 1977). Thus, whether transport is energy-linked via the proton gradient or by direct hydrolysis of ATP, the energy is derived from ATP

generated by glycolysis or by respiration in the mitochondria.

Eddy and co-workers (reviewed in Eddy, 1980) have shown that the transport of amino acids in Saccharomyces species is apparently driven by the proton gradient. Thus in cells of Saccharomyces carlsbergensis in which the ATP levels had been drastically reduced by pre-incubation with 2-deoxyglucose and antimycin, there was still significant uptake of glycine (Eddy et al., 1970). Studies with ion-sensitive electrodes showed that the uptake of Gly or Phe under these conditions was accompanied by the uptake of 2 equivalents of protons, and the extrusion of 2 equivalents of K^+ (Eddy and Nowacki, 1971). The flux of protons is interpreted as the driving force, and the K^+ flux as a method of preserving electroneutrality in the absence of plasmalemma proton pump activity. Studies on amino acid transport mutants of Sacc. cerevisiae showed different proton stoichiometries for the general and specific amino acid permeases (Seaston et al., 1973).

The uptake of cytosine, uracil, and uridine is known to be mediated by three separate systems in Sacc. cerevisiae (Losson et al., 1978). Studies on the effect of inhibitors on these three systems (Losson et al., 1978) showed that all three were sensitive to uncoupling agents, whereas the specific ATP-ase inhibitors Dio 9 and chlorhexidine (1,6-di-4'-chlorophenyldiguanidohexane) inhibited cytosine and uridine uptake severely, but did not affect uracil uptake. It appears, therefore, that cytosine and uridine uptake is energised via the proton gradient, whereas uracil uptake is coupled in another fashion. Reichert et al., (1975) proposed a model for

purine transport in which the uptake was mediated by a proton symporter/K^+ antiporter. Reichert and Foret (1977) showed a 1:1:1 substrate : H^+ : K^+ ratio for the transport of hypoxanthine, which uses the cytosine permease. However, there is no evidence that the K^+ fluxes observed were not a secondary phenomenon, as suggested for amino acid transport.

Sugar uptake in Sacc. cerevisiae occurs via several systems. The constitutive system for glucose, fructose and mannose (Heredia et al., 1968) and the inducible galactose system (Kuo et al., 1970) are facilitated diffusion systems. The inducible maltose system appears to act by a proton symport (Seaston et al., 1973) and requires an energised membrane to act even in a 'downhill' mode (Serrano, 1977).

1-4-3-3 Energy Coupling in Strep. faecalis

Strep. faecalis, in common with Strep. lactis, lacks a membrane-bound electron-transport chain and thus relies on glycolysis for the generation of ATP from glucose (Diebel, 1964). The p.m.f. in Strep. faecalis is generated by a membrane-bound proton-translocating ATP-ase (Harold and Papineau, 1972 a,b; Laris and Pershadsingh, 1974) which is potently inhibited by N,N'-dicyclohexylcarbodiimide (DCCD) (Harold et al., 1969). Generation of the p.m.f. across the cytoplasmic membrane is thus, as in Sacc. cerevisiae, directly dependent on ATP. Unlike E.coli, the endogenous energy reserves of Strep. faecalis are very low (Forrest and Walker, 1965) and substrate accumulation requires a supply of metabolisable energy source.

Asghar et al., (1973) showed that proton-conducting uncouplers abolished the accumulation of neutral amino acids by Strep. faecalis, but that in starving cells, the imposition of an artificial membrane potential could support accumulation. This evidence indicates that neutral amino acid accumulation is energised by the p.m.f. and not directly by ATP hydrolysis. In starving cells, whereas accumulation was abolished, the uncoupler-insensitive exchange of intra- and extracellular neutral amino acids continued (Holden et al., 1968; Asghar et al., 1973). The p.m.f. is also the driving force for the accumulation of galactosides in Strep. lactis (Kashket and Wilson, 1973) and, on the basis of sensitivity to DCCD and proton-conducting ionophores, the uptake of Phe, Met, Tyr and His is similarly linked (quoted in Harold and Spitz, 1975).

Some transport systems in Strep. faecalis are seemingly linked directly to phosphate-bond energy, and are thus insensitive to protonophores and DCCD. The systems for phosphate, dicarboxylic amino acids (Harold and Spitz, 1975) and the extrusion of calcium (Kobayashi et al., 1978) are linked in this way. The transport of potassium requires conditions which permit the presence of both the p.m.f. and intracellular ATP (Bakker and Harold, 1980) although which functions as the driving force for uptake is not clear.

1-4-4 The Energetics of Peptide Transport

1-4-4-1 Is Peptide Transport Active?

It has been established (Section 1-1-2) that peptide transport is independent of hydrolysis in many microorganisms. Evidence will be presented in this thesis that this is the case for Sacc. cerevisiae and Strep. faecalis, and so the possible role of

group translocation mechanisms in peptide transport will not be discussed here.

In a prokaryote, if the intracellular peptidase activity is high relative to the rate of transport, peptide transport could be passive, and 'powered' by the continuing intracellular hydrolysis of substrate. It is thus necessaary to demonstrate intact accumulation of peptide before the transport can be termed active.

In eukaryotes, it is possible to envisage a situation where there is passive peptide transport across the plasmalemma, and active accumulation by some organelle such as the vacuole. In this case, the demonstration of intact peptide accumulation does not necessarily imply active transport across the plasmalemma.

1-4-4-2 Evidence for Active Peptide Transport

There is much indirect evidence for active transport of peptides in bacteria. Studies showing that a metabolisable energy source is needed and/or that metabolic poisons inhibit uptake have been performed in E.coli (De Felice et al., 1973; Cowell, 1974; Payne and Bell, 1979), Lactobacillus casei (Leach and Snell, 1959, 1960), Leuconostoc mesenteroides (Mayshak et al., 1966; Yoder et al., 1965), Pseudomonas putida (Cascieri and Mallette, 1976a), Streptococcus lactis (Rice et al., 1978), Bacteroides ruminicola Pittman et al., 1967), Sacc. cerevisiae (Becker and Naider, 1977), Candida albicans (Logan et al., 1979), and Neurospora crassa (Wolfinbarger and Marzluf, 1975a). The dependence on metabolic energy in these cases is not, however, unambiguous evidence for active transport, as the high levels of intracellular peptidase activity did not allow intact peptide accumulation to be

demonstrated. For example, if uptake is 'downhill' via a facilitated diffusion system, it is possible to envisage that the rate of translocation may be much reduced in a membrane lacking a p.m.f., due to, for instance, changes in affinity of carrier for substrate.

Active transport has clearly been indicated by the demonstration in E.coli of intact accumulation of the peptidase-resistant ϵ-derivatized lysine peptides (Payne, 1972a), glycyl-sarcosine (Payne and Bell, 1977b), triornithine (Payne and Bell, 1977b, 1979), and of Gly-Gly in a peptidase deficient strain (Kessel and Lubin, 1963). In peptidase-deficient strains of Salmonella typhimurium intact accumulation of Ala-Pro-Gly (Jackson et al., 1976), Gly-Pro (Yang et al., 1977) and a range of other peptides (J.W. Payne, personal communication) has been demonstrated.

Detailed investigations of the mode of energy coupling to peptide transport have only been carried out in E.coli. Cowell (1974) showed that Gly-Gly uptake occurred via a shock-sensitive system, and was linked to phosphate bond energy rather than the p.m.f.. This mode of energy coupling has been confirmed for several di- and tripeptides in a number of E.coli strains (Payne and Bell, 1979).

1-4-4-3 Sacc. cerevisiae and Strep. faecalis

To date there have been no reports of peptides being accumulated against a concentration gradient in Sacc. cerevisiae. Sodium azide, potassium cyanide, 2,4 dinitrophenol, and N-ethyl-maleimide were potent inhibitors of Met-Met-Met uptake, and N,N'-dicyclohexylcarbodiimide was also inhibitory (Becker and

Naider, 1977), although peptidase action did not allow peptide accumulation to be demonstrated. The report that transport was arsenate insensitive (Becker and Naider, 1977) cannot be accepted on the evidence given, as the transport assays were performed in the presence of phosphate, an antagonist of arsenate action.

There are no reports in the literature on the energy coupling of peptide transport in Strep. faecalis.

1-4-4-4 Other Fluxes Associated with Peptide Transport

The exodus of amino acids during peptide uptake and hydrolysis (Section 1-1-3) is a complicating factor in studying the energy coupling of peptide transport. It can be envisaged that the amino acid exodus is passive, active, or even, by a symport mechanism, contributes to the p.m.f., and so may influence the measurement of ion fluxes and energy consumption during transport.

The transport of peptide substrates of varying charge is another factor to be considered. The accumulation of charged substrates may require a different energy input to neutral ones, and there will presumably be associated ion fluxes to preserve electro-neutrality during their uptake. As for amino acid exodus, the influence of charge on the energetics of peptide transport has yet to be investigated experimentally.

CHAPTER 2

METHODS FOR THE ASSAY OF PEPTIDE TRANSPORT

2-1 A Survey of the Available Methods

2-1-1 Introduction

In the present work, use is made of two highly sensitive fluorescence assays of peptide transport, and to a lesser extent of radiotracer assays. However, the literature evidence for peptide transport is often based on rather more indirect methods. It is pertinent to survey the range of methods used, and to highlight their advantages and disadvantages.

2-1-2 Direct methods

As peptide transport systems in microorganisms often display high substrate affinity (Section 1-1-4), any study of kinetic parameters of uptake requires the use of low substrate concentrations. However, conventional reagents for the assay of α-amino nitrogen (e.g. ninhydrin) do not possess the sensitivity to work at such concentrations. Assays of α-amino nitrogen have the further drawback that they do not distinguish between amino acids and peptides. The two fluorescence methods, using dansyl chloride (Section 2-3) and fluorescamine (Section 2-4), employed in the present studies overcome both the problem of sensitivity and that of specificity. The applications of the methods are discussed fully in the relevant sections.

Because of the previous lack of suitable chemical reagents for the assay of peptide uptake, the majority of direct studies to date have used radiolabelled peptides. One major disadvantage of the approach is the lack of commercially available substrates, which has meant that studies are generally restricted to only a few peptides; such a situation is far from ideal when the system being

studied often has a wide substrate specificity. Apart from this obvious disadvantage, it has been shown that there are other problems associated with radiotracer assays, which are fully discussed in Section 2-7.

2-1-3 Indirect methods

2-1-3-1 Growth Tests

The majority of indirect studies of peptide uptake have been performed by monitoring the growth response of an organism to a peptide as a source of amino acids. The most informative studies have used amino acid auxotrophs. Here, the rate of supply needed is much lower than if the substrate is acting as a general nitrogen source, and so the assay is much more sensitive.

The growth of an amino acid auxotroph on a peptide that contains the required amino acid is good evidence for the transport of the peptide, providing extracellular cleavage and uptake as free amino acid can be ruled out by demonstrating lack of inhibition by other amino acids known to compete for uptake with the one required. The converse situation of lack of growth does not always however, correspond to lack of transport. Lack of growth may also occur if a transported peptide cannot subsequently be hydrolysed; it is thus of importance to demonstrate that cytoplasmic enzymes are able to cleave the supplied peptide in every case. The rate of growth obviously depends on the rate of supply of the required amino acid; a slow rate of supply may result in a slow growth rate or in a pronounced lag before growth begins. Such lags are noticed particularly in Sacc. cerevisiae (Marder et al., 1977) and presumably result from the requirement for a minimum pool level of the required amino acid before growth can

begin. If the rate of supply is low, such a level may not be attained in the duration of the assay. The lower limit of detection of peptide transport using such growth assays is therefore hard to define. At the other extreme, if the rate of supply is high, growth is likely to be limited by factors other than the supply of the required amino acid, and so differences in transport rate between peptides cannot be detected.

By testing the growth response of a range of auxotrophs it is possible to obtain information on the transport of a whole range of peptides. Information about substrate specificity and relative affinities can be obtained from the inhibition of the growth response when other peptides that lack the required amino acid are added to the medium. Such inhibition can frequently be attributed to competition for uptake, although competition for cleavage or possible toxic effects of the peptide alone should always be considered. The approach also has other limitations. As the affinity for uptake cannot be known from growth tests alone, it is not possible to tell whether substrates are being supplied above their Km values. If the system is far from saturated, little competition for uptake will occur, even if two substrates share an uptake system. Secondly, if the rate of supply of the required amino acid is much in excess, even a severe inhibition of uptake may not affect the growth response.

In summary, growth tests have the advantage of allowing the use of a wide range of substrates, but, because of their indirect nature, they have the disadvantage of allowing only very limited conclusions about the rate and specificity of transport to be made.

2-1-3-2 The Uptake of Toxic Peptides

The use of toxic peptides to select for peptide-transport-deficient mutants is widely reported, and cross-resistance studies can provide some information about the specificity of a given transport system. However, resistance to a toxic peptide does not necessarily imply total loss of transport ability, but merely a lowering of rate of supply to below toxic levels. Thus, two toxic peptides may share a common mode of uptake, but a mutation reducing uptake by 90% may lead to resistance to one peptide only. If a toxic peptide uses two uptake systems, loss of either could result in resistance if the rate of supply is critical. Such a situation seems to apply for the uptake of Orn-Orn-Orn in E.coli (R. Alves, personal communication). Clearly, studies of resistance to toxic peptides are open to misinterpretation in the absence of other data.

Studies on the toxicity of a wide range of peptides bearing the same toxic residue can give broad outlines of some features of uptake (e.g. size limit), although the influence of cleavage must be considered, and a large number of peptide analogues must be synthesised. Such studies have been performed in a range of organisms for phosphonopeptides (Atherton et al., 1979a).

2-1-3-3 Other Methods

Other methods again rely on the supply of an amino acid in peptide form, but use more rapid and sensitive monitoring than the measurement of growth. In one method, an E.coli amino acid auxotroph is supplied with peptide, β-galactosidase is induced, and the amount of enzyme synthesised is monitored (Bell et al., 1977). The amount of enzyme synthesis is proportional to the amount

of the required amino acid supplied. In another method, an E.coli double amino acid auxotroph is supplied with one radiolabelled free amino acid, and the other unlabelled and in peptide form (Payne & Bell, 1977c). Protein synthesis can only proceed in the presence of both required amino acids, and so, in the presence of excess labelled amino acid, the amount of incorporation of label into protein is proportional to the supply of the unlabelled, peptide amino acid.

These methods have certain advantages over growth tests: less peptide is needed per assay; they are potentially more sensitive; experiments are less time consuming; some rough estimates of kinetic parameters can be obtained. However, they still suffer from the criticism that transport is not being monitored directly.

2-2 Instrumentation and Materials

2-2-1 Instrumentation

For the automated fluorescamine assay, a Perkin Elmer Model 1000 fluorescence spectrophotometer equipped with a flow cell (part no. 5201-9520) was used. For manual fluorescamine assays, either the above instrument (equipped with a cuvette holder), a Baird Atomic Fluoripoint Model FP 100 or a Perkin Elmer Model 204 was used. The intensities of dansyl derivatives on chromatograms were measured using the Baird Atomic Instrument equipped with a TLC plate scanner (Baird Atomic).

Liquid scintillation counting was performed on either a Packard Model 3320 Tri-Carb, Packard Prias P L Tri-Carb, or Nuclear Enterprises Model 8312 instrument. In all cases, detection windows were adjusted to monitor ^{14}C-emission.

2-2-2 Peptides

Ala-[U-^{14}C]Ala, Ala-Ala-[U-^{14}C] Ala and Ala-[^{14}C] aminoethylphosphonic acid (Ala-[^{14}C]Ala (P)) were gifts from Dr. W.J. Lloyd, Roche Products Ltd., Welwyn Garden City. [1-^{14}C] Gly-Phe and Gly-[U-^{14}C] Phe were from the Radiochemical Centre, Amersham. All other peptides were purchased from Sigma (London) Ltd, Poole, or from Uniscience Ltd., Cambridge.

2-2-3 Other Reagents

Dansyl chloride and dansyl-amino acid standards were from BDH Ltd. Polyamide sheets (15 x 15 cm) were from BDH Ltd. or Pierce-Warriner Ltd. Fluorescamine (Fluram) was a gift from Dr. W.J. Lloyd, Roche Products Ltd., Welwyn Garden City, Herts. 2,5 Bis (5-tert-butylbenzoxazol-2-yl) thiophene (BBOT) was from Fisons Scientific Apparatus, Soluene 350 from Packard Instruments Inc., and NE 260 scintillant from Nuclear Enterprises Ltd. All other reagents were of analytical grade.

2-3 Dansyl Chloride Procedure

2-3-1 Introduction

Dansyl chloride (1-dimethylamino-naphthalene-5-sulphonyl chloride) reacts with primary and secondary amines to form fluorescent sulphonamide derivatives. The reagent is extremely sensitive, and nanomole amounts of amines can be detected. The reaction is routinely employed in protein sequencing studies, and a number of chromatographic methods for the separation of dansyl amino acids have been developed (for a review see Seiler, 1970).

Dansyl chloride reacts with the α-amino groups of small peptides. The derivatives can be separated by thin layer chromatography, and quantified by the intensities of the fluorescent spots. Dansyl chloride is thus a suitable reagent for monitoring the disappearance of small peptides from the incubation medium and it allows monitoring of the uptake of several peptides simultaneously, a feature not demonstrable with other assays. Because the chromatographic separation of derivatives is clear and reproducible, the procedure is also very useful for monitoring cell extracts for the accumulation of peptide and changes of amino acid levels.

2-3-2 Standard Method for Monitoring Incubation Media

Samples of incubation media were collected as described in Sections 3-5-2-1 and 4-5-2-2. Samples containing 0-10 nmol peptide (usually 100 µl) were placed in Durham tubes (6 x 30 mm) together with an ornithine or diaminopimelic acid (DAP) standard (10 µl, 0.5 mM), and evaporated in vacuo. Sodium bicarbonate (200 mM in deionised water, 20 µl) was added to each tube to bring the pH to about 9, followed by an equal volume of dansyl chloride (2.5 mg ml^{-1}, in acetone). Tubes were sealed with silicone rubber stoppers and incubated at 45°C for 90 min to allow the dansylation reaction to proceed to completion. The mixture was then evaporated to dryness and the residue redissolved in aqueous pyridine (1 : 1 vol/vol, 10 µl). Samples (5 µl) were spotted near one corner of 15 x 15 cm polyamide sheets and chromatographed for about 45 min (until the solvent had traversed about three-quarters of the plate) in the following solvents: 1) 1st dimension, H_2O:formic acid (98.5 : 1.5, vol/vol): 2) 2nd dimension, acetic acid: toluene (10:90,

vol/vol]: 3) 2nd dimension, methanol: butyl acetate : acetic acid (40 : 60 : 2, by volume). Plates were sometimes finally rerun in the first solvent to 'round-up' the fluorescent spots. Chromatograms were viewed under long wave UV light and the derivatives identified and quantified by reference to the intensities of standards of the same compound and allowance for the intensity of the standard ornithine spot on each plate. A permanent record of each plate was made by photographing 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.

2-3-3 Standard Method for Cell Extracts

Cell extracts were prepared as described in Sections 3-5-2-2 and 4-5-2-5. Samples of 100 µl were normally taken, except in those experiments (e.g. Section 3-5-5-4) where high internal peptide or amino acid concentrations occurred, when smaller volumes (25 or 50 µl) were taken to allow quantitation of these large amounts. DAP (10 µl , 0.5 mM) was used as an internal standard, as ornithine is present in cell extracts. Other methodological details were as in Section 2-3-2.

2-3-4 Discussion

2-3-4-1 Dansylation Reaction

A large excess of dansyl chloride must be used in order to obtain a quantitative reaction with small peptides. In the standard method, if 10 nmol peptide was present there was a 20 fold molar excess of dansyl chloride. Hence, when large amounts of peptide were present in incubation media or extracts smaller

samples were taken to ensure that dansyl chloride was in excess.

The pH at which the dansylation reaction is performed is important. At the stage at which bicarbonate was added, the pH of the tube contents was checked to ensure that it was greater than 9. If the pH was low, the tube contents were evaporated, and more bicarbonate was added. This could be a problem when using incubation buffers such as N-ethylmorpholine, but did not occur routinely.

2-3-4-2 Chromatographic Separation and Identification

Chromatography in the first dimension often gave poor resolution because of retardation by insoluble debris (phosphate and glucose) at the origin. The problem was minimised by using low buffer concentrations in the incubation, although for experiments with yeast it was necessary to have fairly high glucose concentrations (Section 3-5-3-1). When the resolution was poor, chromatograms of incubation media were rerun in the first dimension after being run in the initial three solvents; cell extracts in distilled water did not give rise to the same problems.

Dansyl derivatives could be identified from their positions on a chromatogram relative to the internal standard and the by-products (see Section 2-3-4-3) of the reaction. Identification was aided by running the relevant dansyl derivatives on the reverse side of the polyamide sheet, but after initial identification this was not routinely necessary, as derivatives ran consistently to the same relative positions. Whereas most derivatives fluoresce green, the histidine derivative is orange and tyrosine is yellow, which further aids identification. Free amino acids that contain

derivatizable side-chain groups, e.g. lysine, tyrosine, ornithine, form bis-derivatives with dansyl chloride, in which both the α-amino and side-chain groups are reacted.

The relative positions of dansyl amino acids after chromatography in all three solvents used in these studies are shown in Figure 2-1. Similarly, locations for the dansyl derivatives of the peptides used are given in Appendix 1. Some amino acids, such as proline/valine and leucine/isoleucine, run very close to each other after three solvents. However, by examining plates after two solvents, these amino acids can readily be distinguished. For diagrams showing the location of amino acid derivatives after two solvents, and many peptide derivatives after three solvents, see Higgins (1979). Examples of chromatograms of both incubation media and cell extracts are given in Figures 3-2 and 4-2. It should be emphasised, however, that these monochrome photographs are poor reproductions of the original chromatograms.

2-3-4-3 By-products of the Reaction

There are two main by-products of the dansylation reaction that are detectable on chromatography plates. The reaction of dansyl chloride with water forms dansyl hydroxide, which gives a blue-fluorescent smear in the first dimension. However, this is not in the same final location as any of the amino acid derivatives (Fig 2-1). The dansyl ammonia derivative runs very close to dansyl proline (Fig. 2-1) and so is potentially intrusive; however, under standard conditions, very little is produced. Unreacted dansyl chloride is sometimes detected on chromatography plates, although as it runs to the solvent front in the second dimension it does not interfere

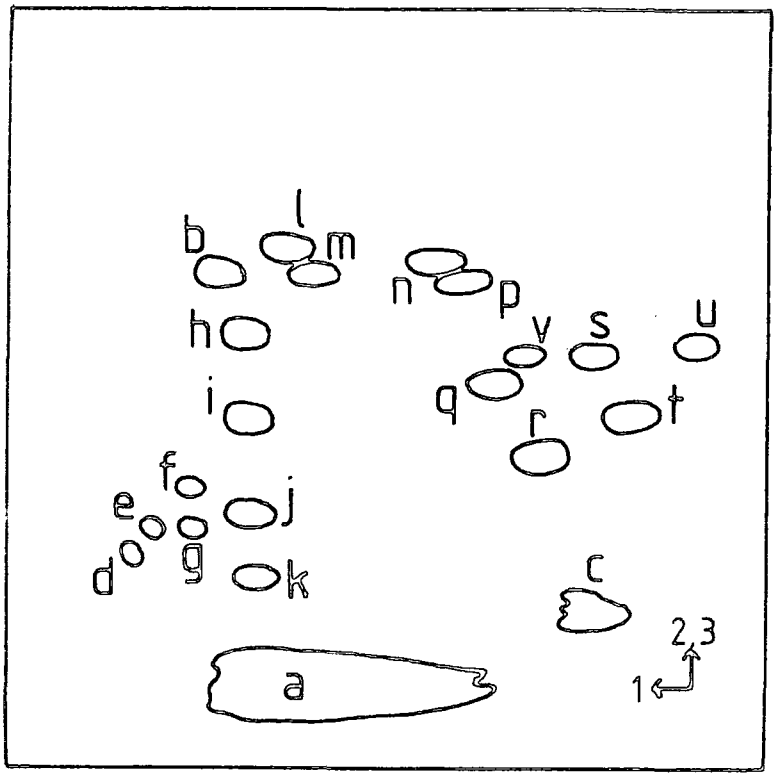


Fig 2-1 Positions of Dansyl-Amino Acids After Chromatography in Three Solvents

a) dansyl hydroxide, b) dansyl ammonia, c) diaminopimelic acid, d) Arg, e) Asn, f) Thr, g) Ser, h) Ala, i) Gly, j) Glu, k) Asp, l) Pro, m) Val, n) Ile, p) Leu, q) Met, r) Orn, s) His, t) Lys, u) Tyr, v) Phe.

with the quantitation of other derivatives.

2-3-4-4 Quantitation of Dansyl Derivatives

We have found that the fluorescence of dansyl derivatives on polyamide thin-layers does not fade significantly over several hours or upon drying, in the light, or over periods of up to a month for dark storage. This stability of the derivatives allows reproducible quantitation without complex procedures.

Routinely, the intensity of a chromatographed derivative was estimated by comparison to a set of known standards of a dansyl amino acid. This value was corrected by reference to the intensity of the internal standard (DAP or ornithine) on each sheet, and that of a standard amount of the derivative in question. This method of quantitation was shown to be accurate to 5-10% (Payne & Bell, 1979).

In some cases, the intensity of a spot on a chromatogram was measured using a thin layer scanner attached to a recording fluorescence spectrophotometer, excitation and emission wavelengths being set to 340 and 485 nm respectively. Results in these cases verified those from visual assessment. Further confirmation of the validity of the method comes from the use of tritiated dansyl chloride (Payne & Bell, 1979). Estimation of radioactivity in chromatographed dansyl derivatives (using a Pannax thin layer scanner, RTLS-1A) corresponded with visual estimation of fluorescence.

2-3-4-5 The Derivation of True Peptide Transport Rates

Using the standard procedure, the rate of disappearance of peptide from the medium is followed. This may not be the true rate of transport if i) there is extracellular cleavage of peptide or

ii) there is extracellular deamination of peptide. Neither process has been detected for organisms studied to date, i.e. E.coli (Payne & Bell, 1979) Salmonella typhimurium (J.W. Payne, personal communication), Sacc. cerevisiae (Chapter 3) or Strep. faecalis (Chapter 4). The fact that decrease in peptide concentration can in many cases be totally abolished by inhibitors of energy metabolism is firm evidence that such reactions are not occurring at detectable rate, and that the rate of disappearance of peptide is representative of the rate of transport.

2-3-5 Concluding Remarks

Because of its sensitivity, the dansylation procedure allows the assay of peptide uptake by microorganisms to be performed at suitable substrate concentrations. As the technique allows peptides and amino acids to be separated easily and quickly, the fate of absorbed peptide can be followed. The rapid exodus of the constituent amino acids of a supplied peptide, a phenomenon that seems widespread in bacteria (Section 1-1-3), was first unambiguously shown in E.coli using the dansylation procedure (Payne & Bell, 1979), and is clearly demonstrated here for Strep. faecalis (Section 4-5-3).

Whereas the dansylation procedure can be used to assay the rate of peptide transport, the quantitation of low rates is difficult, due to the errors involved, and such rates are often better derived from other assays such as that using fluorescamine (Section 2-4). The dansylation technique is, however, invaluable in studying the process of peptide transport as it allows the simultaneous monitoring of both peptide and amino acid levels inside and outside the cell.

2-4 Fluorescamine - A Manual Assay of Peptide Concentration

2-4-1 Introduction

Fluorescamine, first synthesised by Weigele et al., (1972), reacts with picomole amounts of primary amines to form fluorescent derivatives (Udenfriend et al., 1972). The reaction shows a marked pH dependence, with the optimum for amino acids at about pH 9 and that for small peptides at pH 7-8 (Udenfriend et al., 1972 ; Perrett et al., 1975). As it is the non-protonated primary amine that reacts (DeBernado et al., 1974) the greater reaction of peptides at lower pH values can be attributed to a lower pKa value for the terminal $-NH_2$ group. At high pH values, inactivation of fluorescamine by hydrolysis significantly lowers yields.

The reaction sequence of fluorescamine with primary amines is complex. Stein et al., (1974) proposed that the reaction took place by a reversible addition of the amine across the double bond of fluorescamine followed by an irreversible multi-step rearrangement to the final fluorophor (Fig. 2-2). At pH 9, the reaction is of first order with respect to primary amine and the inactivation of fluorescamine by hydrolysis is relatively slow (Stein et al., 1974). Later work (Chen et al., 1978) indicated that the reactions involved in the formation of fluorophors and non-fluorescent hydrolysis products were very complex.

At pH 6, the yield of the reaction of fluorescamine with primary amines is considerably lower than at pH 9, but the yields of small peptides are much greater than those of free amino acids. This allows the assay of peptides in the presence of amino acids (Perrett et al., 1975). The assay used in the present study was performed at

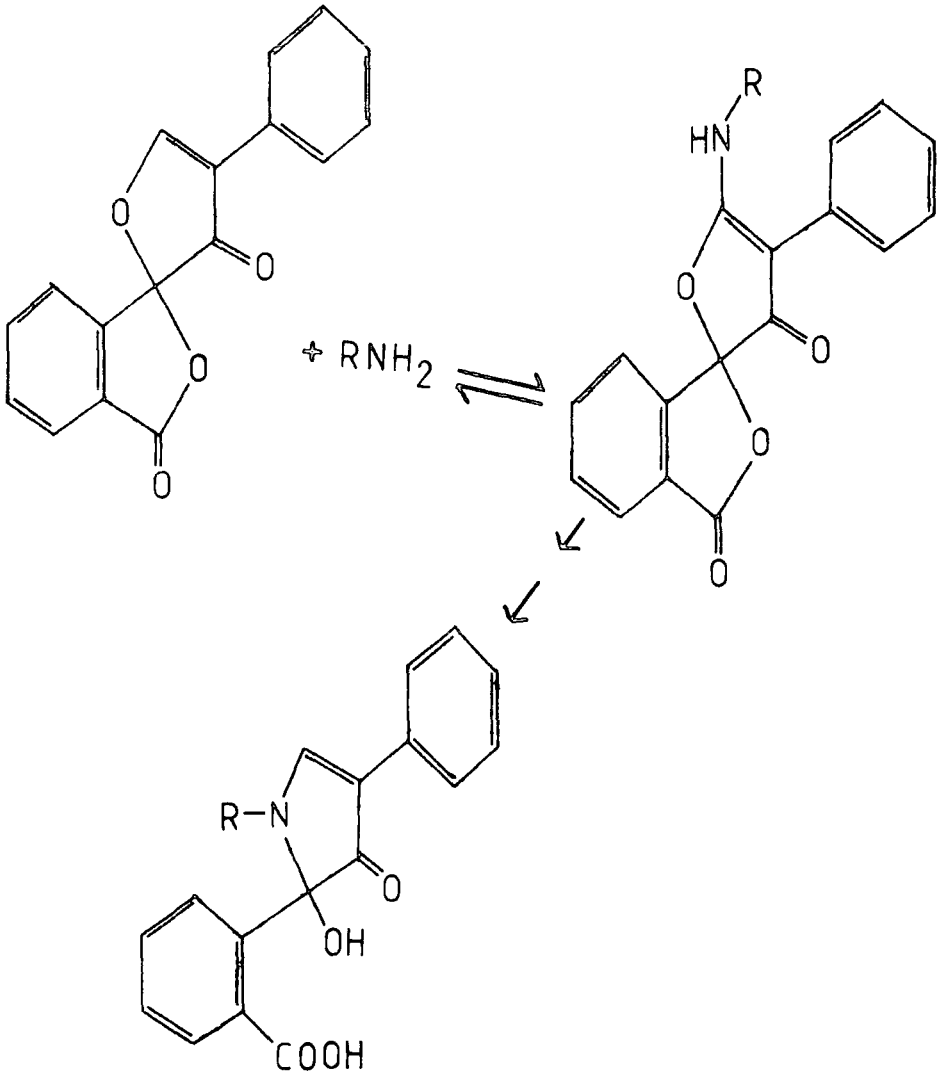


Fig 2-2 The Reaction Sequence of Fluorescamine with Primary Amines

(after Stein et al., 1974)

pH 6.2, where interference by amino acids is negligible.

Fluorescamine reacts with nucleophiles other than primary amines to form non-fluorescent products. The reaction with proline is rapid and irreversible (Chen et al., 1978); that with alcohols such as methanol (DeBernado et al., 1974) and glycerol(Castell et al., 1979) is reversible, and so the presence of such an alcohol leads merely to a lower rate of reaction of fluorescamine and primary amine, and not to a lower yield (Castell et al., 1979).

Whilst the brief discussion above makes it clear that the reactions of fluorescamine are very complex and still not completely understood, it should be stressed that under controlled conditions, fluorescamine can be used as a reagent for the quantitative assay of amino acids and peptides. It has been used in a detection system for an amino acid analyser (Stein et al., 1973) and for the assay of dipeptides (Perrett et al., 1975). The data presented in the remainder of this section show that the procedure used in the present studies is a sensitive and quantitative tool for the assay of small peptides.

2-4-2 Standard Method

All glassware was washed carefully, including four final rinses in distilled water. Duplicate aliquots (50 µl) of supernatant solutions from peptide uptake incubations (0-5 nmol peptide) were added to di-sodium tetraborate/HCl buffer (2.5 ml, 0.1 M in deionised water, pH 6.2) in test-tubes (100 x 12 mm). While the contents were rapidly mixed, fluorescamine (0.5 ml, 0.15 mg ml^{-1} in acetone) was added. Samples were left for 2 min at room temperature for the fluorescence to stabilize before reading in a fluorescence spectrophotometer (excitation 390 nm, emission 485 nm). By reference

to control tubes containing known amounts of peptide or incubation medium alone, the fluorescence readings were converted to give peptide concentrations.

2-4-3 Discussion

2-4-3-1 Linearity of Response

The maximum amount of peptide assayed in these studies was 10 nmol per tube (in studies on the affinity for peptide uptake in Sacc. cerevisiae, Section 3-5-4-2). The fluorescent yield of the fluorescamine reaction is linear over a range well in excess of this amount (Fig. 2-3). This result has been confirmed in our laboratory for many peptides.

2-4-3-2 Fluorescence Yield

When assayed by the standard technique (Section 2-4-2), Gly-Gly (0.1 mM) gave a fluorescence yield 4×10^{-1} times that of a 1 μg ml^{-1} solution of quinine sulphate in 0.1 M H_2SO_4.

2-4-3-3 Relation Between Fluorescence Yield and pKa

Fluorescamine will only react with the non-protonated form of the terminal $-NH_2$ group of a peptide or amino acid (DeBernado et. al., 1974). If one makes the assumption that the reaction yield (F) is proportional solely to the concentration of the non-protonated group, and to no other factors, the following theoretical relationship can be derived:

$$F \propto \frac{[-NH_2]}{[NH_2] + [NH_3^+]} \qquad (1)$$

The assay pH of 6.2, is well below the pKa values for the $-NH_2$ groups of most peptides (>7.5), so:

$$[\ -NH_2\ ] \ll [\ -NH_3^+\ ]$$

$$\therefore\ [\ -NH_3^+\ ] \simeq [\ -NH_2\ ] + [\ -NH_3^+\ ]$$

$$\therefore\ \text{From (1)} \qquad F \propto \frac{[\ -NH_2\ ]}{[\ -NH_3^+\ ]}$$

$$\therefore\ \underline{\text{Log } F \propto - \text{ pKa} + \text{constant}}$$

The yields of a range of peptides in the standard assay were determined, and a plot of literature values of pKa (from Perrin, 1965) against log F was made (Fig. 2-4). It is clear that in the majority of cases, the derived relationship holds, suggesting that the availability of the $-NH_2$ species is the most important factor affecting yield at this pH. Whilst the quantum yields of most amino acid derivatives are equivalent, derivatives of tryptophan and cysteine are internally quenched (Chen et al., 1978), which could explain the low yield obtained with Trp-Ala (Fig 2-4). The low yield of Ser-Gly cannot be explained by such quenching, and the literature pKa value was confirmed experimentally (by the Physical Methods Dept., Roche Products Ltd., Welwyn Garden City). Other factors are clearly affecting the yield in this case.

2-4-3-4 Interference by Amino Acids

As the pKa values for the $-NH_2$ groups of most amino acids are high (9-10), at pH 6.2 yields with fluorescamine are very low (see values for Ala and Gly in Figure 2-4), and the presence of amino acids does not affect the yield of peptides. Examples of the effect of the constituent amino acids on the yields of several peptides are given in Table 2-1. In each case the amount of amino acid

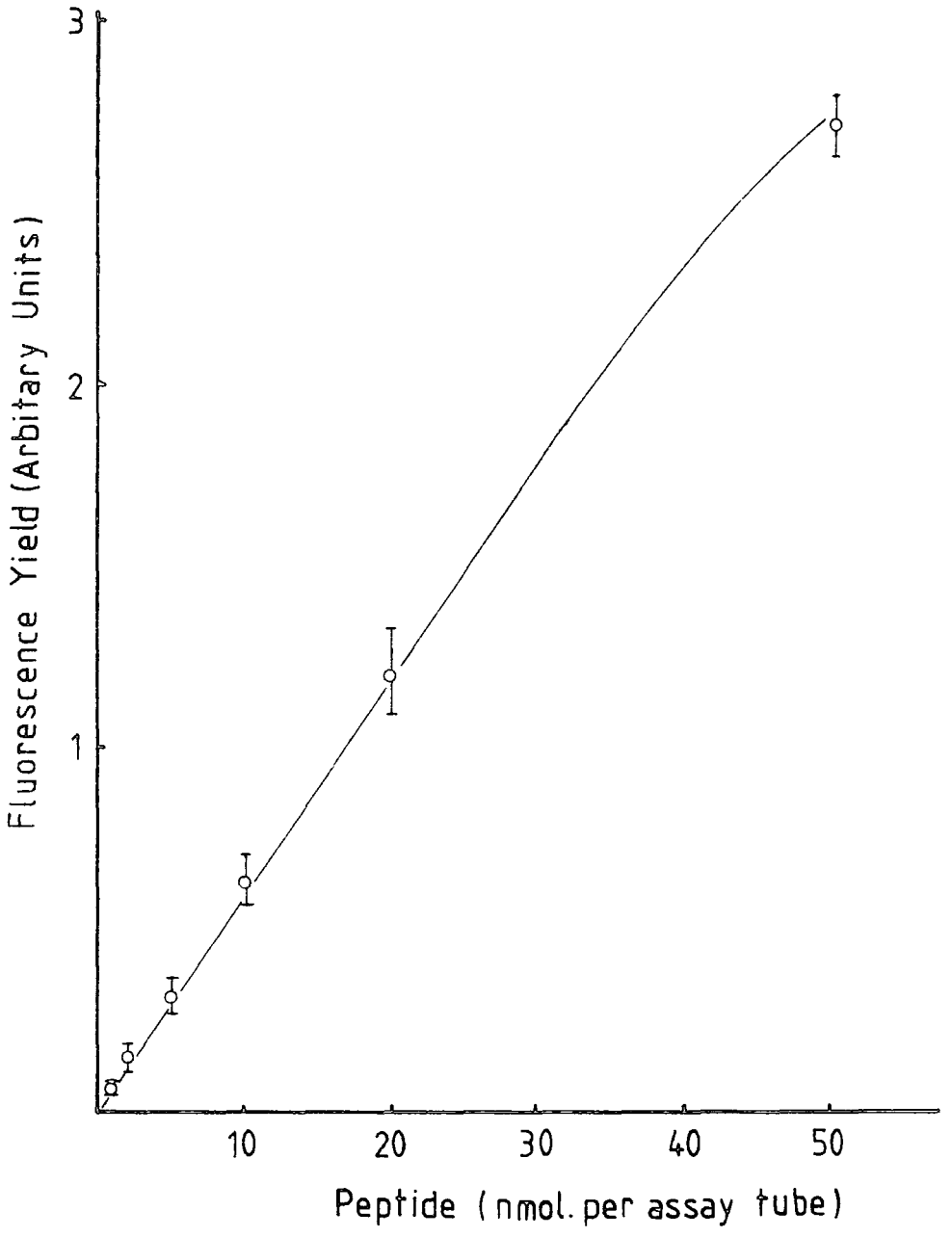


Fig 2-3 Effect of Peptide Concentration on Fluorescence Yield in the Standard Fluorescamine Assay

Varying amounts of Gly-Gly were assayed by the standard method (Section 2-4-2). Values given are the mean and range for 3 determinations.

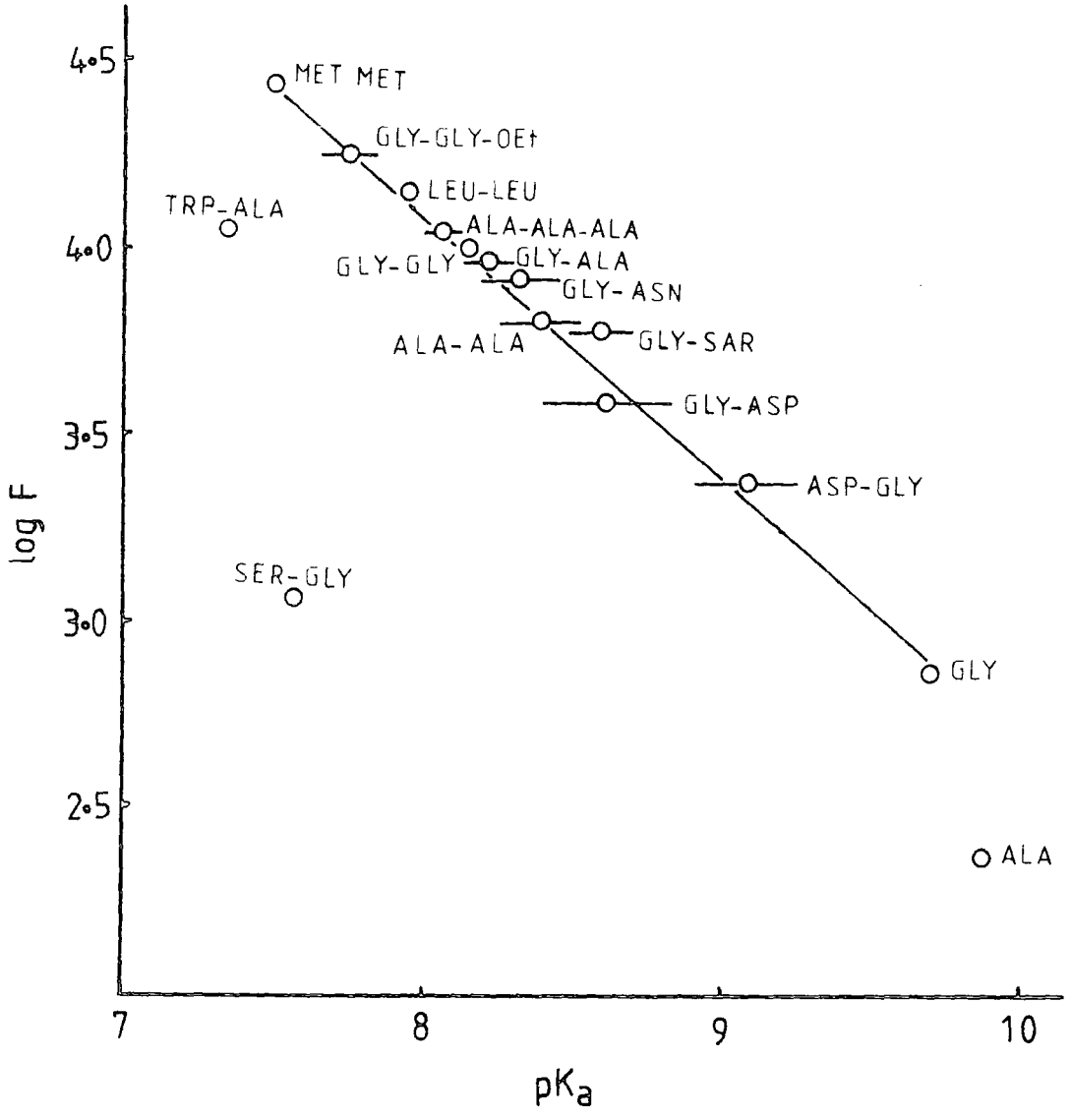


Fig 2-4 The Relation Between Yield with Fluorescamine and pKa of α-amino Group

Peptide (0.1 mM) was assayed by the standard method described in the text. Fluorescence yield was normalised to a value of 10,000 for Gly Gly.

Table 2-1 The Effect of Amino Acids on the Fluorescence Yield of Peptides in the Standard Fluorescamine Assay

Peptide or Amino Acid	Fluorescent Yield (Arbitary units)
Ala-Ala-Ala (5 nmol)	15,250
Ala (15 nmol)	750
Ala-Ala-Ala (5 nmol) + Ala (15 nmol)	15,500
Gly-Gly (5 nmol)	10,000
Gly (10 nmol)	875
Pro (1 nmol)	-150
Gly-Gly (5 nmol) + Gly (10 nmol)	9,875
Gly-Gly (5 nmol) + Pro (1 nmol)	10,312

Amounts of substrates are given per assay tube. Fluorescence yields are given relative to an arbitary value of 10,000 for 5 nmol Gly-Gly, and are the average of 2 determinations.

supplied is the maximum that could result from total cleavage of the peptide. In no case is the interference significant. Clearly, in an uptake study where much peptide is taken up, the exodus of amino acids will lead to significant fluorescence when only, say, 10% of the peptide remains. However, the uptake assays used do not rely on the uptake of such large proportions of the peptide, rates being derived during the uptake of less than 50% of substrate.

Two amino acids, histidine and phenylalanine, have abnormally high fluorescence yields at pH 6.2, and react slowly with fluorescamine (Higgins, 1979). Recently, a reaction mechanism for this has been proposed (Castell et al., 1979). In practical terms, the assay of the uptake of peptides containing these residues in situations where amino acid exodus may occur could lead to misinterpretation of results. In the present studies, such peptides were only used in Sacc. cerevisiae, where no amino acid exodus occurred.

As proline forms a non-fluorescent product with fluorescamine (Chen et al., 1978), and it may be present in low amounts in incubations due to leakage (particularly in the case of Sacc. cerevisiae, where proline is the sole nitrogen source during growth), the effect of proline on the yields of peptides was also assayed (Table 2-1). Again, no significant interference was detected under the standard assay conditions.

2-4-3-5 Other Factors

The fluorescamine reaction will take place in a range of buffers (see Section 2-5-3-4). Comparison between phosphate and borate buffers (Higgins, 1979) showed that 0.1 M di-sodium tetraborate

gave the best yield, and so this was adopted for the standard assay.

Varying the concentration of fluorescamine used in the assay alters the yield considerably (Higgins, 1979), presumably because of competition between peptide and water for the reagent. The concentration (0.15 mg ml^{-1}) used routinely was a compromise between obtaining sufficient yield and the use of what is an expensive reagent.

2-4-4 Concluding Remarks

The standard fluorescamine assay described here is an extremely sensitive method for the assay of small peptides in solution. As interference by free amino acids is minimal, it is especially suited to uptake studies where amino acid exodus is occurring. The assay is rapid, and allows more exact quantitation of low rates of peptide uptake than the dansyl chloride procedure (Section 2-3). Used together, these two assays allow much information about peptide uptake to be derived.

2-4 An Automated Assay for the Uptake of Small Peptides Using Fluorescamine

2-5-1 Introduction

A logical progression from the assay of peptide uptake by the manual fluorescamine procedure (Section 2-4) is the development of a continuous, automated assay, and such a system is described here. Among the advantages of the system are:

a) Continuous monitoring is more informative than discrete sampling.

b) Because the mixing step is vital for reproducible results and the automated assay allows more consistent mixing, the detection of very small changes in peptide concentration which are within the

error of manual measurements is possible.

c) Results are obtained while the uptake assay is in progress.

d) The assay is less time, and labour, consuming.

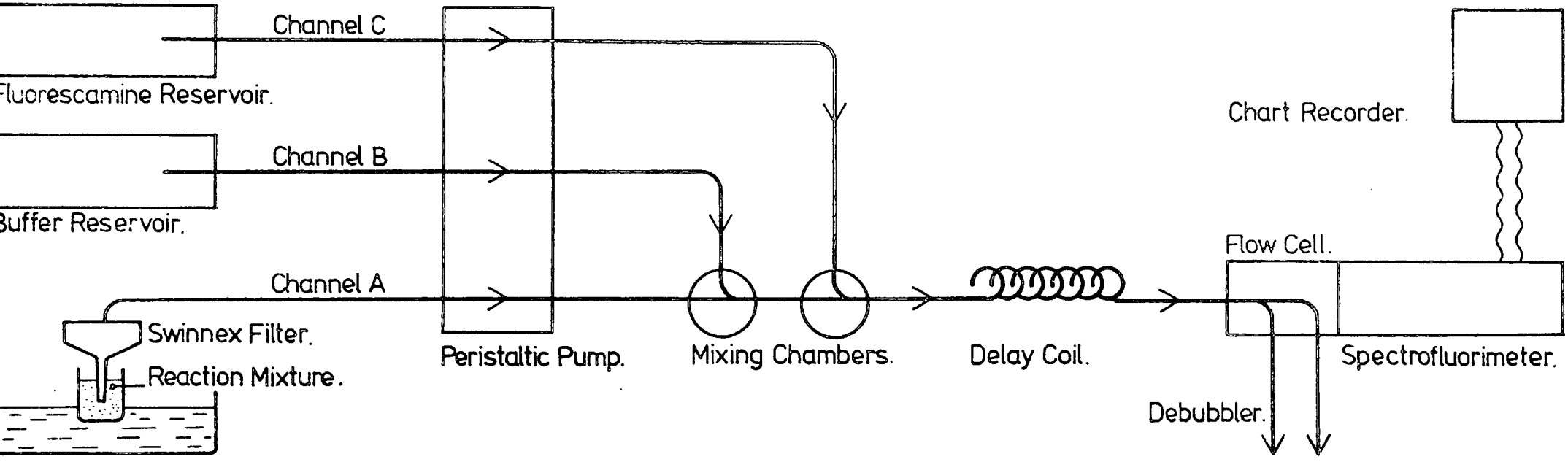
Fluorescamine has previously been used as a detecting agent in automated amino acid analysers, using acetone as a solvent (Stein et al., 1973). However, acetone is incompatible with the material of most peristaltic pump tubing, and so the system described here uses isopropanol (Davidson et al., 1975).

2-5-2 Standard Method

The assay uses a simple system based on a three-channel peristaltic pump (Pharmacia P-3), and a fluorescence spectrophotometer incorporating a flow cell. A schematic diagram of the apparatus is given in Figure 2-5 and all nomenclature in the following description is that used therein.

The incubation vessel (usually a small glass vial) equipped with a magnetic stirrer was placed in a thermostatically controlled water bath. The filter holder was adapted from a 25 mm diameter Swinnex (Millipore) by careful insertion of resin to reduce the dead space to a minimum whilst retaining adequate flow, and contained a membrane filter (25 mm diameter, 0.45 um pore size, Oxoid) which was changed after each assay. Peristaltic pump tubing (0.1 mm. internal diameter) was from Pharmacia. Mixing chambers were adapted from glass junctions with three outlet arms (Technicon). Small,bent-metal, resin-coated, fleas were fabricated and inserted via one arm, which was then sealed in a flame. The flea was then trapped at the junction of the remaining arms, and spun when the junction was attached to a magnetic stirrer.

Fig 2-5 Diagrammatic Representation of the Apparatus for the Automated Fluorescamine Assay



For assays of peptide uptake, a suspension of microorganisms was placed in the incubation vessel and pumped through channel A (0.9 ml min^{-1}), the microorganisms being removed by the filter. The resulting solution was mixed with a second buffer solution (Table 2-2) (Channel B, 0.9 ml min^{-1}) to bring the pH to 6.2 - 6.8 (see Section 2-5-3-4), and fluorescamine (0.15 mg ml^{-1} in isopropanol) was introduced (Channel C, 0.9 ml min^{-1}). The resulting solution was passed through a delay coil (5.7 m) to allow fluorescence to stabilise, and the fluorescence then monitored, any air bubbles by-passing the flow cell via the waste gate.

In a standard assay, the system was run for several minutes with incubation buffer passing through Channel A, to allow a stable baseline to be attained. The pump was then stopped momentarily, the needle transferred to the incubation vessel containing a suspension of microorganisms, peptide added, and the pump restarted. When the suspension was exhausted, the needle was placed in incubation medium again to stop air entering the system.

2-5-3 Discussion

2-5-3-1 Delays in Response to Change in Peptide Concentration

Because of the internal volume of the system, fluid takes 2.8 min to pass from the incubation vessel to the flow cell. Thus events portrayed on the recorder trace actually occurred 2.8 min beforehand.

There is also some longitudinal mixing in the tubing and filter holder. Thus if peptide is added to the incubation vessel at time X, an instantaneous rise is not registered on the chart recorder at time X + 2.8 min, but rather a rise lasting 2 min (Fig. 2-6). In

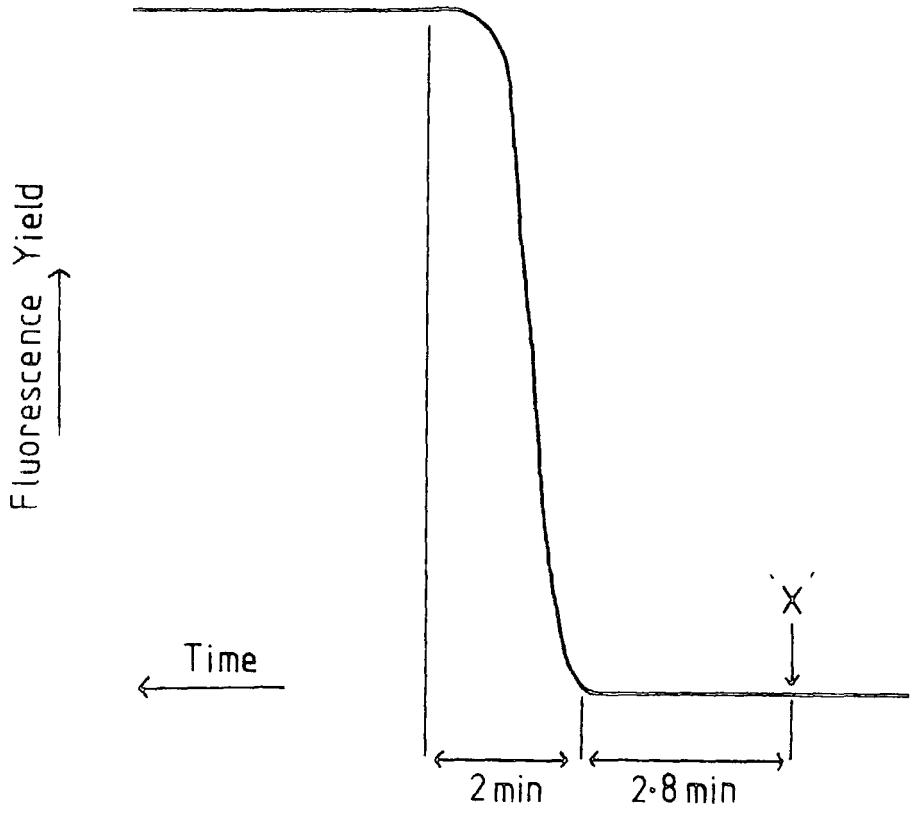


Fig 2-6 Delays in Response of the Automated Fluorescamine Procedure

A diagrammatic representation of the trace on the chart recorder upon addition of peptide to incubation vessel. For explanation see Section 2-5-3-1

cases where peptide uptake is being followed, this lag makes it difficult to determine the rate of uptake in the first 2 min directly. This information can be obtained by comparing the profile of the rising phase of the recorded trace with a corresponding curve in which no uptake is occuring, but such a procedure is tedious, and in many cases superfluous. Routinely, if the rate after 2-3 min is extrapolated backwards to zero time, the peptide concentration corresponds to that added, suggesting that the rate is identical over the first 2 min and the next several minutes (except when the peptide in the medium is greatly depleted during this time). The interfacing of a programmed microprocessor to the output of the fluorescence spectrophotometer (J.W. Payne, work in progress) should allow the routine, direct determination of initial rates.

2-5-3-2 Linearity of Response to Peptide Concentration

Extensive studies (J.W. Payne and G. Bell, personal communication) have shown that the system gives a linear response to peptide concentration over the range 2-250 μM (in the incubation vessel). Studies in which peptide uptake was simulated by the continual dilution of a peptide solution in the incubation vessel (Payne and Nisbet, 1980c; J.W. Payne personal communication) confirm that the measured rate of change of peptide concentration is real.

2-5-3-3 Fluorescence Yield

When assayed by the standard technique (Section 2-5-2) a solution of Gly-Gly (0.1 mM in KH_2PO_4/K_2HPO_4 buffer, 50 mM, pH 6.9) gave a fluorescence yield of 3 times that of the same solution assayed by the manual method (Section 2-4-2). (Yields were measured relative

to a standard preparation of a fluorescamine - peptide derivative in water/isopropanol, as the use of quinine sulphate in 0.1 M H_2SO_4 as a standard was not feasible in the automated system because of potential attack by the acid against elements of the pumping system).

2-5-3-4 The Use of Different Incubation and Reaction Buffers

As the final reaction pH must be in the range 6.2 - 6.8 to avoid interference from amino acids (Higgins, 1979), the use of different incubation buffers with different organisms requires the use of different reaction buffers in channel B (Table 2-2). Citrate phosphate buffer was routinely used as the reaction buffer as it buffers strongly in the required range, and allows adequate fluorescence to be detected. Monitoring the pH of the solution at the flow cell gave values about 0.4 units higher than those in Table 2-2. It is not clear how much this is due to the effect of isopropanol on the electrode used for monitoring pH, but even if the effect on pH is real, the solution will still be buffered in the correct range.

2-5-3-5 The Influence of Metabolic Inhibitors on Fluorescence Yield

In some of the studies reported here (Chapter 5) metabolic inhibitors were added to peptide uptake incubations. These compounds may affect the yield of the fluorescamine reaction in several ways: by reacting to form a fluorescent product; by reacting to form a non-fluorescent product; by quenching fluorescence; by altering the reaction pH. Studies of the effect of the inhibitors used on fluorescence yield (Table 2-3) show that in no case was the effect large.

Table 2-2 The Use of Different Incubation and Reaction Buffers in the Automated Fluorescamine Procedure

Incubation Buffer (Channel A)	Organism	Reaction Buffer (Channel B)	Combined pH
K_2HPO_4/KH_2PO_4 50 mM, pH 6.9	Strep. faecalis	Citrate-Phosphate 0.2 M, pH 6.0	6.3
K_2HPO_4/KH_2PO_4 50 mM, pH 7.2	E.coli	Citrate-Phosphate 0.2 M, pH 5.8	6.2
Citrate-Phosphate 25 mM, pH 4.5	Sacc. cerevisiae	Citrate-Phosphate 0.2 M, pH 6.5	6.2
Dimethyl-glutaric acid/KOH 25 mM, pH 4.5	Sacc. cerevisiae	Citrate-Phosphate 0.2 M, pH 6.5	6.3

Measurements of pH were made using a pH meter. The combined pH quoted is that of a mix of equal amounts of the two buffers. Addition of isopropanol alters the pH in the automated procedure (see text).

Table 2-3 Effect of Metabolic Inhibitors on Fluorescence Yield in the Automated Fluorescamine Assay

Inhibitor	Conc (mM)	Fluorescence Yield (%)
None		100
DCCD	0.1	97
CCCP	0.1	95
arsenate	10	99
azide	10	108

The effect of metabolic inhibitors on the fluorescence yield of Ala-Ala (0.1 mM) in K_2HPO_4/KH_2PO_4 buffer (pH 6.9, 50 mM) was assayed using the standard automated fluorescamine technique. Results are expressed relative to a control with no inhibitor.

2-5-3-6 Filter Capacity and Rate of Pumping

The volume of a suspension of microorganisms that can be pumped through the system is clearly limited by the capacity of the filter before blocking. The maximum amounts that could be pumped varied, being 5 ml of a 0.2 mg ml^{-1} suspension of E.coli (J.W. Payne, personal communication), 5 ml of a 0.3 mg ml^{-1} suspension for Strep. faecalis, and 10 ml of a 1.5 mg ml^{-1} suspension for Sacc. cerevisiae. At the rate of pumping routinely employed (0.9 ml min^{-1}), these densities of resuspension represent a pumping time of approximately 5 min for bacteria and 10 min for yeast. Whilst these were normally adequate, if longer incubations were required (e.g. Section 5-4) the pump speed was halved, although the problems of lag in machine response (Section 2-5-3-1) were then obviously increased. Alternatively, the substitution of a glass-fibre filter (Whatman GF/C) gave a larger capacity before blocking.

2-5-3-7 The Assay of Amino Acid Uptake

The method has been successfully used to study amino acid uptake (Payne and Nisbet, 1980c; J.W. Payne, personal communication). By using 0.2 M K_2HPO_4 or 0.1 M borax as the buffer in channel B the reaction pH can be raised to 8.5 - 9.0. Many amino acids give high fluorescence yields at this pH, and so amino acid uptake can be followed.

In the case of bacteria where exchange of internal and external amino acids can occur (e.g. for neutral amino acids in Strep. faecalis, Section 1-4-3-3) the uptake of one amino acid may be accompanied by exodus of others; this will clearly interfere with the uptake assay. The method is thus particularly suitable in organisms such as

Sacc. cerevisiae, where such exodus is normally indetectable (Section 1-2-4-4)

2-5-4 Concluding Remarks

The automated assay described here has been demonstrated to be sensitive, reproducible, and applicable to a range of microorganisms. The problems associated with lag in response should be largely eliminated by the interfacing of a microprocessor to the output of the fluorescence spectrophotometer (J.W. Payne, work in progress).

2-6 The Assay of the Uptake of Radiolabelled Peptides by Strep. faecalis

2-6-1 Introduction

Radiolabelled peptides have been used in the majority of direct studies of peptide transport reported in the literature. The methods reported here can give real rates of uptake into Strep. faecalis, although problems of amino acid exodus are highlighted. However, it is clear (Section 2-7) that, in several other microorganisms, radiotracer assays may give gross underestimates of transport rates.

2-6-2 Standard Method

Suspensions (500 µl) of Strep. faecalis containing radiolabel were harvested on filters as described in Section 4-5-2-4. Filters were placed in scintillation vials and dried at 80°C for at least 45 min. Scintillant (10 ml BBOT in toluene, 4 g l^{-1}) was added to each vial, and these were counted for ^{14}C for 1 min in a scintillation counter. Duplicate 50 µl samples of the whole incubation medium were spotted on filters which were placed in vials and dried and treated as above. Thus, as these controls represent 10% of the activity of each 500 µl sample, the percentage

accumulation in each filtered cell sample could be obtained.

2-6-3 Discussion

2-6-3-1 Linearity of Counting Response

Samples (50 µl) of a wide concentration range of Ala-[^{14}C]Ala solutions were spotted on filters and counted by the standard technique. A linear relationship between amount present and counts detected was found over the range 1 - 250 nmol peptide (data not shown).

2-6-3-2 Absence of Quenching by Bacterial Cells

The external standard ratio (and sample channels ratio) for scintillation counting was identical in samples with and without bacteria, which implies that there is no significant quenching of the scintillation by the presence of bacteria. Efficiency of counting averaged 70%.

In the scintillant system used, cells are not solubilised, and so there is a possibility of some attenuation of the β-emission from label inside the cells. This possibility was checked in a control experiment: Ala-Ala was added to a suspension of Strep. faecalis ATCC 9790, and also to the suspension buffer alone, to a final concentration of 0.1 mM (3.09×10^{-2} µCi ml^{-1}). After 15 min incubation at 37°C, duplicate 50 µl samples were removed, spotted on filters and treated by the standard method. Counts recovered were 2309 min^{-1} (efficiency 67%) in the presence of bacteria and 2217 min^{-1} (efficiency 65%) in their absence, suggesting that no detectable attenuation of β-emission is occuring. This is consistent with the report (Zarybnicky and Reich, 1980) that attenuation of β-emission from accumulated label by whole bacterial cells should not exceed 4%.

2-6-3-3 Exodus of Amino Acids

The time course of accumulation of label from Ala [^{14}C] Ala by Strep. faecalis ATCC 9790 showed an initial steep rise, followed by a fall to a plateau level (Fig 2-7). This can be interpreted in terms of rapid peptide uptake and cleavage followed by amino acid exodus (which is clearly demonstrable by the dansylation technique, Section 4-5). The final plateau is consistent with the maintenance of a pool of accumulated alanine once the peptide in the medium has been exhausted. The uptake profile obtained shows that it is necessary, for peptides whose constituent amino acids undergo rapid exodus, to sample very rapidly to obtain a true rate of uptake. For the labelled alanyl peptides used in these studies (Section 4-5) sampling at 8s intervals for the first 48s after addition of peptide to the Strep. faecalis suspension allowed the derivation of an initial rate of uptake uninfluenced by amino acid exodus (see Section 2-6-3-4 below). However, there is evidence that Ala-Ala is less rapidly cleaved by strain ATCC 9790 than some other peptides (J.W. Payne, personal communication) and so even 8s samples may be influenced by exodus in the case of some labelled substrates.

2-6-3-4 Comparison with Fluorescamine Assay

The uptake of Ala-[^{14}C]Ala was followed simultaneously by radiotracer and manual fluorescamine assays (Fig. 2-8). The initial rates are comparable, but the rate of label accumulation soon declines, whereas the loss of peptide from the medium continues until the concentration falls to near zero.

Comparison of the rates obtained for the uptake of several peptides in two strains of Strep. faecalis (Table 2-4) show that the

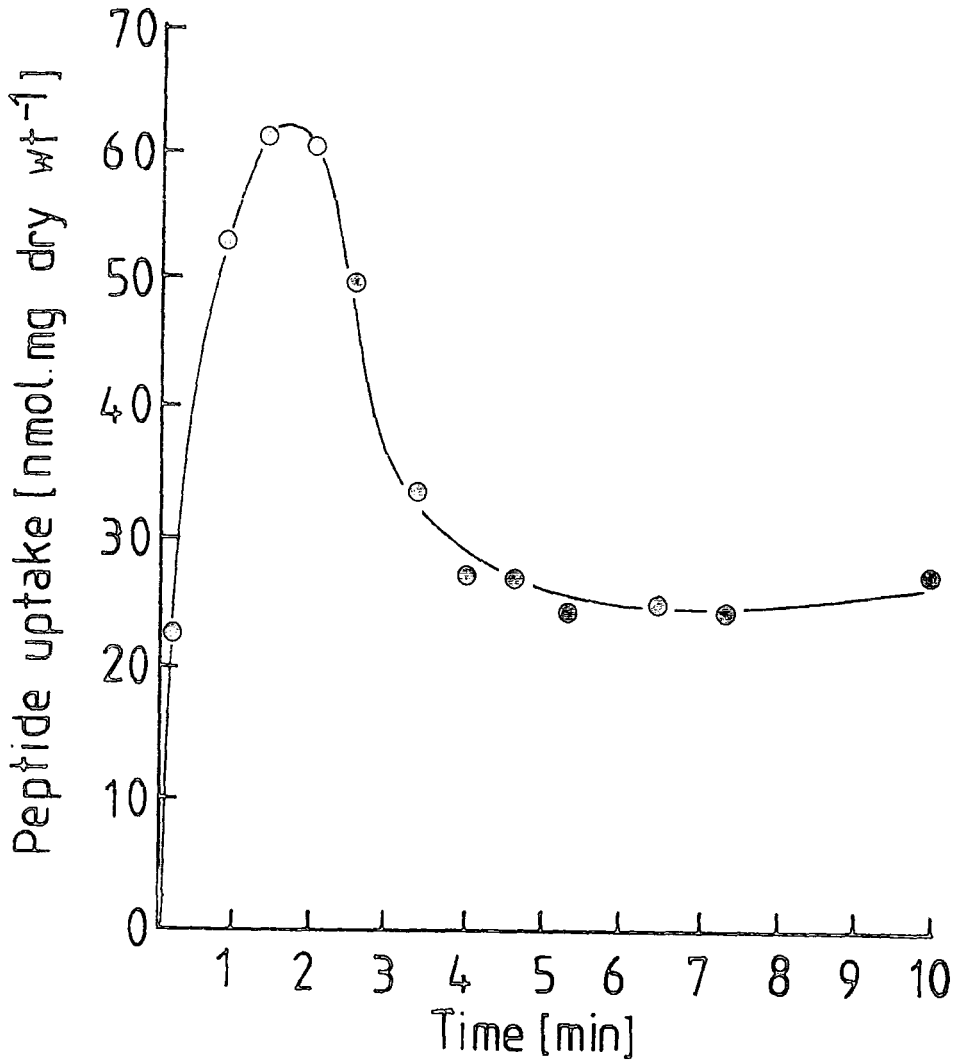


Fig 2-7 Uptake of Label from Ala-[^{14}C] Ala by Strep. faecalis (faecium) strain ATCC 9790

Strain ATCC 9790 was resuspended in RST 2 medium to a density of 0.36 mg dry wt ml^{-1}. Uptake of Ala-[^{14}C] Ala (0.1 mM, 3.09 x 10^{-2} µCi ml^{-1}) was assayed by the standard radiotracer technique.

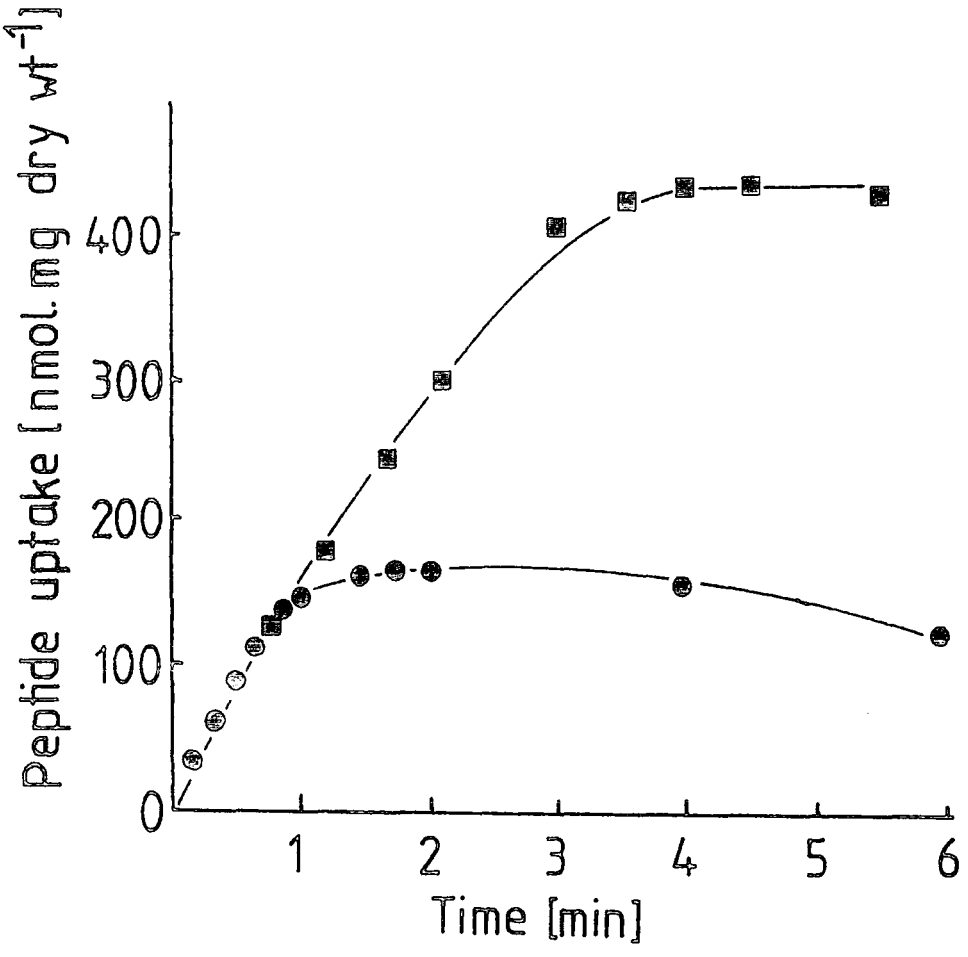


Fig 2-8 Comparison of Radiotracer and Fluorescamine Methods for Monitoring Peptide Uptake in Strep. faecalis (faecium)

Strain ATCC 9790 was resuspended in potassium phosphate (pH 6.9, 50 mM) with glucose (0.2% w/v) to a density of 0.22 mg dry wt ml^{-1}. Uptake of Ala- [^{14}C] Ala (0.1 mM, 3.09 x 10^{-2} μCi ml^{-1}) was assayed by the standard radiotracer (●) and fluorescamine (■) methods.

Table 2-4 The Uptake of Peptides by Strep. faecalis Measured by the Standard Radiotracer and Automated Fluorescamine Assays

Strain	Peptide	Rate (nmol min^{-1} mg dry wt^{-1})	
		Radiotracer Assay	Fluorescamine Assay
ATCC 9790	Ala-Ala	99(3)	98(5)
	Ala-Ala-Ala	25(2)	26(4)
FS5	Ala-Ala	44(2)	52(1)
	Ala-Ala-Ala	94(1)	82(1)
	Ala-Ala-Ala-Ala	89(1)	110(1)

Values given are the mean of the number of readings in parentheses. Uptake of labelled peptides was measured over the first 30s and of unlabelled peptides over the first 3-4 min.

radiotracer and fluorescamine assays give similar rates.

2-6-4 Concluding Remarks

The data presented demonstrate that the application of a conventional radiotracer assay for the study of peptide uptake by Strep. faecalis gives valid rates for the substrates used, provided samples are taken at very early times if the exodus of labelled amino acids occurs. However, this is not necessarily true for more rapidly cleaved substrates and, as the data presented in Section 2-7 show, is certainly not true for other microorganisms.

2-7 Problems Associated with the Assay of Radiolabelled Peptide Uptake in Microorganisms

2-7-1 Introduction

The assay of labelled alanyl peptide uptake by Strep. faecalis has been shown to give meaningful results (Section 2-6). The data presented in the current section, however, show that there are problems associated with the metabolism and exodus of amino acids that can lead to gross underestimates of the transport rates of labelled peptides in other organisms.

2-7-2 Methods

2-7-2-1 Organisms, Growth Conditions and Harvesting

E.coli W M2626 lys was grown in A + C medium (Davis and Mingioli, 1950) with lysine (30 μg ml^{-1}), E.coli B MRE 160 in A + C medium, and Salmonella typhimurium Leu 485 in E medium (Vogel and Bonner, 1956) with leucine (30 μg ml^{-1}), at 37°C with shaking. These organisms were harvested during exponential growth and resuspended in K_2HPO_4/KH_2PO_4 buffer (pH 6.9, 50 mM phosphate, 0.2% w/v glucose). Sacc. cerevisiae and Strep. faecalis were grown,

harvested and resuspended as in Sections 3-5-2 and 4-5-2 respectively. Organisms were pre-incubated at 37°C (28°C for Sacc. cerevisiae) for 10 min before addition of labelled peptide (0.1 mM).

2-7-2-2 Assays of Label Uptake

i) Assay of accumulated counts: After addition of peptide, samples (500 µl) were removed periodically, filtered under vacuum (Whatman GF/C) and immediately washed with saline (0.9% w/v, approx. 10 ml, room temp.). Radioactivity associated with filters was assayed by liquid scintillation counting.

ii) Assay of peptide or counts in medium: After addition of peptide, samples of the incubation medium were taken periodically and freed from microorganisms by membrane filtration (Oxoid, 0.45 µm pore size). Peptide concentration in the filtrate was determined by using the manual fluorescamine assay, and radioactivity was determined by liquid scintillation counting on 50 µl samples.

iii) Assay of total counts present: Organisms were incubated with peptide for, routinely, 15 min. Samples (50 µl) were then removed and spotted directly onto filters (Whatman GF/C), and the counts present assayed by liquid scintillation counting. Samples of a solution of labelled peptide at the same initial concentration as in the incubation were also taken and assayed in the same way.

2-7-2-3 Liquid Scintillation Counting

Two methods for counting were used:

i) Filters were dried and counted as in Section 2-6-2, using BBOT as a scintillant.

ii) Soluene 350 tissue solubilizer (2 ml) was added to filters in

vials. These were then capped, incubated overnight at 45°C, NE 260 micellular scintillant (10 ml) was added, and counted after at least 6 h at room temperature (to decrease chemiluminescence).

2-7-3 Results and Discussion

2-7-3-1 Absence of Quenching by Bacterial Cells

Control experiments in which samples of bacterial suspensions were dried onto filters before the addition of peptide solutions (0.1 mM, 50 µl) showed that the presence of E.coli M2626 lys (50 µl of a 0.5 mg dry wt ml^{-1} suspension) or Strep. faecalis ATCC 9790 (50 µl of a 0.25 mg dry wt ml^{-1} suspension) did not affect the efficiency of counting of the (extracellular) label, in either scintillant system. Efficiencies of ^{14}C -counting were 70% for both the BBOT/ toluene system and the Soluene/NE 260 system.

2-7-3-2 The Monitoring of Label in the Cells and Medium During Ala- [^{14}C] Ala Uptake

The amount of label associated with the cells and with the incubation medium was assayed simultaneously for the uptake of Ala- [^{14}C] Ala by E.coli M2626 lys and Strep. faecalis ATCC 9790 using the BBOT/toluene scintillant system (Fig. 2-9). In contrast to the situation for Strep. faecalis, for E.coli there is a decline in the aggregate amount of label monitored as the incubation proceeds. This decline also occurred in the presence of chloramphenicol (data not shown). There are two possible explanations for this phenomenon:

i) Labelled alanine is being metabolised in such a way that label is being lost (e.g. as $^{14}CO_2$)

ii) Label associated with E.coli cells is not counted as efficiently as that in the medium.

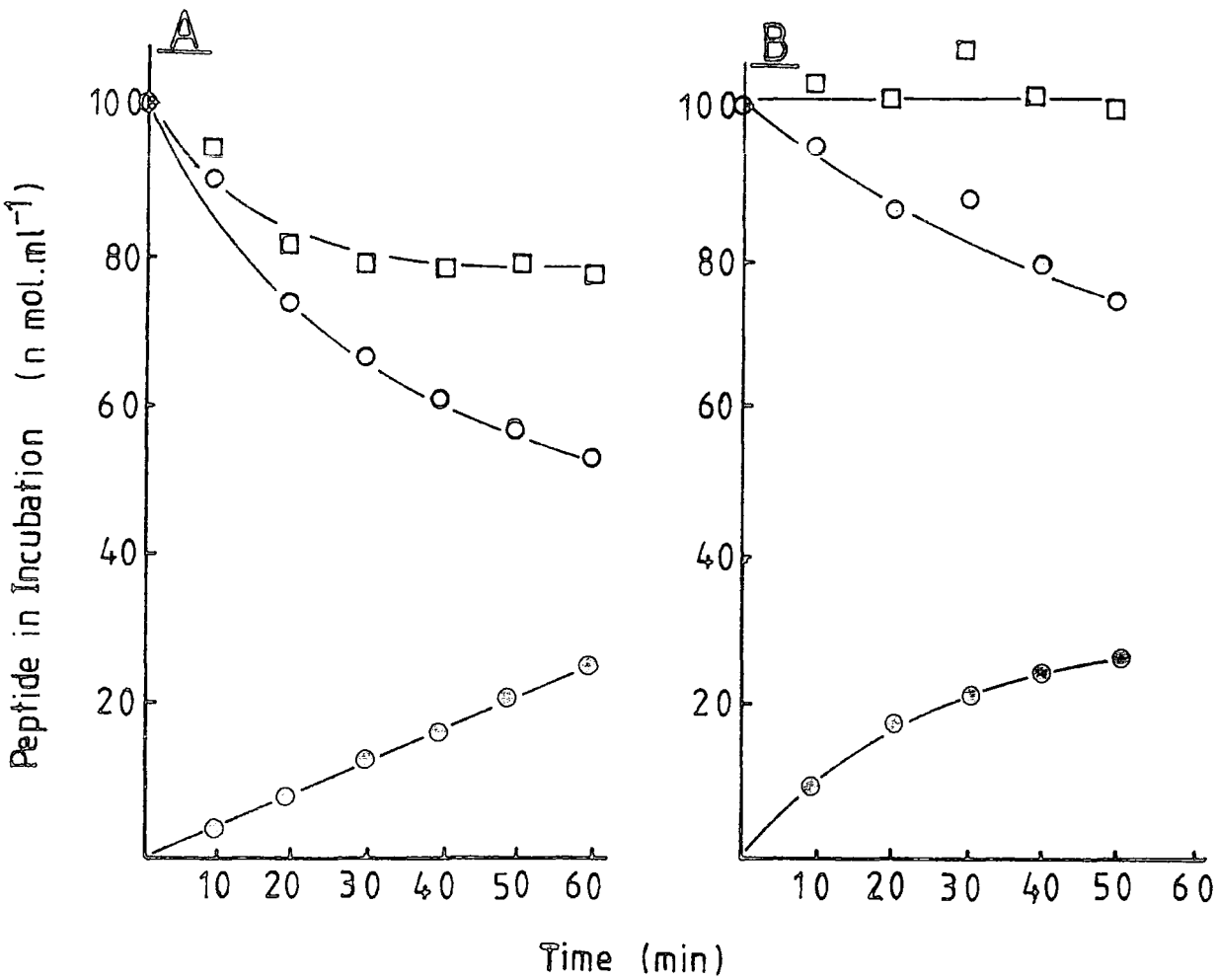


Fig 2-9 Distribution of Label During Ala-[^{14}C] Ala Uptake in E.coli W M2626 lys and Strep. faecalis (faecium) ATCC 9790.

The radiolabel present in samples of bacteria (●) and incubation medium (o) during uptake of 0.1 mM Ala-[^{14}C] Ala was assayed as described in the text. Total counts present (□) were obtained by addition.

A) E.coli W M2626 lys — 0·4 mg dry wt ml^{-1}

B) Strep. faecalis (faecium) ATCC 9790 — 0·25 mg dry wt ml^{-1}

It has already been shown (Section 2-6-3-2) that label inside Strep. faecalis cells is counted as efficiently as that in the medium, and logically the same should apply for E.coli. The β-emission of ^{14}C, with a peak at 156 KeV, should not be significantly attenuated by materials of the density found in bacterial cells, and the molecular form of the label (e.g. incorporation into protein) should have no effect; attenuation of ^{14}C emission in whole cells of E.coli has been calculated as less than 4% (Zarybnicky and Reich, 1980). For the data obtained here (Fig. 2-9) to be explained in these terms, attenuation in the order of 50% would be needed; this seems unlikely in the extreme. Whilst the likely explanation of the loss of counts is thus as in i) above, in further experiments a solubilizing scintillant system was used so that explanation ii) could be totally eliminated.

2-7-3-3 Loss of Label from Peptides in a Range of Microorganisms

Incubations of microorganisms with peptides were set up and sampled as in Section 2-7-2-2 iii). Comparisons of the counts present in 50 µl samples with or without microorganisms (Table 2-5) show that, except in Strep. faecalis, incubation with peptides containing [^{14}C] Ala, Gly or Phe leads to the loss of label from the incubation. As Soluene/NE 260 scintillant was used in most cases, there can be no question of attenuation of β-emission. It seems likely that the losses are as $^{14}CO_2$, as has been reported for [1-^{14}C] Gly-Phe and Gly-[U^{14}C]Phe uptake in Staph. aureus (Perry and Abraham, 1979). It is interesting to note that for Ala-[^{14}C]Ala (P), no loss of label was detected (Table 2-5). Whilst this result may be due in part to low uptake rate, the free aminoethylphosphonic acid (Ala(P))

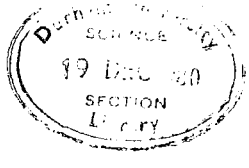


Table 2-5 The Loss of Counts During Incubations with Labelled Peptides

Organism	Peptide	Counts Present (%)
E.coli M2626 lys	Ala-Ala	66
	Ala-Ala(P)	100
E.coli MRE 160	Ala-Ala	69
S. typhimurium Leu 485	Ala-Ala	88
Sacc. cerevisiae Σ1278b	Ala-Ala	87
Strep. faecalis ATCC 9790 (faecium)	Ala-Ala	100
	Ala-Ala(P)	100

The counts present in samples of 15 min incubations with 0.1 mM peptide (Ala-Ala, 3.09×10^{-2} μCi ml^{-1}; Ala-Ala(P), 2.21×10^{-2} μCi ml^{-1}) with and without microorganisms were assayed by scintillation counting using Soluene/NE260 scintillant (BBOT/toluene for Strep. faecalis). Results are expressed as the percentage of total added counts which were detected after incubation with microorganisms.

moiety does not undergo extensive metabolism (Atherton et al., 1979b) and so presumably no loss of label occurs.

2-7-3-4 The Uptake of Labelled Peptides by E.coli - Monitoring by Fluorescamine and Label Uptake

The uptake of Ala-[$U^{14}C$]Ala, Ala-Ala- [$U^{14}C$] Ala (Fig 2-10), Gly-[$U^{14}C$] Phe and [1-^{14}C] Gly-Phe (Fig 2-11) by E.coli. W M2626 lys was monitored simultaneously by scintillation counting of label in cells (using Soluene/ NE260 scintillant) and by manual fluorescamine assay of supernatant solutions. In each case, the initial rate from the radiotracer assay was considerably lower. For alanyl peptides (Fig. 2-10) both metabolic loss of $^{14}CO_2$ and exodus of [^{14}C] Ala may contribute to this lower apparent rate; exodus of Ala has been detected by dansylation very early in similar assays (J.W. Payne, personal communication). Whereas the initial rate of accumulation of label from [1 - ^{14}C] Gly-Phe was 6 nmol min^{-1} mg dry wt^{-1} and from Gly-[$U^{14}C$]- Phe was 12 nmol min^{-1} mg dry wt^{-1}. the rates of uptake determined from the fluorescamine assay were identical (15 nmol mg dry wt^{-1} min^{-1}). This could in part be due to more rapid exodus of Gly than Phe (Payne and Nisbet, 1980b) and partly to the greater availability of the label in [1-^{14}C] Gly for decarboxylation, as has been demonstrated for Staph. aureus (Perry and Abraham, 1979).

2-7-3-5 The Uptake of Ala- [^{14}C] Ala by Sacc. cerevisiae - Monitoring by Fluorescamine and Label Uptake.

The uptake of Ala-[^{14}C] Ala by Sacc. cerevisiae Σ1278b was monitored by fluorescamine and label accumulation (Fig. 2-12). The lower rate of uptake detected by label accumulation could be due in part to the exodus of deaminated derivatives (Woodward and Cirillo, 1977) and partly to decarboxylation reactions following transamination of the

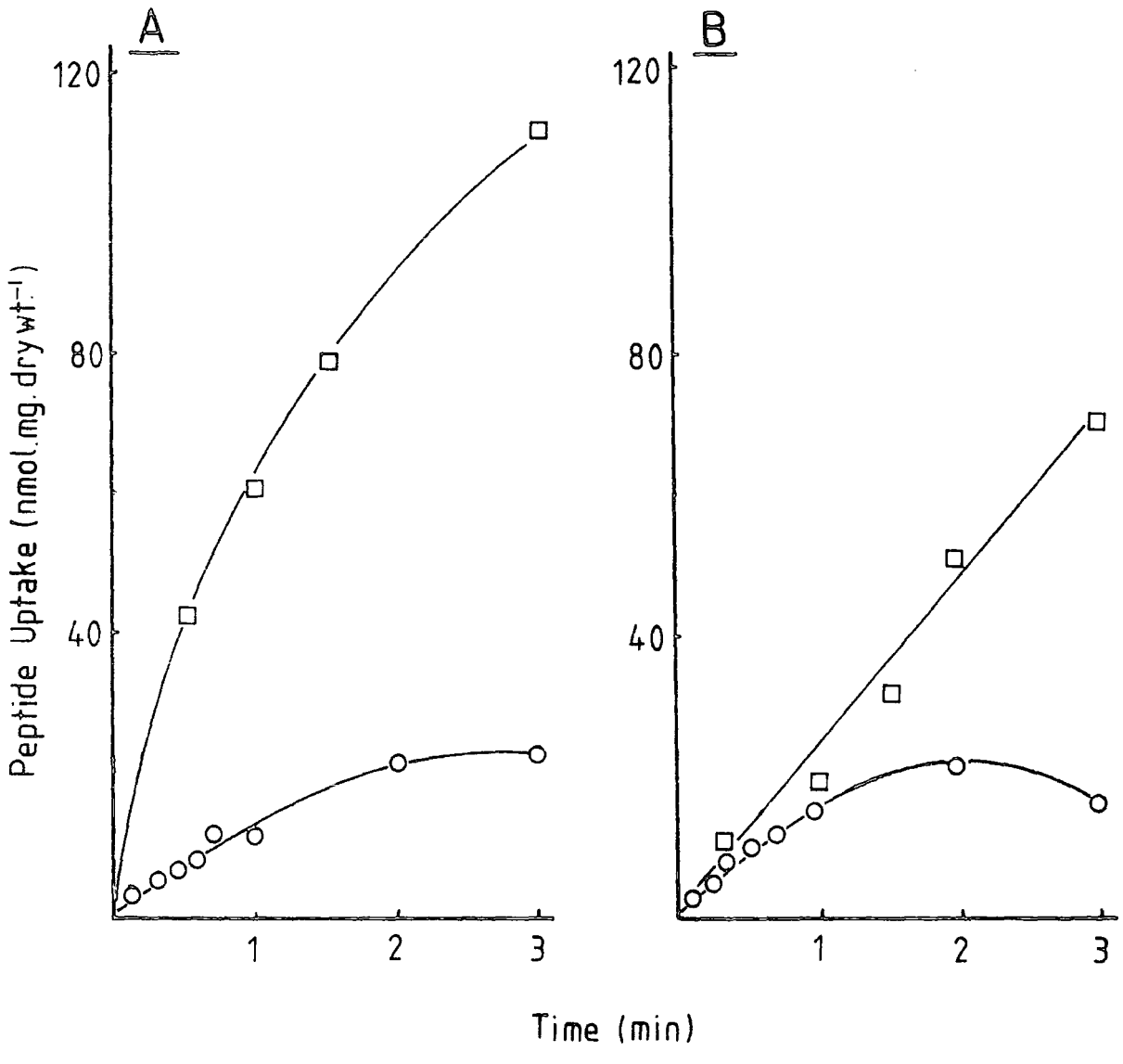


Fig 2-10 Comparison of Radiotracer and Fluorescamine Methods for the Assay of Peptide Uptake in E.coli.

E.coli W M2626 lys was resuspended in phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v) to a density of, A) 0.33 mg dry wt ml^{-1}, B) 0.5 mg dry wt ml^{-1}. Uptake of, A) Ala-[^{14}C]Ala and B) Ala-Ala-[^{14}C]Ala was assayed by the standard radiotracer (O) and fluorescamine (□) methods.

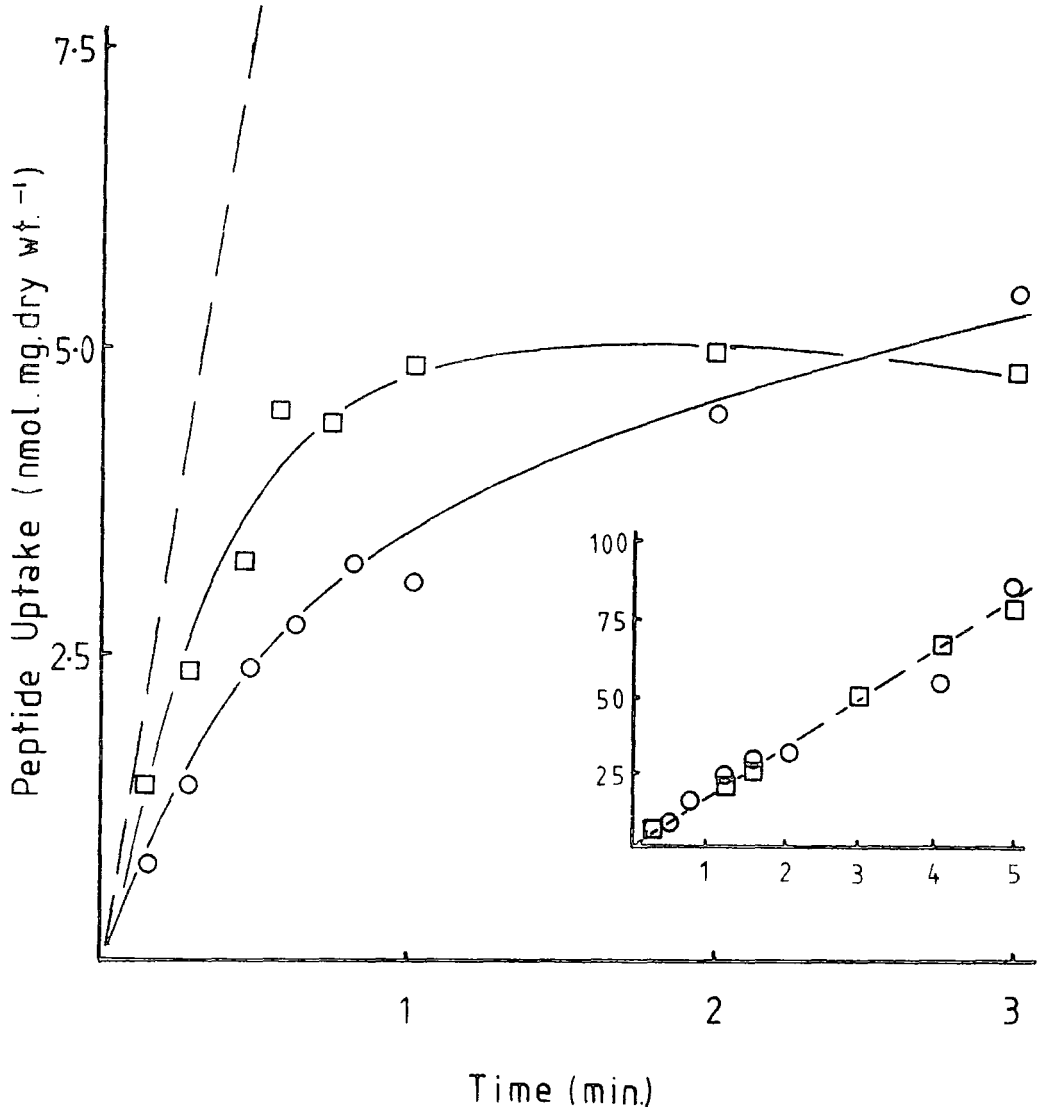


Fig. 2-11 Comparison of Radiotracer and Fluorescamine Methods for the Assay of Peptide Uptake by E.coli.

E.coli W M2626 lys was resuspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v) to a density of 0.4 mg dry wt ml^{-1}. Uptake of Gly-[$U^{14}C$] Phe (□) and [$1^{14}C$] Gly-Phe (○) was assayed by the standard radiotracer (main figure) and fluorescamine (inset) methods. (---: rate from fluorescamine assay)

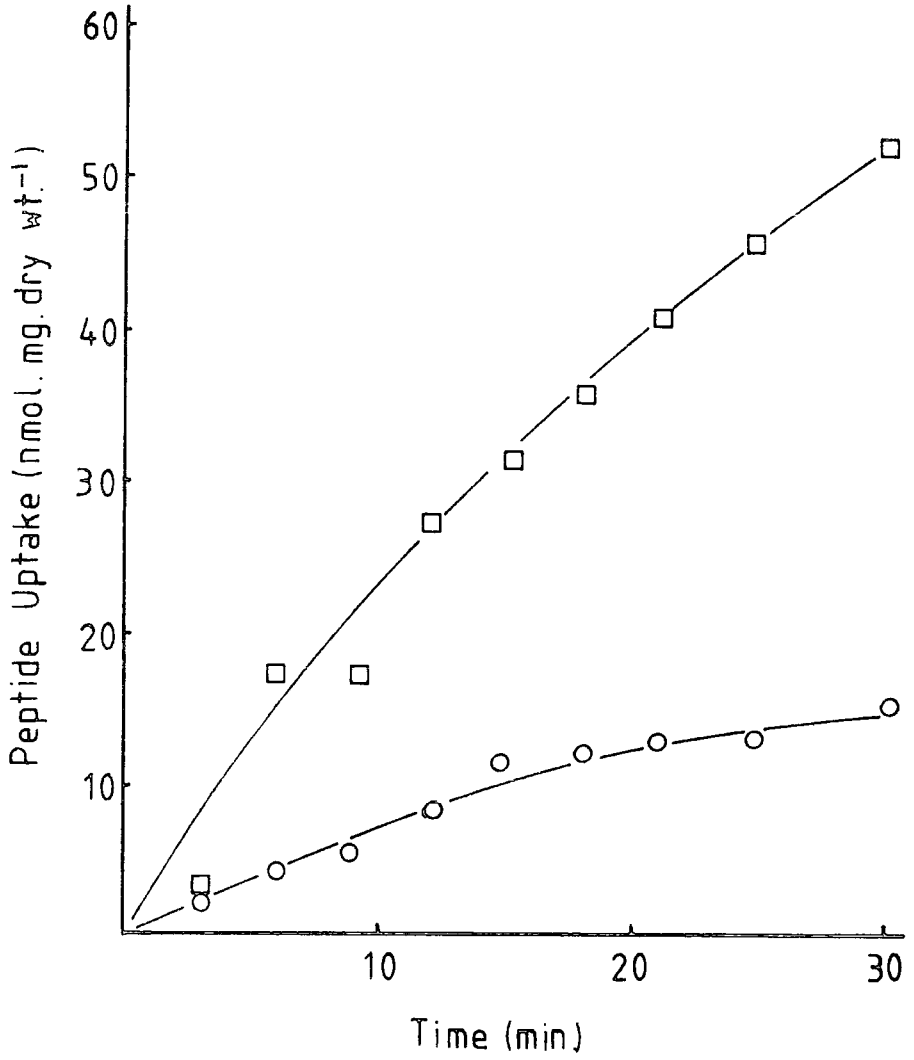


Fig 2-12 Comparison of Radiotracer and Fluorescamine Methods for the Assay of Peptide Uptake in Sacc. cerevisiae.

Strain Σ1278b was resuspended in citrate-phosphate (pH 4.5, 25 mM) with glucose (1% w/v) to a density of 1.5 mg. dry wt. ml^{-1}. Uptake of Ala-[^{14}C]Ala (0.1 mM, 3.09 x 10^{-2} μCi ml^{-1}) was assayed by the radiotracer (○) or fluorescamine (□) methods.

intracellularly liberated Ala (Section 1-2-4-3).

2-7-4 Concluding Remarks

The data presented show that the methods commonly used for assaying peptide uptake by label accumulation are open to misinterpretation. The problem of amino acid exodus can in part be overcome by very rapid sampling, but in organisms such as E.coli exodus may be significant after even a few seconds. The loss of label by reactions such as decarboxylation is potentially more serious. Although in standard radiotracer uptakes samples are taken very rapidly, organisms, once filtered, are generally left for several minutes before inactivation (by heat or solubilization) and metabolism can clearly continue during this period.

These problems of metabolism and exodus as other molecules, or loss of label to the gas phase,are clearly not likely to be confined to peptides; gross underestimates of rate of uptake and accumulation of any substrate could be made on the basis of radiotracer studies unless the substrate studied is metabolically inert. Although investigators are commonly aware of this potential difficulty, comparative methods to evaluate its significance are not normally available. This problem is clearly of great relevance to studies in which kinetic parameters of uptake are determined, or the stoichiometry of energy coupling is investigated.

CHAPTER 3

PEPTIDE TRANSPORT IN SACCHAROMYCES CEREVISIAE

3-1 Introduction

Peptide transport in Sacc. cerevisiae has been previously investigated (Section 1-2-2) but, when the present work was undertaken, some aspects were poorly understood. In particular, the number of major permeases and their specificities were unknown, and there had been no demonstration of intact peptide accumulation.

In this chapter, it is shown by a combination of studies of competition for uptake and the isolation of a transport-deficient-mutant that there is one major peptide permease in Sacc. cerevisiae, and its substrate specificity is investigated. Intact accumulation of sarcosyl peptides is also demonstrated.

3-2 Materials

Bacilysin was a gift from Prof. E.P. Abraham, Oxford University; Lys-Lys and His-His were gifts from Dr. J. Morley, ICI Pharmaceutical Division, Alderley Park, Cheshire. Glycyl-sarcosyl-sarcosine (Gly-Sar-Sar) was a gift from Dr. S. Wilkinson, Wellcome Research Laboratories. All other peptides were purchased from Sigma (London) Ltd., Poole, Dorset, or Uniscience Ltd., Cambridge. Reagents for the assay of peptides were as in Section 2-2. All other reagents were of analytical grade.

3-3 Organisms and Culture Conditions

3-3-1 Organisms

Sacc. cerevisiae haploid wild-type strain Σ1278b (mating type α) was used as the test organism in these studies. Mutants lacking amino-acid permeases but otherwise isogenic with strain Σ1278b were of the following genotypes: strain 2512c, gap (general amino-acid permease deficient); strain MG 276, met-pI (methionine permease

deficient); strain 5156d, gap, met-pI; strain CR 10, gap, arg-pI (general amino acid and arginine permease deficient). Mutants 2512c, MG 276 and 5156d were isolated by Gits and Grenson (1967) and Grenson et al.,(1970). Mutant CR 10 (previously referred to as strain D) was isolated by P. Earnshaw (Seaston et al., 1973). All cultures were kindly provided by Prof. A.A. Eddy, Dept. of Biochemistry, UMIST, Manchester.

3-3-2 Media and Growth Conditions

Cultures were maintained on 1.5% (w/v) agar slopes containing glucose (2%, w/v), peptone (2%, w/v), yeast extract (1%, w/v) and a Yeast Nitrogen Base lacking amino acids (Difco 0919-15), in which ammonium sulphate (0.5% w/v) was the nitrogen source. Subculturing was routinely carried out every 6 months, and slopes were stored at $4^{\circ}C$.

Cultures were transferred from slopes to the same medium without agar, grown overnight at $28^{\circ}C$, with shaking, and were then subcultured into liquid medium without peptone or yeast extract and regrown. Cells for transport assays were routinely grown in 50 ml batches, in a medium containing glucose (2%, w/v), proline (4 mg ml^{-1}, as sole nitrogen source) and a Yeast Nitrogen Base lacking amino acids and ammonium sulphate (Difco 0335-15) (referred to as proline medium), as it has been shown that the rate of peptide transport is approximately ten times greater in proline-grown cells than in ammonia-grown ones (Becker and Naider, 1977; Section 3-5-3-2). After the initial subculturings, cells were kept in liquid proline medium at $4^{\circ}C$ for up to two weeks and subcultured from this stock as required.

Growth of liquid cultures was monitored by measuring the

absorbance of a portion of the culture 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 up to an A_{660} of 0.5. Determinations of dry weight and viable count showed that an A_{660} of 0.1 was equivalent to 0.1 mg dry wt ml^{-1} and 2.3×10^6 colony forming units ml^{-1}.

Growth curves in both ammonia and proline liquid medium were obtained. These yielded a generation time of 2.4 h on ammonia, which is in agreement with a previous figure for this strain (Dubois et al., 1974), and 3.6 h on proline. The slower growth on proline is again in accord with literature information (Watson, 1976).

3-4 The Response of Strain Σ1278b to Potentially Toxic Peptides, and Isolation of a Bacilysin-Resistant Mutant

3-4-1 Introduction

The studies described in this section were carried out to discover a peptide that is toxic to Sacc. cerevisiae, and to use such a peptide to isolate a resistant mutant. Such resistant mutants may be altered in peptide transport, altered in the target site of the toxic moiety or, in the case of a peptide containing a toxic amino acid, defective in peptidase action. Previous experience with other organisms has suggested that transport-deficient mutants are the most frequently obtained in response to toxic peptides (Section 1-1-9).

The peptides used as potential antibiotics were as follows: a) peptides containing norleucine or norvaline residues; b) Lys-Lys; and c) bacilysin. Norleucine acts as an antagonist of methionine in bacteria, and norvaline is an antagonist of valine. Peptides containing these residues are therefore toxic to bacteria, e.g. E.coli.

The growth of Sacc. cerevisiae on poor nitrogen sources such as proline is inhibited by the addition of basic amino acids to the medium (Sumrada and Cooper, 1976). This effect occurs because of depletion of other basic amino acid pools, the addition of lysine, for instance, causing severe depletion of arginine and histidine (Sumrada and Cooper, 1978). In the present studies Lys-Lys was used as a source of lysine.

The dipeptide antibiotic bacilysin (Figure 3-1) is a natural product of Bacillus subtilis (Walker and Abraham, 1970a,b). The C-terminal amino acid, anticapsin, acts as a potent inhibitor of glucosamine sythetase (Kenig et al., 1976). Bacilysin is active against Candida albicans and a range of bacteria, and its action is antagonised by various dipeptides (Kenig and Abraham, 1976), suggesting a common uptake route. Competition for uptake between bacilysin and natural peptides has been shown in Staphylococcus aureus (Perry and Abraham, 1979).

3-4-2 Methods

Growth in liquid medium in the presence of inhibitors was followed by standard methods (Section 3-3-2). Between 5 and 10 ml of proline medium in test tubes was routinely used. Potential inhibitors were also assayed by plate methods. An agar plate (1.5% w/v agar) of proline medium was spread with 0.1 ml of an overnight culture of strain Σ1278b (approx. 2×10^6 cells). A sterile filter paper disc (4 mm diameter) was placed in the centre of the plate, and a solution of the peptide to be assayed (10 µl) was pipetted onto the disc. In some experiments, crystals of the peptide were placed directly onto the spread plate in order to

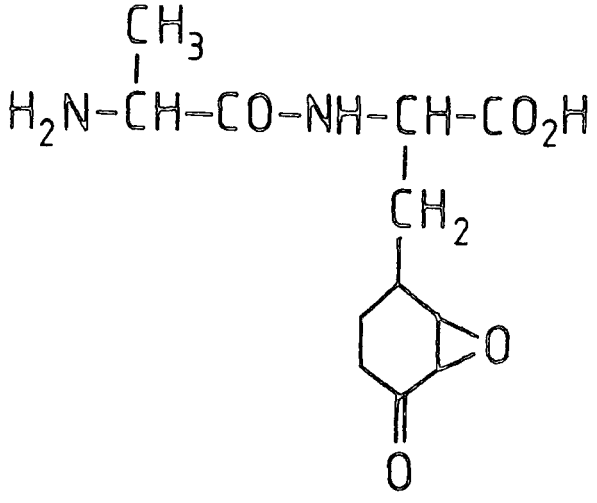


Fig 3-1 The Structure of the Dipeptide-Mimetic-Antibiotic Bacilysin

Bacilysin (L-alanyl-anticapsin) acts as a 'warhead delivery' antibiotic.

achieve sufficiently high concentrations. Plates were routinely incubated at 28°C for 48 h, after which time a lawn of cells had grown. A clear zone at the centre of the lawn was observed if the peptide added was growth inhibitory.

For counter-selection of presumptive peptide-transport deficient mutants, agar plates containing no nitrogen source were used. After the spreading of a lawn of cells, crystals of Ala-Ala were placed at the centre of the plate. After incubation, a zone of growth was observed if the cells were able to utilize Ala-Ala as a nitrogen source.

3-4-3 Results

3-4-3-1 Growth Inhibition Studies

The generation times of strain Σ1278b in liquid culture in the presence of potential inhibitors (Table 3-1) show that of the peptides tested, only L-norleucyl-L-norvaline was growth inhibitory. Free L-lysine was extremely growth inhibitory, thus confirming published results (Sumrada and Cooper, 1976).

Results from plate tests (Table 3-2) show that of the peptides tested, only bacilysin was sufficiently inhibitory to give a pronounced inhibition zone.

3-4-3-2 Isolation of Peptide-Transport Deficient Mutant

Bacilysin crystals (0.2 - 0.3 mg, 50 - 75 units) were placed in the centre of an agar plate of proline medium spread with strain Σ1278b (approx. 2×10^6 cells). After incubation at 28°C for 3 days, there was a pronounced inhibition zone (50 mm diameter) which contained 15 separate colonies. Eight colonies were picked off the plate and grown separately in liquid medium for 2-3 days to a cell density of 2×10^7 cells ml^{-1}. Samples (0.1 ml) of each presumptive mutant were

Table 3-1 Inhibition of Growth of Strain Σ1278b

Potential Inhibitor	Concentration (mM)	Generation Time (hrs)
None	-	3-4
Gly-Gly-Nle	2	4
Gly-Nle	2	4
Ala-Nva	2	4
Nva-Gly-Gly	2	4
Nle-Nva	2	4
	10*	10*
Lys	0.125	9
	1.25	> 24
	12.5	No Growth

The generation time of strain Σ1278b in the presence of potential inhibitors of growth was measured. Cells were grown in liquid proline medium at 28°C.

(*Cells previously grown in the presence of 2 mM L-norleucyl-L-norvaline and then subcultured).

Table 3-2 Inhibition of Growth of Strain Σ1278b

Peptide	Amount Added to Plate (nmol)	Diameter of Inhibition Zone (cm)
Gly-Gly-Nle	100	None
Nle-Nva	100	None
Lys-Lys	250	None
	750	Slight Halo Around Disc
Bacilysin	18	None
	≈ 900*	5

Solutions of peptides (10 µl) were added to filter paper discs at the centre of agar plates spread with strain Σ1278b (* peptide added directly to plate as solid). Plates were incubated for 2-3 days at 28°C.

then spread onto plates containing no nitrogen source, and as a counterselection procedure crystals of Ala-Ala(approx 2 mg, as a nitrogen source) were placed in the centre of the plates. After incubation at 28°C for 3 days, the presumptive mutants showed very little growth, whereas the parent strain Σ1278b gave a growth lawn. After a further 3 days incubation, plates spread with presumptive mutants showed approximately 10^2 - 10^3 isolated colonies, which were presumably of parental phenotype. (These isolated colonies were only noticeable after this long incubation period because of very slow growth on Ala-Ala as a nitrogen source, whereas in the case of the parent strain a lawn was noticeable after a shorter incubation, even though individual colonies were presumably still very small). As each plate was spread with approximately 2 x 10^6 cells, the isolated colonies on plates spread with presumptive mutants represent between 0.005 and 0.05% of the total. It seems likely that these were present as contaminants when the original colonies were picked out of the bacilysin inhibition zone, although they could have arisen as revertants during the initial liquid culture of the colonies.

One of the presumptive mutant colonies showing a low level of contamination was chosen, and 0.1 ml of a 10^{-4} dilution of the stored liquid culture was spread in duplicate onto proline plates. Crystals of bacilysin (approx. 0.5 mg) were placed in the centre of one plate, and both were incubated at 25°C for 4 days. Growth rate of cells was equivalent on both plates, no inhibition zone was evident and each plate contained approx. 50 colonies. A colony was picked from the centre of the bacilysin-treated plate, and was grown up in proline liquid medium. This mutant was subsequently shown to be deficient in

the uptake of small peptides (Section 3-5-4-4) and was designated Σ1278b gpp⁻ (general-peptide-permease deficient).

3-4-4 Discussion

It was demonstrated (Table 3-1) that L-norleucyl-L-norvaline was growth inhibitory to strain Σ1278b. The toxic effect, however, was only obtained during the long exposure to the compound at high concentration. In contrast, in liquid cultures of E.coli, 0.5 mM L-norleucyl-L-norvaline and 0.2 mM glycyl-L-norleucine were both very growth inhibitory (R. Alves, personal communication).

Lysine proved to be very growth inhibitory to strain Σ1278b, when assayed in liquid culture (Table 3-1). Lack of sufficient quantities of Lys-Lys precluded its use in a similar way, and when assayed by a plate method it was not growth inbibitory (Table 3-2). If larger quantities of Lys-Lys could have been used, it seems likely that growth inhibition would have been observable, although the rate of uptake and/or cleavage may not have proved to be sufficient to parallel the effects of free lysine.

The inhibitory effects of the above peptides were not investigated further, as bacilysin proved to be sufficiently toxic to strain Σ1278b to allow the isolation of a resistant strain by a one-step procedure.

3-5 The Characterization of Peptide Uptake in Strain Σ1278b and Some Isogenic Mutants

3-5-1 Introduction

The uptake of peptides in Sacc. cerevisiae was assayed using the two fluorescence methods described in detail in Chapter 2. Disappearance of peptide from the medium was followed using both

dansyl chloride and fluorescamine. In addition, the presence of peptides and amino acids in cell extracts was examined by dansylation.

The uptake of a wide range of natural peptides was monitored by their disappearance from the medium. Because of the high intracellular peptidase levels, intact accumulation could not be demonstrated. However, peptides containing sarcosine are very resistant to enzymic hydrolysis, and intact accumulation of these substrates was detectable. Intact sarcosine peptide uptake has previously been demonstrated in E.coli (Payne and Bell, 1977b, 1979), mammalian intestine (Addison et al., 1972), and germinating barley embryos (Higgins and Payne, 1977).

Direct uptake assays and competition studies using both natural and sarcosine peptides were carried out in strain Σ1278b and its amino acid and peptide-transport-deficient mutants. As a result, it was possible to demonstrate a single major peptide permease, and to investigate its substrate specificity.

3-5-2 Methods

3-5-2-1 Monitoring Peptide In Medium

Cultures were routinely grown at 28°C in liquid proline medium to a cell density of 0.2 - 0.7 mg dry wt ml^{-1}. Organisms were harvested by membrane filtration (25 mm diameter, 0.45 μm pore size, Oxoid), washed on the filter with 4 volumes of citrate/phosphate buffer, pH 4.5 (20 mM with respect to phosphate) at room temperature, and resuspended in the same buffer so that a ten-fold dilution of the suspension gave a cell density of 0.2 - 0.7 mg dry wt ml^{-1}.

Portions of this suspension were added to tubes containing

distilled water and glucose, to give a final buffer concentration of 10 mM phosphate, and a final glucose concentration of 0.8% w/v. Final volumes for uptake assays were routinely 1 or 2 ml.

Uptake assays were carried out at 28°C in a shaking waterbath. After 10 min pre-incubation, peptide was added (from a 1 mM stock solution in distilled water) to a concentration of 0.1 mM (except where indicated in results). After appropriate intervals (routinely every 5 min for 20 min) samples (0.3 - 0.4 ml) were removed in a 1 ml syringe and immediately freed from yeast by passing them through a membrane filter (13 mm diameter, 0.45 µm pore size, Oxoid) using a Swinnex (Millipore) filter holder. Filtrates were collected in small vials, and were stored at -20°C.

Examination of the samples of incubation medium by dansylation was performed on 100 µl aliquots of the filtrates, treated by the standard method (Section 2-3-2). Assay of peptide concentration by fluorescamine was performed on 50 µl aliquots, again by using the standard method (Section 2-4-2).

3-5-2-2 Examination of Cell Extracts

Harvesting and incubation with peptide was performed as in Section 3-5-2-1. After incubation with peptide, a 1 ml sample of the yeast suspension was harvested on a membrane filter under water vaccuum, immediately washed with distilled water (40 ml., room temp.) 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 debris, and the filtrate was stored at -20°C. Portions (100 µl) of the filtrate were dansylated by the standard procedure.

3-5-3 Results and Discussion - General Features

3-5-3-1 Effect of Glucose Concentration on Peptide Uptake

The effect of glucose concentration on the rate of Leu-Leu uptake by strain Σ1278b was followed by the dansylation of medium samples (Table 3-3). There was little uptake in the absence of glucose, and uptake increased with glucose concentration up to 0.4% glucose; above this level the rate of Leu-Leu uptake did not increase. A glucose concentration of 0.8% was therefore used in subsequent experiments, this representing 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 (Section 2-3-4-2).

3-5-3-2 Effect of Nitrogen Source for Growth on Peptide Uptake

The rate of Ala-Ala uptake in ammonia-grown cells was followed by the standard fluorescamine procedure, and was found to be 0.17 nmol min^{-1} mg dry wt^{-1}. This compares with values in proline-grown cells of 1.0 - 4.3 nmol min^{-1} mg dry wt^{-1} (Section 3-5-4-1). This is in agreement with the ratio of transport rates found for Met-Met-Met uptake (Becker and Naider, 1977). It seems that peptide uptake, like many functions of amino acid metabolism in Sacc. cerevisiae, is under ammonia repression (see Section 1-2-4-4).

3-5-3-3 Uptake of Peptides is not Accompanied by Amino Acid Exodus

The uptake of a range of homo di- and tripeptides by strain Σ1278b was followed by the dansylation method (Table 3-4). In no case was there any evidence of exodus of the constituent amino acids of the peptide taken up (see for example Fig. 3-2, A-C). This is in contrast to the observations from similar assays in E.coli

Table 3-3 The Effect of Glucose Concentration on Leu-Leu Uptake by Strain Σ 1278b

Glucose Con. (% w/v)	Rate of Leu-Leu Uptake (nmol min^{-1} mg dry wt^{-1})
0	0.3
0.2	0.7
0.4	1.8
0.8	1.8

Uptake of Leu-Leu (0.1 mM) was monitored by periodic dansylation of medium samples during a 40 min incubation. Cell density 1.8 mg dry wt ml^{-1}.

Table 3-4 Uptake of Peptides by Strain Σ1278b

Peptide	Rate of Uptake (nmol min^{-1} mg dry wt^{-1})
Ala-Ala	2.4
Leu-Leu	1.0 - 2.5*
Val-Val	2.0
Phe-Phe	ND
Gly-Gly	ND
Ala-Ala-Ala	1.2
Leu-Leu-Leu	1.0
Val-Val-Val	0.3

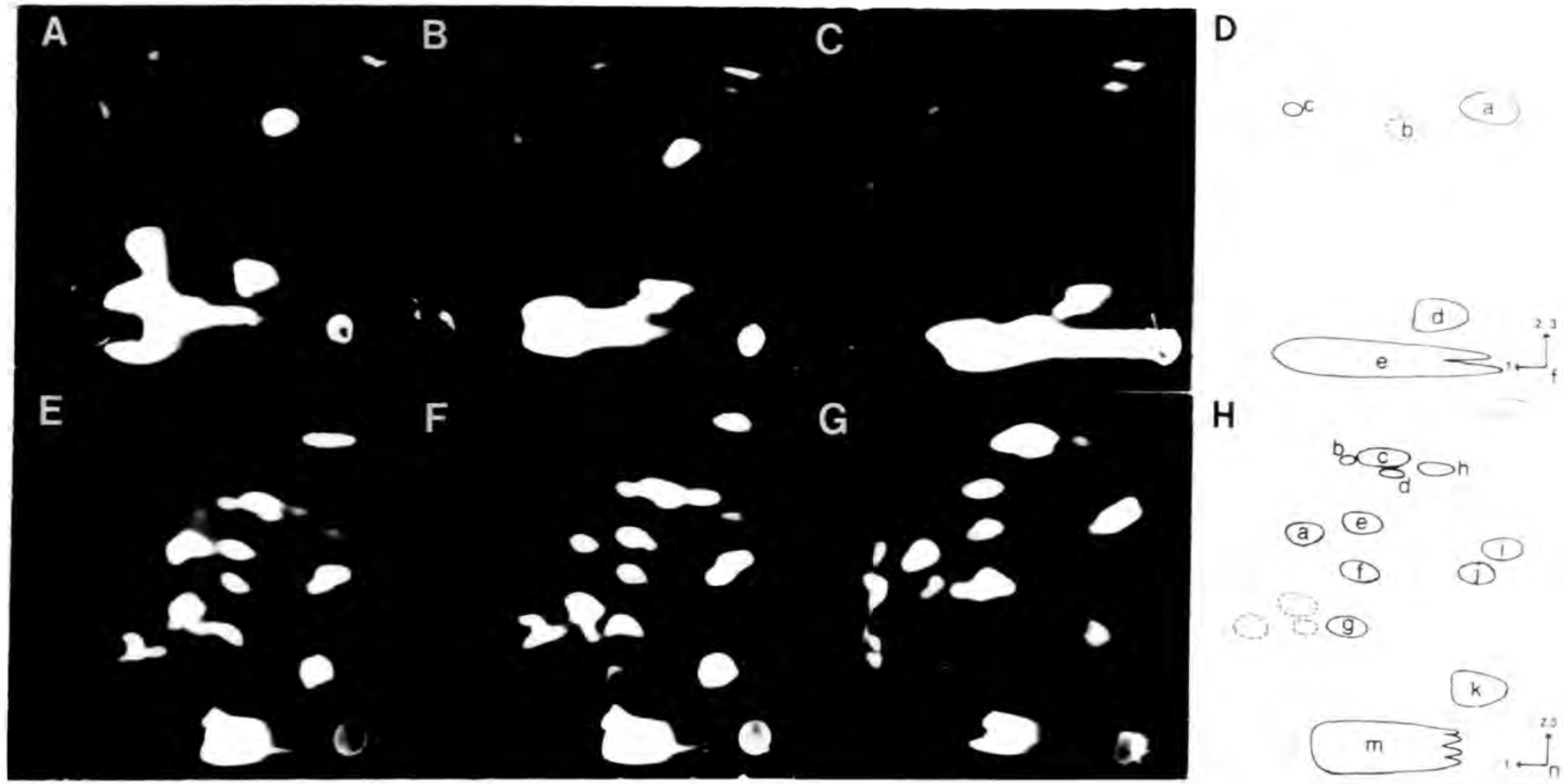
Uptake of peptide (0.1 mM) was monitored by dansylation of medium samples during 30 min incubations. Cell density 0.85 - 3.2 mg dry wt ml^{-1}.

(ND, not detectable; *, 3 determinations)

Fig 3-2 Chromatograms of Dansyl Peptide and Amino Acids from Incubation Media and Cell Extracts of Sacc. cerevisiae

A to C. Samples of medium during incubation of Strain Σ1278b (0.85 mg dry wt ml^{-1}) with Leu-Leu (0.1 mM). A) 0 min, B) 30 min, C) 60 min, D) Key to derivatives:
a) Leu-Leu, b) position of Leu, c) dansyl ammonia, d) diaminopimelic acid (standard), e) dansyl-hydroxide, f) origin.

E to G. Cell extracts of Strain Σ1278b (1.3 mg dry wt ml^{-1}) after 1 h incubation with: E) Gly-Sar (5 mM); F) Gly-Sar (5 mM) plus Leu-Leu (4 mM); G) no peptide, H) key to derivatives:
a) Gly-Sar, b) dansyl ammonia, c) Pro, d) Val, e) Ala, f) Gly, g) Glu, h) Leu, i) Lys, j) Orn, k) diaminop imelic acid (standard), m) dansyl hydroxide, n) origin.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(Payne and Bell, 1979), Strep. faecalis (Section 4-5) and Salmonella typhimurium (J.W. Payne, unpublished results).

The lack of exodus of amino acids from Sacc. cerevisiae may be due to several factors. Firstly, the intracellular volume is high relative to the rate of peptide transport, and hence such large intracellular concentrations do not arise as quickly as in, for instance, E.coli. Secondly, Saccharomyces species can accumulate large amounts of amino acids intracellularly. Studies on Sacc. uvarum (Indge et al., 1977) showed that [^{14}C] glycine could be accumulated to a level of 1 μmol mg dry wt^{-1} with little glycine entering the vacuole, and that preliminary nitrogen starvation increased accumulation to 2 μmol mg dry wt^{-1}, with much of the label being associated with the vacuole. (For a discussion of the role of the vacuole in the maintenance of amino acid pools see Section 1-2-4-5). Thirdly, it has been shown that some amino acids are metabolised rapidly by Sacc. cerevisiae and undergo exodus as deaminated derivatives (Section 1-2-4-3), which would presumably not react with the fluorescent labels used in these studies. In summary, it seems that, under the assay conditions used here, not enough amino acid is introduced to exceed the capacity of the cells, and that if any exodus occurs it is likely to be of deaminated derivatives.

3-5-3-4 Effect of Cell Density at Harvesting on Peptide Transport Activity

The rate of Ala-Ala uptake by strain Σ1278b did not show a correlation with the density at which organisms were routinely harvested, over the range 0.2 - 0.7 mg dry wt ml^{-1} (Fig. 3-3).

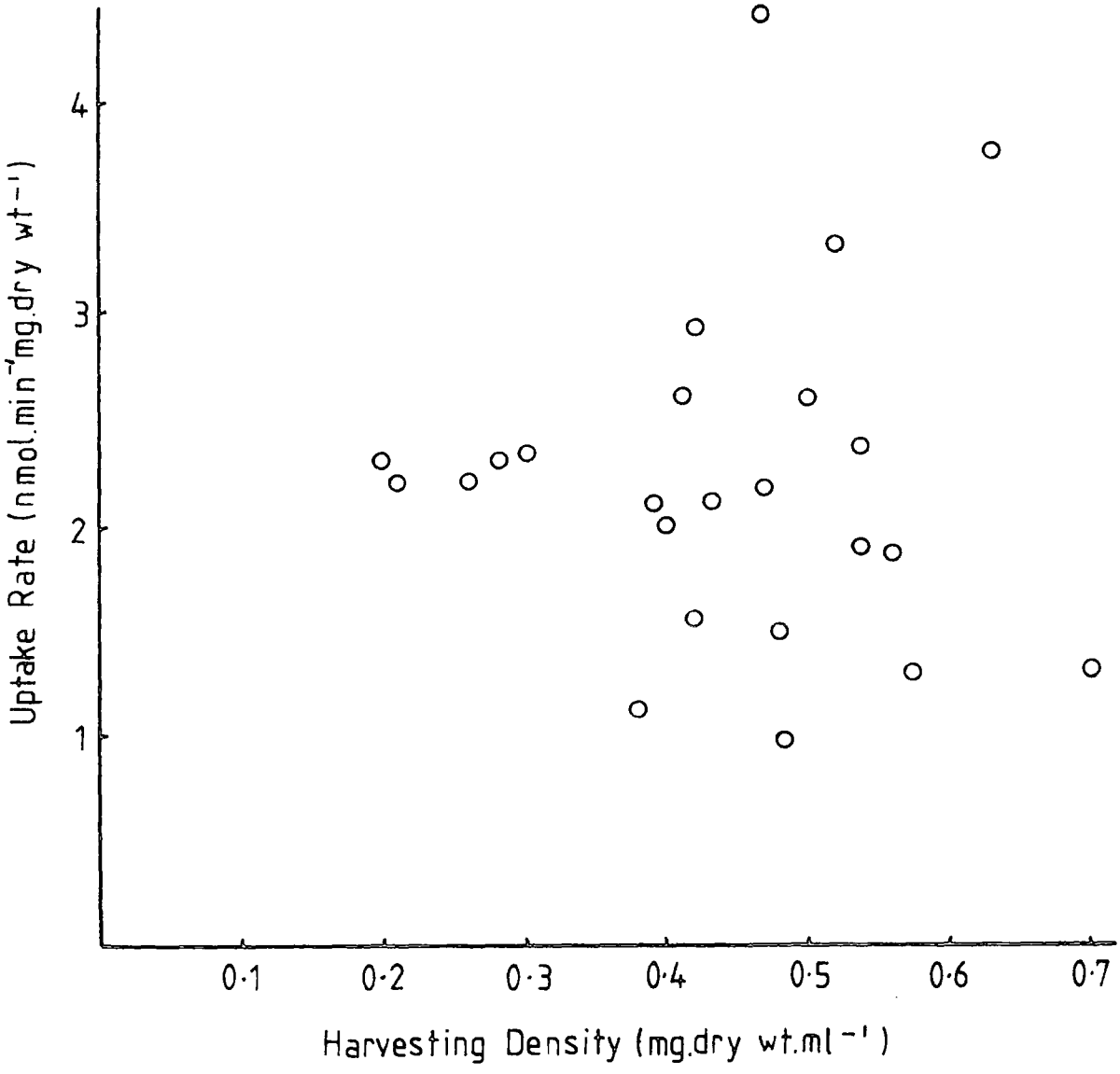


Fig 3-3 Effect of Cell Density at Harvesting on Peptide Transport Activity

Uptake of Ala-Ala (0.1 mM) by strain Σ1278b was followed by the standard fluorescamine method during 20 min incubations.

3-5-4 Results and Discussion - Uptake of Natural Peptides

3-5-4-1 The Specificity of Peptide Uptake by Strain Σ1278b

Using the standard fluorecamine procedure, the uptake of a wide range of peptides by strain Σ1278b was assayed (Table 3-5). The rate of uptake of Ala-Ala was variable (1.0 - 4.3 nmol. min^{-1} mg dry wt^{-1}) when measured for batches of yeast grown and harvested on different days (Fig. 3-3). 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.

The following characteristics of peptide uptake can be deduced from the results in Table 3-5:

a) Strain Σ1278b takes up a wide range of di- and tripeptides. However, the uptake of tetra- and pentaalanine was indetectable. This size limit is in agreement with that found in strain Z1-2D (Marder et al., 1977) but lower than that in some other strains (Section 1-2-2-4).

b) The transport system has a pronounced specificity for L-stereoisomers, uptake of peptides containing D-residues being indetectable. This is in agreement with literature evidence (Section 1-2-2-5).

c) Dipeptides with the sequence Ala-X are generally taken up more quickly than the corresponding X-Ala form.

d) Peptides containing basic amino acids (lysine and histidine) are transported rapidly, whereas those with acidic residues (glutamic acid) are poor substrates. Interestingly, whereas Ala-Lys and Lys-Ala were both transported more quickly than Ala-Ala, Lys-Lys was taken up more slowly.

e) Although Lys-Lys was taken up well by strain Σ1278b, it has been reported as being unable to support the growth of auxotrophic strains

Table 3-5 Uptake of Peptides by Strain Σ1278b

Peptide	Rate	Peptide	Rate	Peptide	Rate
L-Ala-L-Ala	100	Ala-Glu	ND	Glu-Ala	30-40
L-Ala-D-Ala	ND	Ala-Gly	30-50	Gly-Ala	<15
D-Ala-L-Ala	ND	Ala-His	135-180	His-Ala	80-160
D-Ala-D-Ala	ND	Ala-Leu	70-80	Leu-Ala	35-55
L-Ala-L-Ala-L-Ala	70-100	Ala-Lys	190-210	Lys-Ala	150-165
L-Ala-L-Ala-D-Ala	ND	Ala-Met	100-120	Met-Ala	55-65
L-Ala-D-Ala-L-Ala	ND	Ala-Phe	50-60	Phe-Ala	ND
D-Ala-D-Ala-D-Ala	ND	Ala-Pro	50-60	Pro-Ala	*60-80
Ala-Ala-Gly	30-60	Ala-Ser	50-70	Ser-Ala	50-70
Ala-Gly-Ala	100-120	Ala-Tyr	65-90	Tyr-Ala	45-55
Gly-Ala-Ala	55-60	Ala-Trp	60-100		
Glu-Ala-Ala	*ND	Arg-Arg	ND	Leu-Leu	40-60
Lys-Ala-Ala	100-120	Glu-Glu	*ND	Lys-Lys	45-60
Lys-Lys-Lys	ND	Gly-Gly	ND	Met-Met	50-70
Ala-Ala-Ala-Ala	ND	His-His	80-130	Phe-Phe	ND
Ala-Ala-Ala-Ala-Ala	ND				

Rates of uptake of peptide (0.1 mM) were determined by fluorescamine analysis of medium samples during 20 min incubations. Cell density 1.0 - 2.5 mg dry wt ml^{-1}. Rates are expressed relative to that of an Ala-Ala control (1.0 - 4.3 nmol min^{-1} mg dry wt^{-1}) determined for each separate experiment. Values given are the range of 2-3 determinations.

(N.D, not detectable; *, rate determined by dansylation method)

(Marder et al., 1977; Becker et al., 1973). This may reflect differences in the transport characteristics of the strains, or the difficulty in making firm statements about transport from the results of growth tests (Section 2-1).

f) Peptides containing glycine at the C- or N-terminus are generally poor substrates, whereas Ala-Gly-Ala is transported at a rate comparable to Ala-Ala and Ala-Ala-Ala.

3-5-4-2 Affinity of Peptides for Uptake in Strain Σ1278b

Uptake of Leu-Leu and Ala-Ala-Ala at different concentrations was studied using the fluorescamine method. Double reciprocal plots of the data (Fig. 3-4) yield K_m values of the order of 10^{-5} M for Leu-Leu and 10^{-4} M for Ala-Ala-Ala . These values are comparable with the value of 7.7×10^{-5} M obtained for Met-Met-Met by Becker and Naider (1977).

3-5-4-3 Competition for Peptide Uptake in Strain Σ1278b

The uptake of one peptide in the presence of another can readily be studied using the dansylation method. Thus, competition for uptake can be studied without using radiolabelled substrates. Results of such competition studies (Table 3-6) allow the following conclusions:

a) Di- and tripeptides display mutual inhibition of uptake. This implies that they share an uptake system, although such inhibition could arise from, say, competition for a common energy source. It is not possible to show that the inhibition is strictly kinetically competitive, as the error on the data points is too large for commonly used tests to be sensibly applied.

b) Leu-Leu is a much better inhibitor of Ala-Ala-Ala uptake than

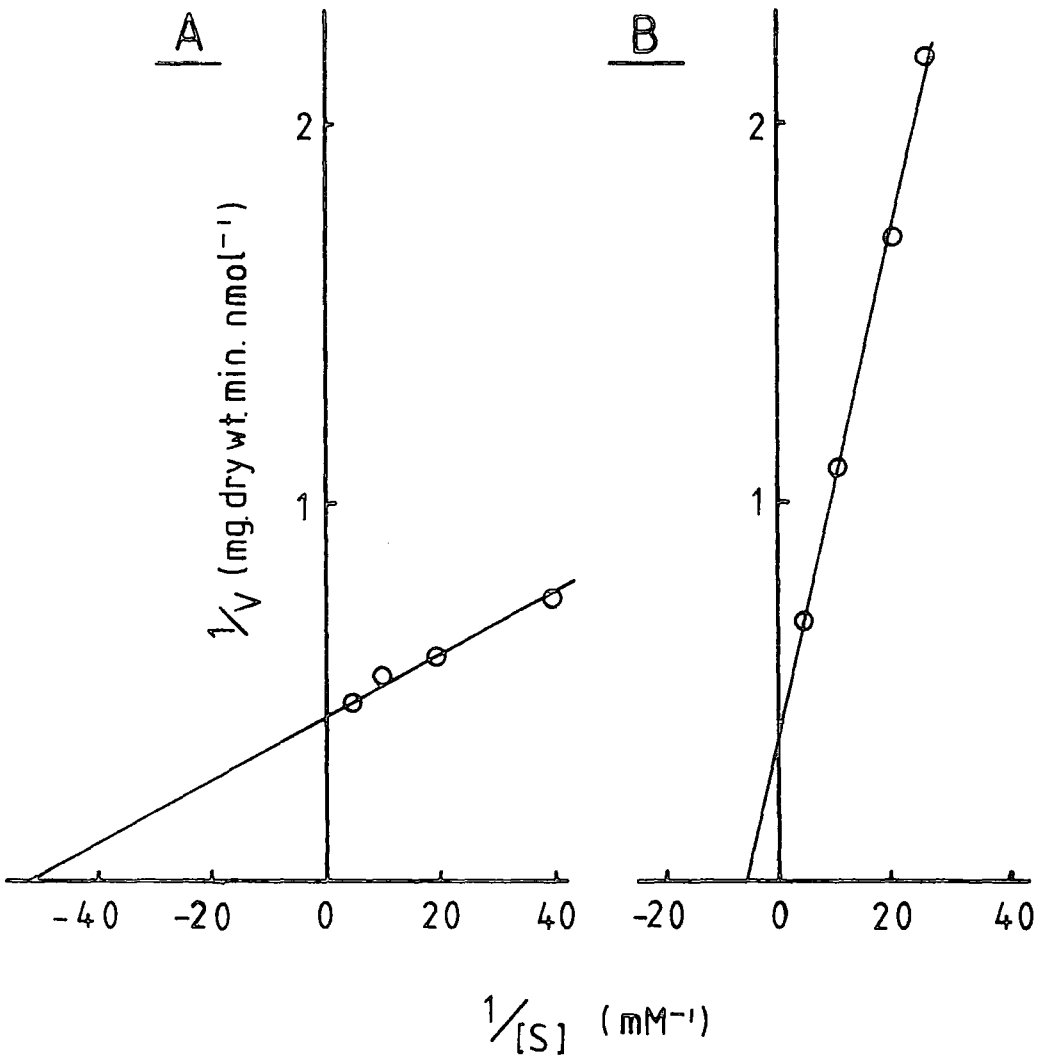


Fig 3-4 Affinity of Leu-Leu and Ala-Ala-Ala for Uptake by Strain Σ 1278b

Uptake rates of A) Leu-Leu and B) Ala-Ala-Ala were determined by the standard fluorescamine procedure.

Table 3-6 Competition for Peptide Uptake in Strain Σ1278b

Substrate	Inhibitor	Inhibitor Concentration (mM)	% Inhibition of Uptake
Ala_2	Met_2	0.1	60
	Leu_3	0.1	50
		1.0	100
Met_2	Ala_2	0.1	25
Leu_2	Ala_3	0.1	0
		0.2	15
		0.3	35
		0.4	70
Ala_3	Leu_2	0.1	50
		0.2	100
		0.3	100
		0.4	100
Leu_3	Ala_2	0.1	0

Uptake of peptide (0.1 mM) was followed by dansylation of medium samples during 30 min incubations. Cell density 1.5 - 2.0 mg dry wt ml^{-1}. Uptake inhibition is expressed relative to a control without inhibitor.

Ala-Ala-Ala is of Leu-Leu uptake. This in agreement with the uptake affinities measured directly (Section 3-5-4-2). However at 0.1 mM, Ala-Ala-Ala is transported quicker than Leu-Leu. It is clear, therefore, than Leu-Leu, although having a higher affinity for transport than Ala-Ala-Ala, has a lower maximum rate of transport. A similar situation exists for Ala-Ala and Met-Met. Whereas Met-Met is the better inhibitor, Ala-Ala is transported more quickly at the concentrations used. This important point means that care must be taken in interpreting data such as those in Table 3-5. In some cases, slow transport could reflect a low affinity, or in other cases a low maximal velocity.

3-5-4-4 Peptide Uptake in the Bacilysin-Resistant-Strain Σ1278b gpp$^-$

The uptake of a range of potentially rapidly transported peptides was monitored in strain Σ1278b gpp$^-$ by the standard fluorescamine procedure. No uptake of Ala-Ala, Ala-Ala-Ala, Leu-Leu, Leu-Leu-Leu, Ala-Lys or His-His was detectable.

By scanning cell extracts, it is possible to monitor the accumulation of the constituent amino acids of peptides after uptake and cleavage. Peptide uptake from high external concentrations can thus be detected, although the possible metabolism of intracellular amino acids must be considered. Strain Σ1278b gpp$^-$ was incubated for 30 min with Ala-Ala, Ala-Met, Ala-Lys, Ala-Ala-Ala, Met-Met or Lys-Lys (all at 1 mm) and cell extracts prepared. In no case were any increases in the relevant pool amino acids detectable, whereas in the parent strain Σ1278b, considerable rises in pool levels were noted.

In all the above assays using strain Σ1278b gpp$^-$, peptide uptake

was indetectable, suggesting that this strain is peptide-transport-deficient. The lack of uptake from 1 mM peptide solutions indicates that the lack of detectable uptake in standard assays cannot be ascribed merely to a ten-fold reduction in the affinity for uptake. The simultaneous loss of the uptake of di- and tripeptides is good evidence for the existence of a single major peptide permease, although this is also consistent with the loss of a common factor from two permeases. Competition data (Sections 3-5-4-3 and 3-5-5-3) indicate, however, that there is a single permease. The mutation in strain Σ1278b gpp$^-$ seems to be confined to peptide transport, as the uptake of alanine, methionine, and lysine therein is still rapid (results not shown).

3-5-4-5 Peptide Uptake in Amino Acid Permease Deficient Strains

The uptake of Ala-Ala in a range of amino acid permease deficient strains (Section 3-3-1) was monitored by the standard fluorescamine assay (Table 3-7). In no case was the rate of uptake in a permease deficient strain less than that in the parent strain Σ1278b.

The uptake of a range of peptides in the parent and in the permease-deficient strain 5156d was compared, again by the standard fluorescamine assay (Table 3-8). The relative rates of peptide uptake were identical in each strain. In no case, therefore, was an amino acid permease deficient strain deficient in peptide uptake, a situation which is consistent with the distinction of the amino acid and peptide permeases.

3-5-5 Results and Discussion - Uptake of Sarcosine Peptides

3-5-5-1 Experimental Conditions

By dansylation of cell extracts, it was possible to detect

Table 3-7 Ala-Ala Uptake in Strain Σ1278b and Amino Acid Permease Deficient Mutants

Organism	Rate of uptake (nmol min^{-1} mg dry wt^{-1}) Range	Mean
Σ1278b	1.0-4.3(17)	2.2
2512c	2.3-2.4(3)	2.4
MG276	2.5-3.1(3)	2.7
5156d	3.7-5.9(4)	4.7
CR10	2.7-4.0(3)	3.6

Uptake of Ala-Ala (0.1 mM) was followed by fluorescamine analysis of medium samples during 20 min. incubations. Cell density 1.0 - 2.5 mg dry wt ml^{-1}.

Table 3-8 Uptake of Peptides by Strains Σ1278b and 5156d

Peptide	Rate of Uptake (% rate of Ala-Ala uptake)	
	Strain Σ1278b	Strain 5156d
Ala-Ala	100	100
Ala-Ala-Ala	70-100	70-90
Met-Met	50-70	50-60
Ala-Met	100-120	100-120
Gly-Gly	ND	ND

Uptake of peptide (0.1 mM) was followed by fluorescamine analysis of medium samples during 20 min incubations. Cell density 1.0 - 2.5 mg dry wt ml^{-1}. Rates are expressed relative to Ala-Ala uptake (1.0 - 4.3 nmol min^{-1} mg dry wt^{-1} in strain Σ1278b, and 3.8 - 4.3 nmol min^{-1} mg dry wt^{-1} in strain 5156d) determined in the same experiment, and are the range for at least 2 determinations.

intact peptide accumulation after incubation of yeast cells with sarcosine peptides. Figure 3-2 E-G show some examples of chromatograms of cell extracts obtained in this way.

Initial experiments showed that the affinity for uptake of sarcosine peptides is low compared with that of natural peptides. Uptake assays were therefore routinely performed with 5 mM sarcosine peptide, and extracts were made after incubation for 1 h at 28°C. Accumulation was shown to be linear over this time, and no free sarcosine was detected in extracts, implying that the rate of intracellular peptide hydrolysis was very low. In control experiments, free sarcosine was rapidly accumulated from the medium but not detectably metabolised, which implies that if appreciable amounts of sarcosine were liberated by intracellular peptide hydrolysis they would be detectable. It was possible to detect very low levels (≈ 2 nmol mg dry wt^{-1}) of sarcosine peptides in cell extracts. Control experiments showed that this could not be attributed to inadequate washing of cells prior to extraction; carry-over of peptide during washing was indetectable.

3-5-5-2 Accumulation of Sarcosine Peptides by Strain Σ1278b

The assay of glycyl-sarcosine (Gly-Sar) and Gly-Sar-Sar uptake by strain Σ1278b shows accumulation of intact peptide (Fig. 3-5). Here, and in further discussions of accumulation, an intracellular volume of 1.5 µl mg dry wt^{-1} will be assumed (Appendix 2), and the cell will be considered as a single compartment. This result (Fig 3-5) is the first demonstration of active accumulation of peptide by Sacc. cerevisiae.

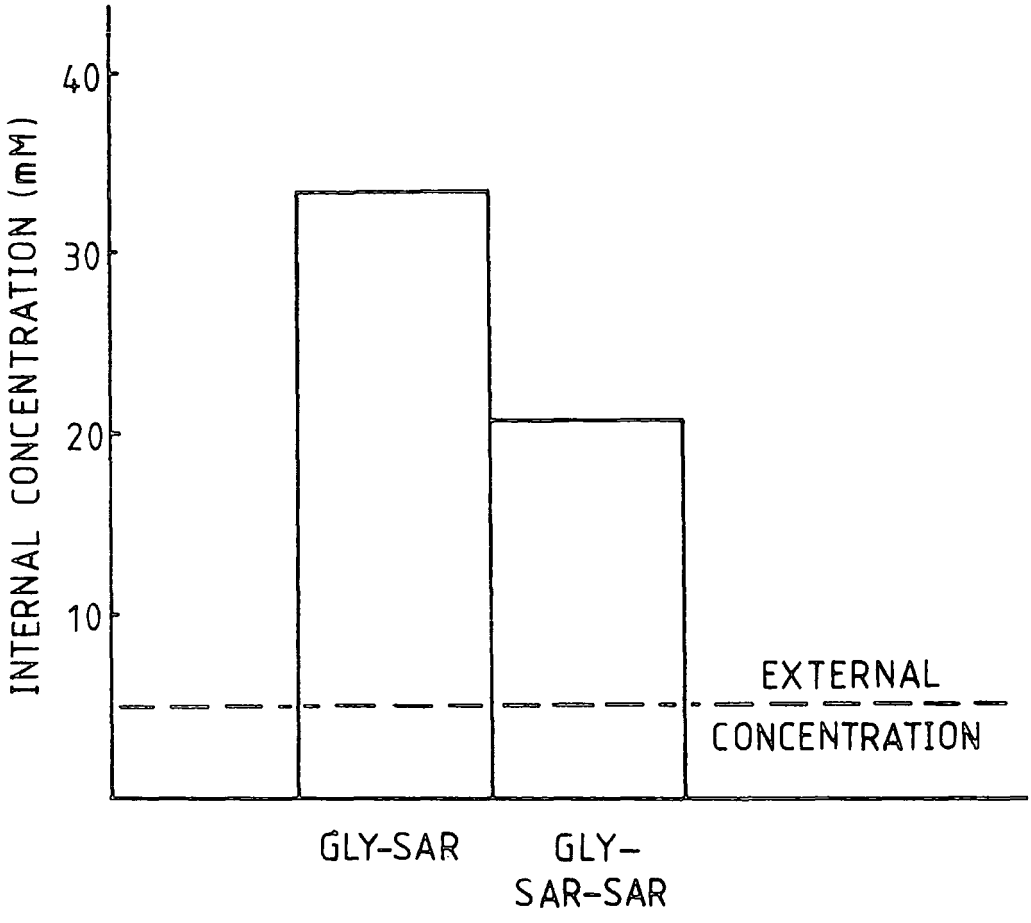


Fig 3-5 The Accumulation of Sarcosine Peptides by Strain Σ 1278b

Cells were harvested and resuspended as in Methods to a final density of 1 mg.dry wt.ml^{-1}. Incubations with peptide (5 mM) were for 1 hr. Extracts were then made and dansylated, and the amount of intracellular peptide present estimated by the standard method.

3-5-5-3 Competition Against Sarcosine Peptide Uptake

The uptake of Gly-Sar and Gly-Sar-Sar by strain Σ1278b in the presence of varying amounts of Leu-Leu and Ala-Ala-Ala was assayed (Fig. 3-6). Leu-Leu proved a better inhibitor than did Ala-Ala-Ala of uptake of both Gly-Sar and Gly-Sar-Sar, which is consistent with their affinities for uptake assayed directly (Section 3-5-4-2). In each case, Gly-Sar-Sar uptake was more susceptible to inhibition than was Gly-Sar uptake. The data are consistent with Gly-Sar, Gly-Sar-Sar, Leu-Leu, and Ala-Ala-Ala sharing a common mode of uptake, although such a conclusion is not kinetically rigorous. Unfortunately, the variation on the data points means that the application of commonly used transformations to distinguish between one and two transport systems does not allow a firmer statement to be made (see Appendix 3).

3-5-5-4 Affinity of Gly-Sar for Uptake

The accumulation of Gly-Sar by strain Σ1278b from a range of external concentrations was assayed (Fig. 3-7). A Lineweaver-Burk plot of the data in Fig. 3-7 yields a K_m of the order of 10^{-2} M. There is, however, evidence for a mode of uptake of Gly-Sar other than via the peptide permease (Section 3-5-5-5); the presence of such uptake would distort the K_m value obtained, particularly if it were more significant at high external concentrations. Although the data in Fig 3-7 imply that Gly-Sar uptake saturates at high concentrations, the K_m value derived probably does not reflect affinity for the major peptide permease.

3-5-5-5 A Second Mode of Uptake for Sarcosine Peptides

Strain Σ1278b gpp$^-$, although deficient in the uptake of those natural peptides tested (Section 3-5-4-4) still takes up Gly-Sar

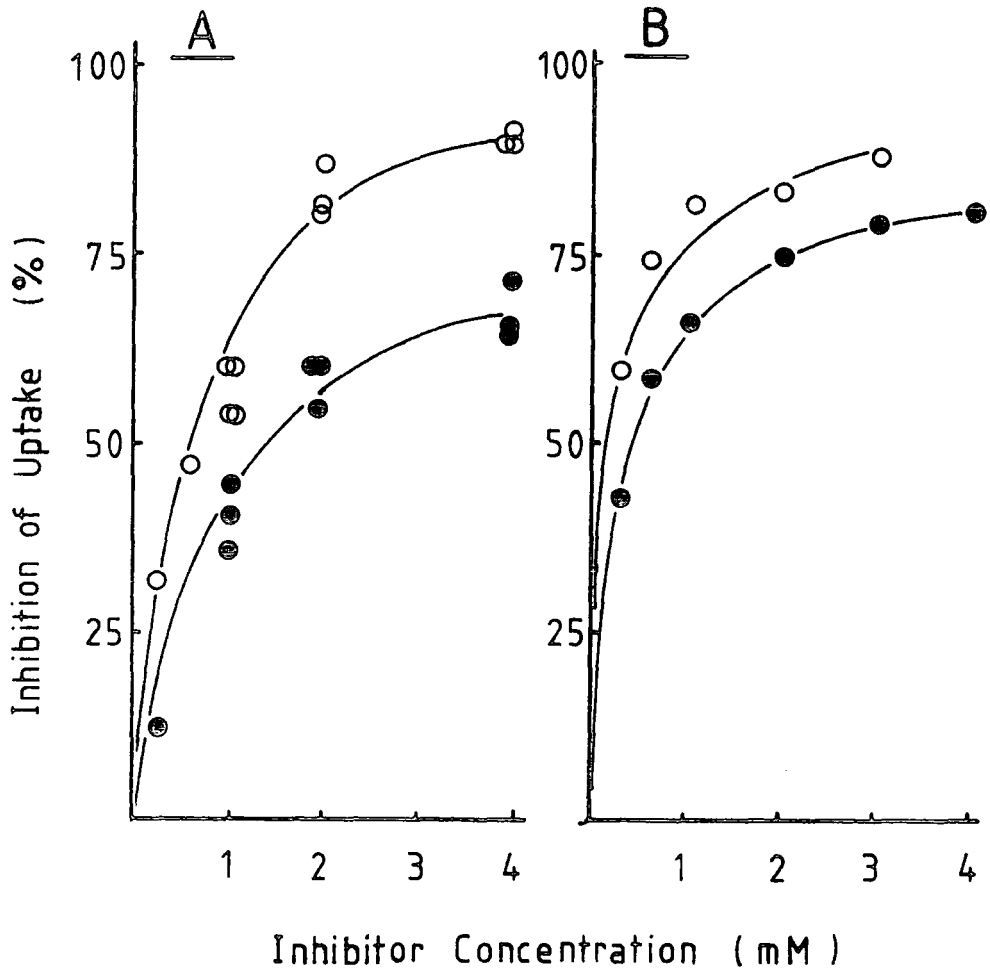


Fig 3-6 Inhibition of Sarcosine Peptide Uptake

Inhibition of A) Gly-Sar uptake (0.6 - 1.4 nmol min^{-1} mg.dry $wt.^{-1}$), and B) Gly-Sar-Sar uptake (0.6 - 0.7 nmol min^{-1} mg.dry $wt.^{-1}$), in strain Σ1278b by Leu-Leu (o) and Ala-Ala-Ala (●). Accumulation of substrate (5 mM) over 1h was estimated by dansylation of cell extracts. Cell density 1.1 - 2.2 mg.dry wt. ml^{-1}.

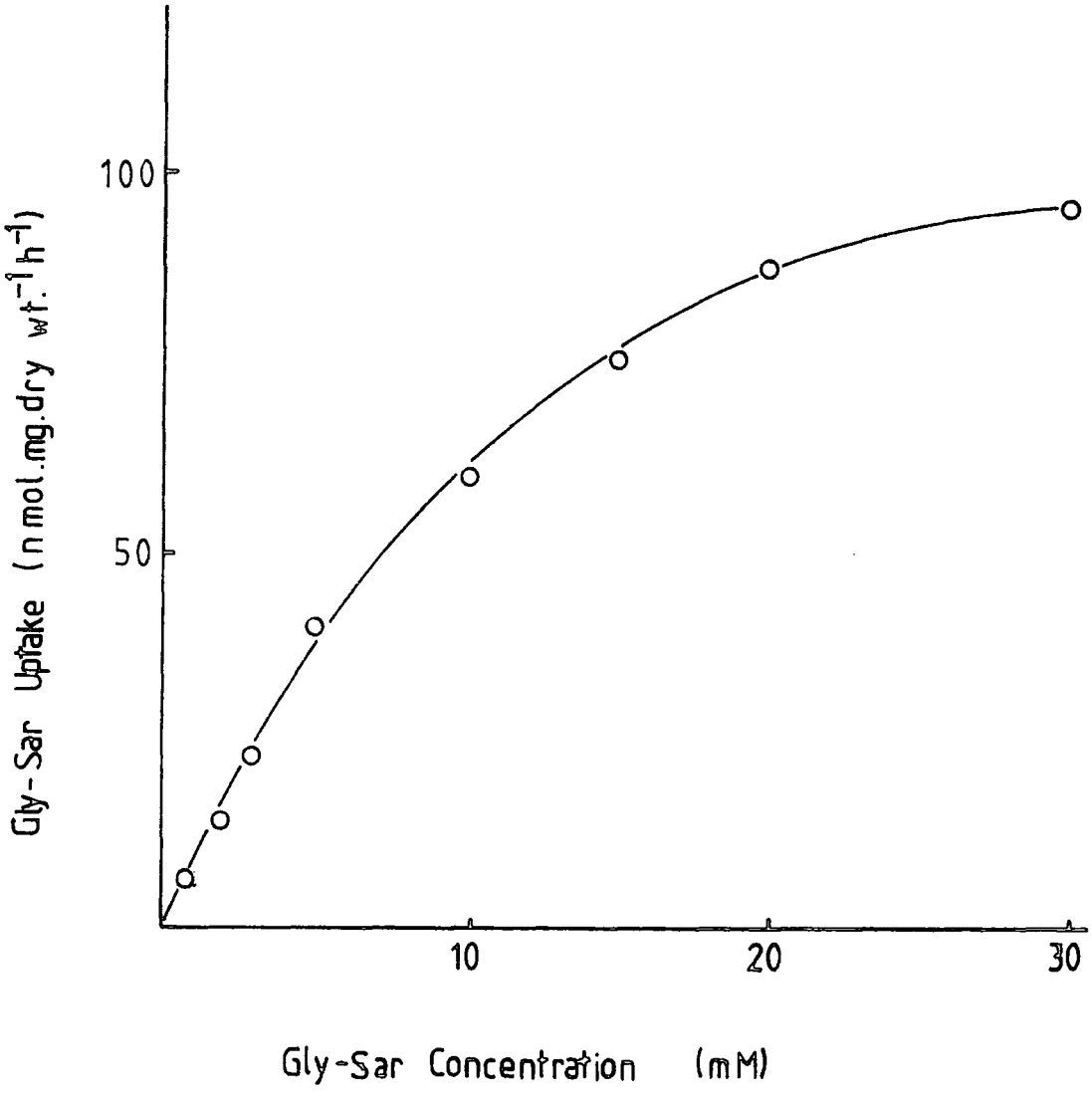


Fig 3-7 Effect of Gly-Sar Concentration on Rate of Uptake

The uptake of Gly-Sar by strain Σ1278b (0.95 mg dry wt ml^{-1}) was assayed by dansylation of cell extracts after 1h incubation.

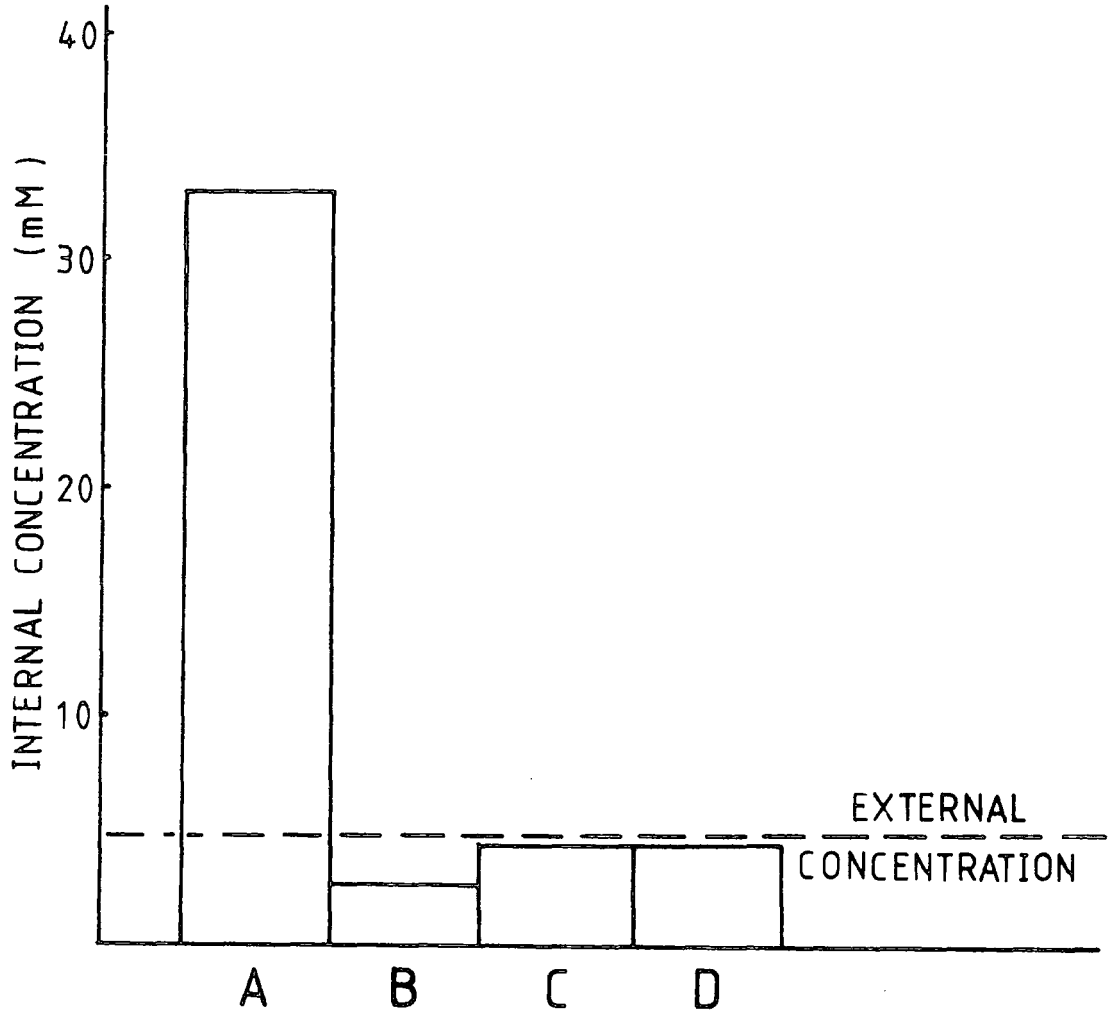


Fig 3-8 The Uptake of Gly-Sar by strains Σ1278b and Σ1278b gpp⁻

Cells were harvested and resuspended as in Methods to a final density of 1.5 - 1.8 mg dry wt ml^{-1}. Incubations with Gly-Sar (5 mM) were for 1 hr. Extracts were then made and dansylated, and the amount of intracellular peptide present estimated by the standard method. A) Gly-Sar in strain Σ1278b, B) As A, but no glucose, C) Gly-Sar in strain Σ1278b gpp⁻, D)As C, but with 4 mM Leu-Leu.

(Fig 3-8C). This uptake is not subject to Leu-Leu inhibition (Fig 3-8D) and it not accumulative. The amount of uptake is comparable to that in energy starved cells of the parent strain (Fig. 3-8B).

There is clearly a second mode of uptake of Gly-Sar operating in strain Σ1278b, other than the major peptide permease. The lack of inhibition by Leu-Leu of this residual uptake suggests that it is either occurring by non-mediated diffusion, or via a mediated system with a very low affinity for Leu-Leu. This component makes kinetic analysis of sarcosine peptide uptake difficult (Section 3-5-5-4) but seems to be indetectable for all those natural peptides investigated (Section 3-5-4-4).

3-6 Concluding Discussion

3-6-1 Extracellular Cleavage Does not Play a Role in Peptide Uptake

Sarcosine peptides are accumulated intact in strain Σ1278b by the route for the uptake of natural peptides; loss of this system in strain Σ1278b gpp$^-$ leads to deficiency in uptake of both natural and sarcosine peptides. The presence of an uptake system for peptides as such is therefore not in doubt. Loss of amino acid permeases does not result in a deficiency in peptide uptake, and vice-versa, facts which are also inconsistent with a major role for external peptidases. Finally, in the absence of glucose, rate of loss of peptide from incubations is very low, and the addition of metabolic inhibitors (Section 5-4-1) completely abolishes peptide loss. In summary, the evidence obtained in these studies indicates that peptide uptake in Sacc. cerevisiae is independent of extracellular peptidase action.

3-6-2 Strain Σ1278b Has One Main Peptide Permease

Studies on peptide uptake in strain Σ1278b show that only di- and tripeptide uptake is detectable by the methods used. Competition for uptake between representative di- and tripeptides, and the loss of all detectable uptake of natural peptides in the bacilysin-resistant strain Σ1278b gpp$^-$, indicates that there is a single main route for peptide uptake in this strain. A similar conclusion was reached from studies on a transport-deficient mutant of another strain, isolated as resistant to L-ethionyl-L-alanine (Marder et al., 1978). The proposal of a single main peptide permease as a general feature of the species would, however, be premature (see Section 3-6-4).

3-6-3 A Secondary Mode of Peptide Uptake in Strain Σ1278b

Studies using the transport-deficient strain Σ1278b gpp$^-$ show abolition of natural peptide uptake, but not of Gly-Sar uptake. Possible explanations for this are as follows:-

a) The residual uptake of Gly-Sar (about 15%) is via the main peptide permease, the mutant not being totally defective in this system, or possessing a system of modified specificity.

b) The residual uptake of Gly-Sar is non-mediated.

c) There is a second, mediated uptake system which will handle peptides in strain Σ1278b.

If there is a residual activity of the main peptide permease (proposal (a)), uptake of other, rapidly transported, peptides should be detectable, and the residual uptake of Gly-Sar should be sensitive to Leu Leu inhibition. As neither of these features are found, this explanation seems unlikely. However, the proposal that the main peptide permease is altered in its specificity cannot be

discounted on the basis of the available evidence.

As sarcosine peptides are supplied at high (5 mM) external concentrations in the assay used, it is possible that uptake by diffusion across the membrane occurs at a detectable rate. If this were the case, uptake should not be saturable, but should increase linearly with external concentration.

Although non-mediated uptake is a plausible explanation for the residual uptake in strain Σ1278b gpp$^-$, the alternative explanation of a second, mediated system cannot be discounted. Such a system would have a different substrate specificity from the main system, and operate at a very low rate. It should be considered that such a system need not be a peptide permease as such, but could be, for example, an amino acid permease which accepts some peptides as very poor substrates. That uptake via such a second mediated system cannot be demonstrated for natural peptides (uptake being abolished in strain Σ1278b gpp$^-$) does not preclude its existence, but merely indicates that it operates at a rate too low to be detected by the assay methods used; in one of the strains of Strep. faecalis used in these studies, uptake of natural peptides via one of the permeases could not be demonstrated directly.

If kinetic characteristics of Gly-Sar uptake in strain Σ1278b gpp$^-$ were to be studied, and other peptides were to be screened for ability to inhibit the uptake, mediated and non-mediated uptake should be distinguishable. If the uptake were to prove to be mediated, it would be of interest to study whether uptake is active or passive (as only "downhill" transport of Gly-Sar has so far been demonstrated in strain Σ1278b gpp$^-$), and to establish whether the system is a

peptide permease per se, or is primarily for some other class of substrate.

3-6-4 Specificity of Peptide Uptake

The specificity of the peptide permease with respect to amino acid side chains has been extensively studied for dipeptides in the present work. The uptake of peptides containing acidic, neutral, and basic amino acids has been demonstrated, and certain conclusions about the specificity of the permease have been drawn (Section 3-5-4-1).

The size limit for peptide uptake in strain Σ1278b seems to occur above tripeptides, although this conclusion rests on studies with homo- alanyl peptides only, and so it is possible that the uptake of other large peptides occurs. The reported size limits in other strains vary from the tripeptide to at least pentapeptide level (Section 1-2-2-4). If this variation is real, and does not merely reflect differing sensitivities of indirect growth assays in different strains, it could be explained by:

a) The presence of only one main permease in all strains, of differing specificity from strain to strain.

b) The presence in all strains of a permease for di- and tripeptides similar to that in strain Σ1278b, plus an oligopeptide permease of appreciable activity in some strains only.

c) Variation in the porosity of the cell envelope to peptides between strains.

The question of whether one or two permeases are present in strains which transport larger peptides could be resolved if similar studies to those performed here for strain Σ1278b were to be carried out

using such a strain.

The possibility that variations in cell envelope porosity to peptides may occur would also bear investigation. In the present studies transport has been considered as a single-step event from the medium to the inside of the cell. However, the porosity of the cell wall may restrict access to a plasmamembrane transport system, and the existence of specific receptors or pores in the cell wall cannot be ruled out. Strain differences in peptide transport may therefore arise from differences in the cell envelope.

3-6-5 Variation in Transport Rates in Different Cell Batches

In the studies reported here, considerable variation in peptide transport rate was detected for batches of cells grown and harvested on different occasions (e.g. Section 3-5-4-1); this rate variation was also noted for studies on Met-Met-Met uptake (Becker and Naider, 1977). Uptake rate does not correlate with the stage of exponential growth at which cells were harvested, although more variation was found at higher harvest densities (Fig. 3-3). As peptide transport is subject to ammonia repression (Section 1-2-4-4), variations in rate can perhaps be ascribed to fairly small variations in the internal and external levels of nitrogenous metabolites. This could also explain the apparently higher rates of peptide uptake obtained with amino acid permease deficient mutants, compared with the parent strain Σ1278b (Table 3-7).

CHAPTER 4

PEPTIDE TRANSPORT IN *STREPTOCOCCUS FAECALIS*

4-1 Introduction

Peptide transport is known to occur in Strep. faecalis (Section 1-3-2), but there is very little information on the specificity of uptake, intact uptake has not been demonstrated, and the number of uptake systems has not been deduced. In this chapter, the characteristics of peptide uptake in two strains of Strep. faecalis are described, and intact accumulation of peptide and evidence for the distinction of peptide and amino acid uptake is presented. The isolation of peptide-transport-, and peptidase-deficient mutants is described, and in one strain the presence of two peptide permeases of differing specificity is demonstrated. Exodus of amino acid residues, apparently occurring via an amino acid permease, is shown subsequent to peptide uptake.

4-2 Materials

Roche Sensitivity Test Medium Type 2 (RST 2) was a gift from Dr. M.J. Hall, Roche Products Ltd., Welwyn Garden City, Herts.

Ala-L-aminoethylphosphonic acid (Ala-Ala(P)), Ala-Ala-Ala(P), Ala-Ala-Ala-Ala(P), Ala(P), Ala-[U-^{14}C]Ala(P), Ala-Ala-[U^{14}C]Ala(P), Ala-Ala-Ala-[U-^{14}C]Ala(P), Ala-[U-^{14}C]Ala, Ala-Ala-[U^{-14}C]Ala and Ala-Ala-Ala-[U^{14}C]Ala were gifts from Dr. P.S. Ringrose or Dr. W.J. Lloyd, Roche Products Ltd., Welwyn Garden City, Herts. Ala-L-aminoxypropionic acid (AX1), Pro-Ala-L-aminoxypropionic acid (AX5), and L-aminoxypropionic acid (AXOL) were gifts from Dr. J.S. Morley, ICI Pharmaceuticals, Alderley Park, Cheshire. All other peptides and amino acids were purchased from Sigma (London) Ltd., Poole, Dorset, or Uniscience Ltd., Cambridge. All other reagents were of analytical grade.

4-3 Organisms and Growth Conditions

4-3-1 Organisms

Strep. faecalis (faecium) strain ATCC 9790 was obtained from Prof. F.M. Harold, National Jewish Hospital, Denver, Colorado. Strains OG-FS5 and NCIB 6459 were supplied by Dr. P.S. Ringrose, Roche Products Ltd., Welwyn Garden City, Herts., the former strain having been originally isolated from a clinical source by Prof. F.W. O'Grady, Department of Microbiology, Nottingham University.

4-3-2 Media and Growth Conditions

In initial studies, strain ATCC 9790 was maintained on a medium containing tryptose (Oxoid, 1% w/v), D-glucose (0.5% w/v), sodium chloride (0.5% w/v), beef extract (Bovril, 0.4% w/v) and agar (1.5% w/v). In liquid culture, cells were grown in 50 ml batches of either the above medium without agar, or the defined, peptide-free medium SF 35 (Andrew, 1973).

In the majority of studies reported here, organisms were maintained on Roche Sensitivity Test Medium Type 2 (RST 2) (Atherton et al., 1979a) with a vitamin supplement (Allen et al., 1979) (see Table 4-1) containing agar (1% w/v), and were grown in liquid culture in the same medium without agar. This medium was prepared by autoclaving at 15 p.s.i. for 15 min, the vitamin supplement being prepared at 100 times final strength, filter sterilized, and added after autoclaving of the medium. The autoclaving step, usually not desirable because of reactions between components of the medium, is possible for RST 2 medium as only low amounts of substances such as glucose are present. However, some browning of the medium during autoclaving was detected.

Table 4-1 Contents of RST 2 Medium

The medium was autoclaved at 15 p.s.i. for 15 min. The vitamin supplement was made up at 100X concentration, filter sterilized, and added to the medium immediately prior to use. All amounts are given as $g.l^{-1}$ in the final single strength medium. The complete single strength medium has a pH of 6.8

Table 4-1 Contents of RST 2 Medium

Medium			
Na_2HPO_4	4.5	L-Cystine	0.2
KH_2PO_4	1.5	Cys	0.3
D-Glucose	2.0	Ile	0.3
NaCl	2.0	Thr	0.2
$(NH_4)_2SO_4$	1.0	Trp	0.025
Sodium pyruvate	1.0	Tyr	0.1
Sodium citrate	0.75	Arg	0.2
$FeSO_4.7H_2O$	0.001	His	0.05
Folic acid	0.005	Leu	0.5
$ZnSO_4.7H_2O$	0.001	Met	0.025
$MnSO_4.4H_2O$	0.001	Pro	0.2
$CuSO_4.5H_2O$	0.001	Phe	0.4
D-Biotin	0.005	Lys-HCl	0.2
Uracil	0.010	Asp	0.1
Guanine	0.010	Ser	0.05
Cytosine	0.010	Ala	0.025
Adenine	0.010	Glu	0.5
Magnesium glycerophosphate	0.2	Gly	0.2
		Val	0.5

Vitamin Supplement			
Choline chloride	0.005	Thiamine HCl	0.005
D-Calcium pantothenate	0.005	Riboflavin phosphate	0.0005
Nicotinamide	0.005	i- Inositol	0.009
Pyridoxal HCl	0.005	$CaCl_2.6H_2O$	0.1

In all experiments reported here, RST 2 medium was used unless otherwise stated. The pH of the single-strength medium subsequent to autoclaving is 6.8.

Organisms were maintained on solid medium at 4°C and subcultured every month. Organisms for uptake experiments were grown in liquid culture at 37°C without shaking. Growth was monitored by measuring the absorbance (at 660 nm) of a portion of the culture using a Bausch and Lomb Spectronic 20 or Pye Unicam SP 1800 spectrophotometer, and a 1 cm. diameter-tube, or a 1 cm path length cuvette, respectively. A linear relationship between cell density and absorbance up to an A_{660} of 0.5 (measured on the Spectronic 20) was shown. Measurements of dry weight and comparison of the spectrophotometers gave the relationship : A_{660} (Spectronic 20) 0.10 ≅ A_{660} (SP 1800) 0.22 ≅ 0.055 mg dry wt. ml^{-1}.

The generation time of strain ATCC 9790 in RST 2 medium was approximately 0.5 hr during exponential growth.

4-3-3 Methods of Checking Identity of Cultures of Strep. faecalis

As Strep. faecalis is a fastidious organism, needing to be grown on a medium with many added nutrients, contamination by other microorganisms is potentially a greater problem than when using organisms cultured on minimal media. In particular, when peptide-antibiotic-resistant mutants are isolated (Section 4-4) it is important to establish that these organisms are Strep. faecalis and not contaminants. The following tests were therefore routinely used to type cultures:

a) Microscopic Examination: Cells were examined at X1,000 magnification using a light microscope. Strep. faecalis cells are

small, non-motile cocci, occurring usually in pairs and sometimes in longer chains.

b) Gram Test: Strep. faecalis gives a strong Gram-positive reaction.

c) Aesculin Hydrolysis: Strep. faecalis is capable of aesculin hydrolysis .

d) Haemolysis: The Strep. faecalis strains used were non-haemolytic.

e) Temperature Tolerance: Strep. faecalis will grow at 45°C.

The above characteristics are sufficient to define the organisms tested as being Strep. faecalis or Strep. faecium (Cowan, 1974) which is sufficient for the present studies (see Section 1-3-1).

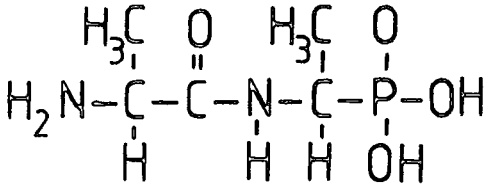
4-4 The Response of Strep. faecalis to Toxic Peptides, and the Isolation of Resistant Mutants

4-4-1 Introduction

The use of peptides as antimicrobial agents has been discussed in Section 1-1-9. In the studies described here, two classes of peptide-mimetic antibiotics, phosphonopeptides and aminoxy-peptides, were used to select for resistant mutants. These mutants were then characterised as transport-defective (Section 4-5-4-4 and 4-5-5-3) or peptidase-defective (Section 4-6).

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, referred to here as Ala-Ala(P)), (Fig 4-1a). 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 (Atherton et al., 1979b). The free Ala(P) moiety is essentially impermeant, and so is

A



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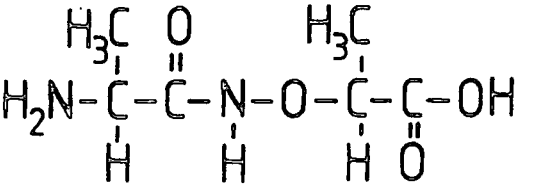


Fig 4-1 Structures of a) alafosfalin, representative of the phosphonopeptides, and b) L-alanyl-L-α-aminoxy-propionic acid, representative of the aminoxypeptides.

inactive if supplied extracellularly, but if supplied in peptide-linked form it can be accumulated to a concentration of 100 to 1,000 fold that of the peptide precursor in the medium (Atherton et al., 1979b), and is potently bactericidal. Many other phosphonopeptides have been shown to have antibacterial action (Atherton et al., 1979a; Allen et al., 1979) and alafosfalin and some related phosphonopeptides have been shown to use the peptide permeases in E.coli (Ringrose and Lloyd, 1979).

Aminoxy-peptides contain an oxygen atom between the amino group and the carbon skeleton of the C-terminal amino acid. Those used in the present studies, of which L-alanyl-L-aminoxypropionic acid (Fig 4-1b) is typical, contain the C-terminal residue L-aminoxy-propionic acid. The mode of antibiotic action of these compounds is not clear but, as the free L-aminoxypropionic acid is less active against Strep. faecalis (Table 4-2), it seems likely that the peptides act as a 'warhead delivery system' (Section 1-1-9) as do the phosphonopeptides.

From experience with other organisms, it was expected that from amongst those mutants of Strep. faecalis isolated as resistant to toxic peptides, the majority would be peptide transport defective (Section 1-1-9). This was found to be so, although peptidase-deficient mutants were also readily isolated (Section 4-6).

4-4-2 Methods

Potential inhibitors of bacterial growth were assayed by plate methods. Plates of SF 35 medium (1.5% w/v agar) or RST 2 medium (1% w/v agar) were spread with a suspension of an exponentially

growing liquid culture of Strep. faecalis (0.1 ml, of the order of 10^8 colony forming units). After allowing the agar surface to dry slightly (20 min at room temp. with dish lid loosened), a solution of the peptide to be assayed (10 µl) was pipetted at various concentrations onto the centre of the plate (for some studies, small filter paper discs which had previously been impregnated with the peptide and then dried, were placed at the centre of the plate).

Plates were incubated for 24 h at 37°C, and the diameter of any zone of growth inhibition was measured. If isolated colonies were found to be growing in the inhibition zone, the plate was incubated for a further 24 h, and a colony picked off and sub-cultured into liquid medium. After being grown at 37°C overnight, the culture was plated (after serial dilution in distilled water to give of the order of 10^2 colonies per plate) in the presence of the same peptide used in isolation, as above, and a colony from near the centre of the plate was taken as a monoculture of the resistant mutant.

4-4-3 Results

4-4-3-1 Response of Strain ATCC 9790 to Toxic Peptides

Strain ATCC 9790 was plated out in the presence of potentially toxic peptides (Tables 4-2, 4-3). Peptides containing norleucine or norvaline are not growth inhibitory under the conditions used: those containing L-α-aminoxypropionic acid (AXOL) give pronounced inhibition zones, greater than for the free AXOL moiety (Table 4-2). Ala-Ala $^{(P)}$ gives a well defined inhibition zone, but higher homologues do not completely inhibit growth, leading to overgrowth of the inhibition zones (Table 4-3).

Table 4-2 The Response of Strain ATCC 9790 to Potentially Toxic Peptides

	Amount Added (nmol)	Diameter of Inhibition Zone (mm)	No. of Colonies in Zone
Gly-D,L-Nle	100	0	-
Gly-D,L-Nva	100	0	-
D,L-Ala-D,L-Nva	100	0	-
L-Nle-L-Nva	100	0	-
AX1	200	30	0
	560	35	0
AX5	200	25	>100
(AXOL	200	15	0)

Strain ATCC 9790 was grown in SF 35 liquid medium and spread on SF 35 agar plates. After addition of peptide (Section 4-4-2) plates were incubated for 24 h at 37°C.

Table 4-3 The Response of Strain ATCC 9790 tc Potentially Toxic Peptides

Peptide	Amount Added µg	Diameter of Inhibition Zone (mm)	No. of Colonies in Zone
Ala-Ala(P)	25	20	60 -70
Ala-Ala-Ala(P)	25	30	complete slow overgrowth
Ala-Ala-Ala-Ala(P)	25	30	complete slow overgrowth

Strain ATCC 9790 was grown in RST 2 liquid medium and spread on RST 2 agar plates. After addition of peptide (Section 4-4-2) plates were incubated at 37°C for 24 hr.

4-4-3-2 Isolation of Resistant Mutants from Strain ATCC 9790

An isolated colony from the inhibition zone on a plate treated with Pro-Ala-L-α-aminoxypropionic acid (AX5) was removed and cultured as described in Methods. This mutant strain was confirmed as being Strep. faecalis (see Section 4-3-3) and was designated strain TN 97902.

Strain TN 97902 is resistant to AX5 but is still sensitive to Ala-L-α-aminoxypropionic acid (AX1) (Table 4-4). In contrast to the parent strain however, isolated colonies grow in the inhibition zone of the latter peptide. One of these colonies was cultured and confirmed as Strep. faecalis (as above). This mutant, which is resistant to both the aminoxypeptides tested, was designated strain TN 979021.

A colony from the inhibition zone of Ala-Ala(P) was also cultured. This mutant was designated strain TN 97901.

The characteristics of the parent strain ATCC 9790 and the three resistant strains are summarised in Table 4-5.

4-4-3-3 Response of Strain OG-FS5 to Phosphonopeptides

Strain OG-FS5 was plated out in the presence of alanyl phosphonopeptides (Table 4-6). All three peptides tested were growth inhibitory, but only in the case of Ala-Ala(P) were isolated colonies present in the inhibition zone.

4-4-3-4 Isolation of Resistant Mutants from Strain OG-FS5

A colony from the inhibition zone of Ala-Ala(P) was cultured and checked as Strep. faecalis. This mutant was designated strain TN FS51, and was resistant to Ala-Ala(P) but not to Ala-Ala-Ala(P) or Ala-Ala-Ala-Ala(P). However, in contrast to the parent, isolated

Table 4-4 The Response of Growth of Strain TN 97902 to Aminoxy-peptides

Peptide	Amount (nmol)	Diameter of Inhibition Zone (mm)	No. of Colonies in Zone
AX1	200	40	20
AX5	200	0	-

Inhibition was assayed by the standard method on SF 35 agar plates.

Table 4-5 Characteristics of Strain ATCC 9790 and Its Derivatives

Strain	Derived From:	Isolated as Resistant To:	Diameter of inhibition zone (mm) Ala-Ala(P)	AX1	AX5
ATCC 9790	-	-	25	30	30
TN 97901	ATCC 9790	Ala-Ala(P)	0	35	30
TN 97902	ATCC 9790	AX5	15	25	0
TN 979021	TN 97902	AX1	0	0	0

Inhibition by peptides (10 µl, 20 mM) was assayed by the standard method on RST 2 plates

Table 4-6 Growth Inhibition of Strain OG-FS5 and Strain TN FS51 by Phosphonopeptides

Strain	Peptide	Diameter of Inhibition Zone (mm)	No. of Colonies in Zone
OG-FS5	Ala-Ala(P)	30	200
	Ala-Ala-Ala(P)	40	0
	Ala-Ala-Ala-Ala(P)	35	0
TN FS51	Ala-Ala(P)	0*	-
	Ala-Ala-Ala(P)	35	100
	Ala-Ala-Ala-Ala(P)	35	100

Inhibition was assayed by the standard method on RST 2 plates. Peptide was added at 25 μg per plate (*: slight clear halo around peptide treated disc).

colonies grew in the inhibition zones of these peptides (Table 4-6). Mutants isolated from the Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P) inhibition zones were designated strain TN FS511 and TN FS512, respectively.

The characteristics of the parent strain OG-FS5 and its derivatives are given in Table 4-7.

4-4-3-5 Stability of Mutant Strains

All strains were maintained on peptide-free RST 2 medium, and so, assuming that the mutants were peptide-transport- or peptidase-deficient, there was no selection pressure for reversion to parental phenotype. All the mutants strains except TN 979021 were of stable phenotype over many subculturings. Strain TN 979021, however, tended to revert to the phenotype of strain 97902.

4-4-4 Discussion

A detailed discussion of the characteristics of the strains isolated is inappropriate at this point, and best awaits the clarification of their peptide transport (Section 4-5) and peptidase (Section 4-6) activities. There are however, several points worthy of note at this juncture:

a) Mutants of strain ATCC 9790 resistant to AX1 could not be obtained directly, whereas they could be obtained easily from strain TN 97902. It clearly requires two mutations (of sufficiently high frequency to be detected by the methods used) to confer resistance to AX1.

b) Strain TN 97901, which is resistant to Ala-Ala(P), is still sensitive to both AX1 and AX5. The mutation is thus not for a common step in the uptake and cleavage of these two classes of antibacterial compounds.

Table 4-7 Characteristics of Strain OG-FS5 and Its Derivatives

Strain	Derived From	Isolated as Resistant to:	Diameter of Inhibition Zone (mm) Ala-Ala(P)	Ala-Ala-Ala(P)	Ala-Ala-Ala-Ala(P)
OG FS5	-	-	30	40	35
TN FS51	OG FS5	Ala-Ala(P)	0*	35	35
TN FS511	TN FS51	Ala-Ala-Ala(P)	0	0	0*
TN FS512	TN FS51	Ala-Ala-Ala-Ala(P)	0	0	0*

Inhibition by peptides (25 μg) was assayed by the standard method on RST 2 plates

(* slight clear halo around peptide treated disc)

c) Strain TN 979021 is resistant to Ala-Ala(P) and to AX1, which suggests the loss of a common step in their uptake and cleavage, as the sites of their intracellular action are presumably distinct.
d) Strains TN FS511 and TN FS512 have identical resistance patterns to the phosphonopeptides tested. This is consistent with resistance to either peptide being conferred by the loss of the same gene product.

4-5 Characterisation of Peptide Transport in Strep. faecalis

4-5-1 Introduction

Peptide transport in Strep. faecalis has not previously been well characterised (Section 1-3-2). In the present studies direct uptake assays, using both radiolabelled peptides (Section 2-6) and fluorescence-labelling assays (Sections 2-3, 2-5), were used to investigate the transport of small peptides in different strains of Strep. faecalis and their derivatives (Section 4-4). The results presented here are divided into three sections: general features of peptide transport in Strep. faecalis (Section 4-5-3); the characterisation of peptide transport in strain ATCC 9790 and its derivatives (Section 4-5-4); the characterisation of peptide transport in strain OG-FS5 and its derivatives (Section 4-5-5).

4-5-2 Methods

4-5-2-1 Harvesting of Organisms

Organisms for transport studies were grown in liquid medium (in early studies SF 35, but routinely RST 2 medium) to a density of 0.1 - 0.3 mg dry wt ml^{-1} and were then harvested and resuspended as follows:

a) Membrane Filtration. Portions (10 ml) of the culture were passed

through a membrane filter (0.45 μm pore size, 25 mm diameter, Oxoid) held in a Swinnex (Millipore) filter holder. Organisms were washed on the filter with 2 volumes of KH_2PO_4/K_2HPO_4 buffer (50 mM, pH 6.9) containing glucose (0.2% w/v) (at room temperature) and resuspended in the same buffer to a density of 0.1 - 0.3 mg dry wt ml^{-1}.

b) Centrifugation. Cultures were transferred to screw top tubes (50 ml, polypropylene, MSE) and accelerated in an MSE High Speed 21 centrifuge up to 18,000 r.p.m. (approx. 26,000 g), using a rotor maintained at 30°C, then rapidly slowed. This process took approximately 5 minutes. The supernatant culture fluid was then decanted, organisms were resuspended using a Vortex mixer in either RST 2 medium, or buffer as above, and centrifuged again. The supernatant wash solution was discarded, amd organisms were resuspended in RST 2 medium or buffer at a density of 0.08 - 0.36 mg dry wt ml^{-1}.

Organisms were routinely harvested by membrane filtration for fluorescence assays of peptide uptake and by centrifugation for radiotracer assays, and these methods of harvesting can be assumed unless stated in the results sections.

4-5-2-2 Monitoring Peptide Uptake from Medium - Dansyl Chloride

Organisms in phosphate buffer with glucose were preincubated at 37°C for 10 min. Peptide (0.1 mM) was added, and portions (0.3 ml) of the suspension were removed after various time intervals, immediately freed from organisms by passing them through a membrane filter (0.45 μm pore size, 13 mm diameter, Oxoid) in a Swinnex (Millipore) filter holder. Filtrates were collected in small vials, which were then placed in a boiling water bath for 10 min (see

Section 4-5-3-4) and stored at -20°C. Samples of the filtrate were then dansylated and examined by the standard method (Section 2-3-2).

4-5-2-3 Monitoring Peptide Uptake from Medium - Fluorescamine

Organisms in phosphate buffer with glucose were pre-incubated at 37°C for 10 min in the incubation vessel of the automated fluorescamine assay system (Section 2-5-2). Peptide (routinely 0.1 mM) was added, and the suspension was pumped through the automated system. The rate of uptake of the peptide over the third to fifth minutes of the incubation was routinely derived from the trace on chart recorder.

4-5-2-4 Monitoring Accumulation of Radiolabel

Organisms in phosphate buffer with glucose or in RST 2 medium were pre-incubated at 37°C for 10 min. Labelled peptide (0.1 mM) was added, and samples (500 µl) were periodically removed, harvested on glass-fibre filters (Whatman GF/C) under vaccuum (filters pre-wetted with saline (0.9% w/v)) and washed with saline (0.9% w/v, 2 x 5 ml). Filters were then dried (80°C, for at least 45 min) and counted by the standard method (Section 2-6-2).

For uptake studies with alanyl homo-peptides, samples were routinely taken every 8s for 48s, filters being held in a multi-position manifold (Millipore). This rapid sampling was necessary to overcome the problems associated with amino acid exodus (Section 2-6). For phosphonopeptides, samples were routinely taken every 2-5 min, filters being mounted on a single-position, sintered-glass holder, and being changed between samples. As the labelled Ala(P) moiety of these peptides does not undergo

detectable exodus, rapid sampling is not a necessity.

For each uptake assay, the following controls were performed : a) A portion (500 µl) of the suspension of organisms alone was harvested under vaccuum, labelled peptide (0.1 mM, in buffer or RST 2 medium, as appropriate, 500 µl) passed through the filter, followed by saline (0.9% v/v, 2 x 5 ml). This zero time control allows for the uptake of label by organisms on the filter, and adsorbtion of label onto the filter.
b) A portion (50 µl) of the incubation with peptide was placed directly onto a dry filter in a scintillation vial. This allowed the total counts present in the incubation to be derived.

Filters from a) and b) above were dried and counted by the standard method. After correction for controls, rates of uptake over the first 30s were derived for rapidly transported peptides, and over 10 - 15 min for those slowly transported.

4-5-2-5 Preparation of Cell Extracts for Analysis by Dansylation

Incubations with peptide were performed in a volume of 1 ml. The whole suspension was then harvested on a membrane filter (0.45 µm pore size, 25 mm diameter, Oxoid) under vaccuum, rapidly washed with distilled water (20 ml), and the filter was then extracted in boiling distilled water (1 ml) containing toluene (50 µl) for 10 - 15 min in a stoppered tube. The resulting suspension was cooled, filtered (0.45 µm pore size) to remove insoluble debris, and the filtrate stored at -20^{o}C. Portions of the extract were dansylated by the standard method (Section 2-3-3).

4-5-2-6 Determining the Intracellular Fate of Radiolabel from Phosphonopeptides

Because of the low specific radioactivity of the phosphonopeptides used to study accumulation of label in cell extracts, it was necessary to use large volumes of incubation, and to concentrate harvested organisms many-fold before extraction, in order to obtain detectable amounts of label in cell extracts.

Incubations were routinely of 20 ml volume in KH_2PO_4/K_2HPO_4 buffer (pH 6.9, 50 mM) with glucose (0.2% w/v), at 37°C. After 10 min pre-incubation, labelled phosphonopeptide (0.1 mM) was added. Samples (5 ml) were taken after various times, and cells pelleted by centrifugation. The supernatant solutions were discarded, remaining moisture was removed with a cotton wool bud, the cells were resuspended in distilled water (100 µl) and extracted by boiling in closed tubes for 15 min. Solid matter was removed by centrifugation and supernatant solutions were stored at -20°C.

The removal of excess supernatant solution with a cotton-bud after centrifugation of cells obviously leaves some interstitial solution. However, this amounts to less than 10 µl (measured by the volume after resuspension in 100 µl water) and in no case does this volume of solution contain a significant proportion of the label present in the pelleted cells. In a typical experiment (Fig 4-8) the label present in the interstitial water represents less than 1 nmol mg dry wt^{-1}.

The phosphonopeptides used were labelled in the Ala(P) moiety and so by separating peptides and Ala(P) by electrophoresis the amount of intracellular cleavage of phosphonopeptide could be determined from the distribution of the label. Samples (50 µl)

of the cell extracts were added to a solution (10 µl) containing unlabelled Ala-Ala(P) and Ala(P) (both 25 mM). Portions (10 µl) of the resulting solution were run on high voltage paper electrophoresis (Shandon Southern Model L24) at 130 Vcm^{-1} for 45 min, using formic acid (pH 1.8, 4% v/v) as the buffer. The paper was sprayed with ninhydrin to visualise the spots, and those corresponding to Ala-Ala(P) and Ala(P) were excised, and counted for ^{14}-C by the standard method (Section 2-6-2).

In initial experiments using strain ATCC 9790, the whole electrophoresis paper was cut up and counted to detect radiolabel. However, the only detectable label comigrated with Ala (P) or Ala-Ala (P), which suggests that metabolism of Ala (P) in this organism is minimal. This confirms previous findings (Atherton et al., 1979b). Routinely, therefore, only those portions of the electrophoresis paper containing Ala(P) or phosphonopeptides were counted for radioactivity. (The peptides Ala-Ala(P) and Ala-Ala-Ala(P) comigrate in the electrophoretic system used).

4-5-3 Results and Discussion - General Features

4-5-3-1 Intact Accumulation of Peptide

The accumulation of glycyl-sarcosine was detected in cell extracts by dansylation (Section 4-5-2-5); that of Ala-Ala(P) was detected as in Section 4-5-2-6. Large accumulation gradients were observed for both peptides in strain ATCC 9790, and for Ala-Ala(P) in strain OG-FS5 (Table 4-8), thus demonstrating that active, intact uptake of peptide occurs in these strains. Here, and in future calculations, a cell-water volume of 1.75 µl mg dry wt^{-1} (Bakker and Harold, 1980) is used. Examples of chromatograms showing

Table 4-8 Accumulation of Peptides by Strep. faecalis

Strain	Peptide	Incubation Medium	Intracellular Concentration* (mM)	Concentration Gradient
ATCC 9790	Gly-Sar	SF 35	115	117X
	Ala-Ala(P)	Phosphate	16	166X
OG FS5	Ala-Ala(P)	Phosphate	70	850X

Organisms were resuspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v), or in SF 35 medium to a density of 0.11 - 0.15 mg dry wt ml^{-1}. Cells were incubated with Gly-Sar (1mM), or Ala-[^{14}C]Ala(P) (0.1mM), for 30 min before extracts were made.

*Assuming an intracellular water space of 1.75 μl mg dry wt^{-1} (Bakker and Harold, 1980).

accumulation of glycyl-sarcosine in cell extracts are given in Fig 4-2 E-G.

4-5-3-2 Amino Acid Exodus During Peptide Uptake

By dansylation of medium samples during uptake of peptides by strain ATCC 9790, exodus of the constituent amino acids of Ala-Ala, Ala-Ala-Ala, Leu-Ala and Met-Met was detected (e.g. Fig 4-2 A-C). Other rapidly transported peptides were not tested in this way, but it seems reasonable to assume that the exodus is a general effect, as in other bacteria (Section 1-1-3).

4-5-3-3 Influence of Amino Acids on Peptide Uptake

The presence in the medium of 1 mM alanine did not affect the initial rate of uptake of label from 0.1 mM Ala-[^{14}C]Ala; the amount of label accumulation at later times in the incubation was, however, lower (Fig 4-3). This is consistent with uptake and cleavage of labelled Ala-[^{14}C] Ala, and exchange of unlabelled extracellular Ala for intracellular [^{14}C] Ala via an amino acid permease (Section 1-3-4-4). Further evidence for the enhanced exit of labelled alanine is obtained if the uptake of label from Ala- [^{14}C] Ala in phosphate buffer and in RST 2 medium is compared (Fig 4-4). The rapid exodus of label in RST 2 medium is consistent with exchange for unlabelled amino acids in the medium.

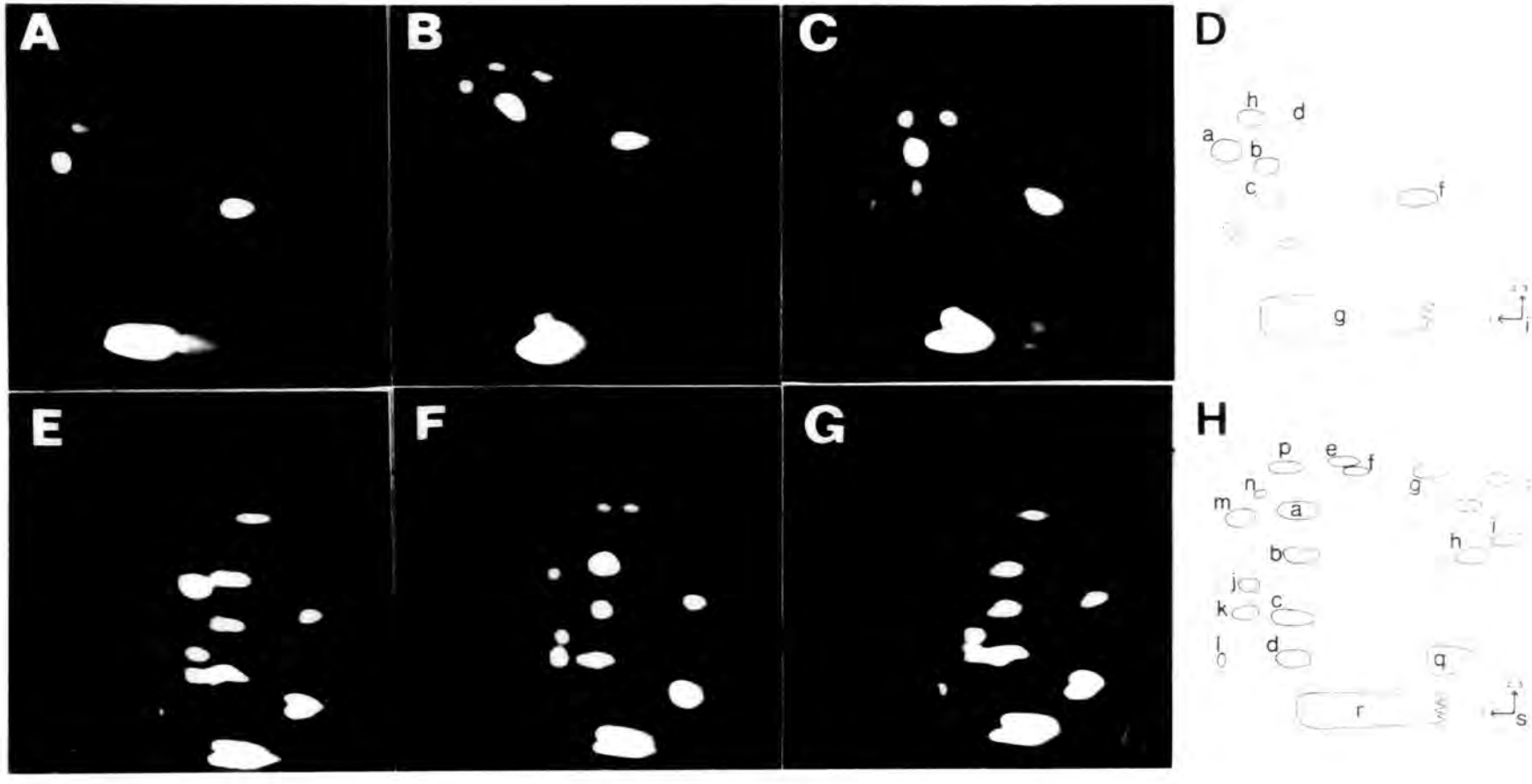
4-5-3-4 Extracellular Peptidase Action

In preliminary studies in which samples of the incubation medium were taken and immediately filtered to remove bacteria (Section 4-5-2-2) it was found that, if the filtrates were left at room temperature, the peptide concentration decreased over several hours. Heating the filtrates to 100°C for 10 min stopped this

Fig 4-2 Chromatograms of Dansyl Peptides and Amino Acids from Incubation Media and Cell Extracts of Strep. faecalis (faecium)

A to C. Samples of medium during incubation of strain ATCC 9790 (0.18 mg dry wt ml^{-1}) with Ala-Ala (0.1 mM). A) 0 min, B) 5 min, C) 10 min, D) Key to derivatives: a) Ala-Ala, b) Ala, c) Gly, d) Pro, f) Orn (standard), g) dansyl hydroxide, h) dansyl ammonia, i) origin

E to G. Cell extracts of strain ATCC 9790 (0.18 mg dry wt ml^{-1}) after 20 min incubation with: A) Gly-Sar (1 mM), b) Gly-Sar (1 mM) + Ala-Ala (1 mM), C) no peptide. D) Key to derivatives: a) Ala, b) Gly, c) Glu, d) Asp, e) Pro, f) Val, g) Leu, h) Orn, i) Lys, j) Thr, k) Ser, l) Arg, m) Gly-Sar, n) Ala-Ala, p) dansyl ammonia, q) diamino-pimelic acid (standard), r) dansyl hydroxide, s) origin.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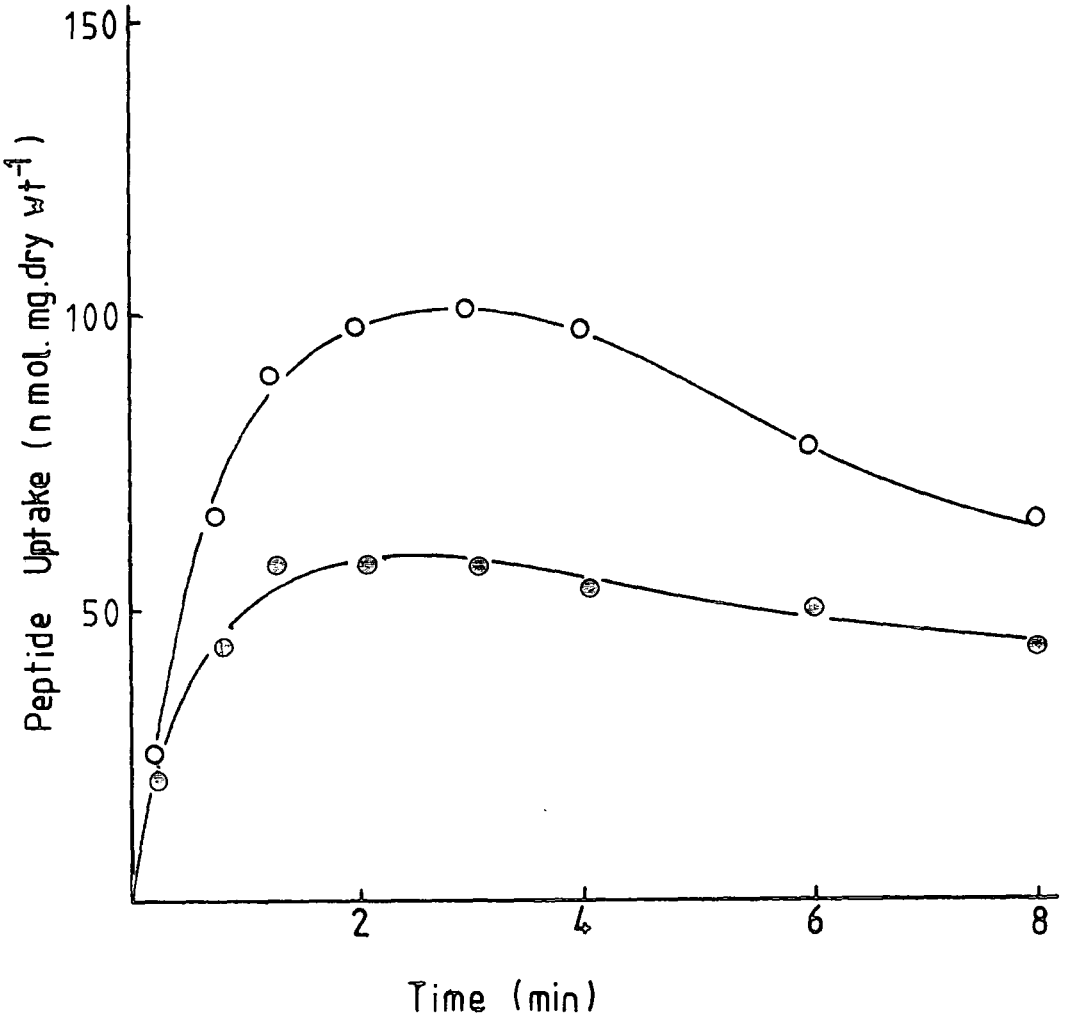


Fig 4-3 The Effect of Ala on Ala-[^{14}C] Ala Uptake by Strep. faecalis (faecium). Strain ATCC 9790 was resuspended in potassium phosphate buffer (pH 6.9, 50 mM) plus glucose (0.2% w/v) to a density of 0.15 mg dry wt ml^{-1}. Uptake of label from Ala-[^{14}C] Ala (0.1 mM, 0.03 μCi ml^{-1}) was followed in the absence (o) and presence (⊛) of unlabelled Ala (1 mM).

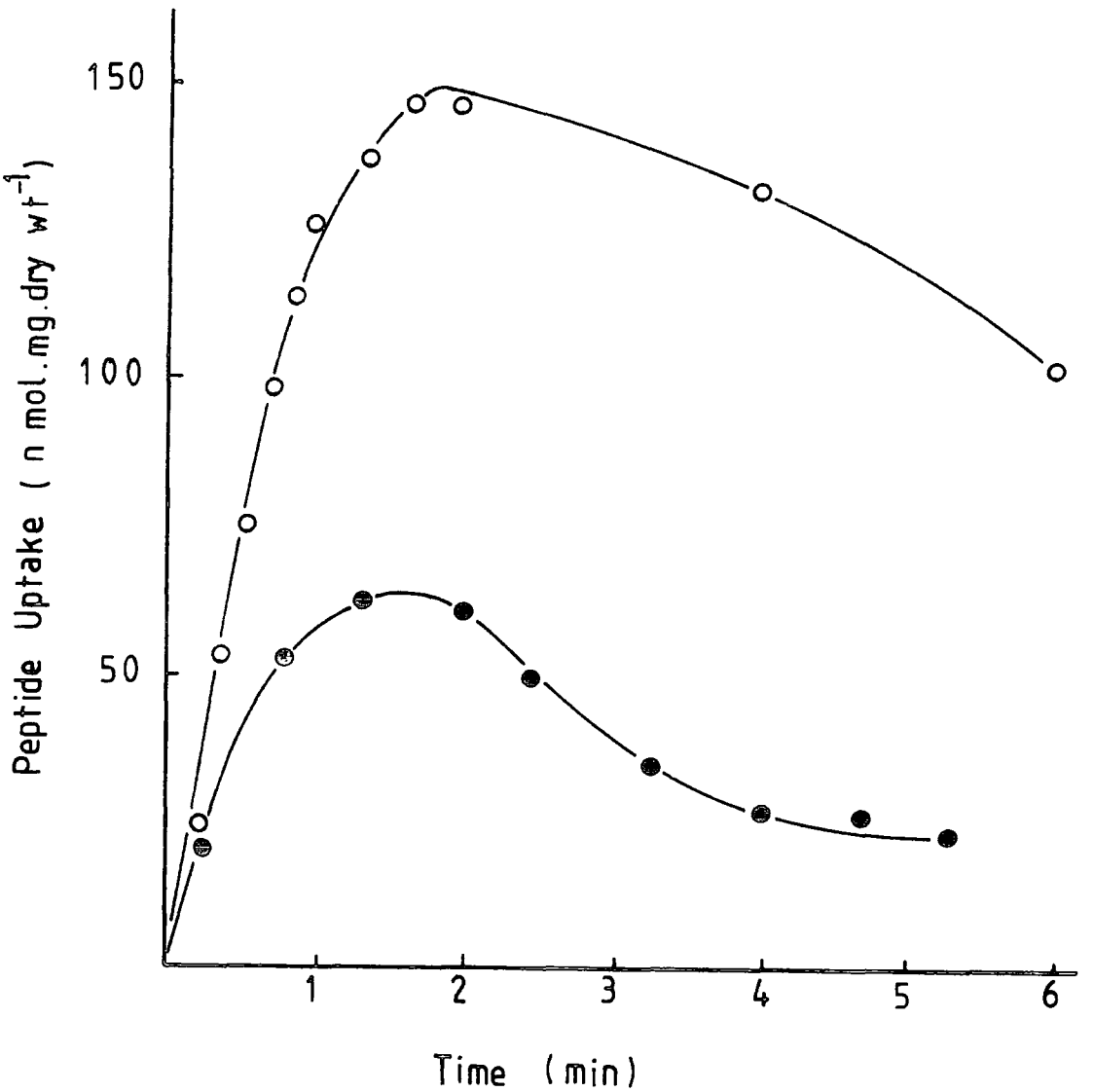


Fig 4-4 Uptake of Label From Ala-[^{14}C]Ala in Phosphate Buffer and RST 2 medium

The uptake of label from Ala-[^{14}C]Ala (0.1 mM, 0.03 μCi ml^{-1}) by strain ATCC 9790 resuspended in: (○) potassium phosphate (pH 6.9, 50 mM) plus glucose (0.2% w/v) at 0.22 mg dry wt ml^{-1}; (●) RST 2 medium (pH 6.8) at 0.36 mg dry wt ml^{-1}.

decrease and so, in all further experiments, filtrates were heat-treated immediately after sampling (Section 4-5-2-2).

In these initial studies, cultures were often harvested late in their growth phase, and subjected to relatively high pressures during filtration of high cell densities. As Strep. faecalis possesses an active autolytic system (Shockman et al., 1974), it seems possible that the apparent release of peptidase activity into the medium may be resulting from the effects of interupting cell growth and of cell lysis. Under the standard conditions adopted for harvesting and sampling, the effect was not detectable. Further, the lack of any uptake or extracellular cleavage of peptide in the absence of an exogenous energy source (Section 4-5-3-5) argues against the presence of extracellular peptidases. In conclusion, the observed effect is consistent with cell lysis under some conditions, but is not detectable under standard conditions. However, where apparent rates of peptide uptake are small, such cleavage should be considered as a possible contributing factor.

4-5-3-5 Peptide Uptake is Dependent on an Exogenous Energy Supply

In experiments in which Strep. faecalis was resuspended in phosphate buffer (50 mM, pH 6.9) without glucose, no decrease in peptide concentration in the suspension medium could be detected by the automated fluorescamine procedure. Addition of metabolic inhibitors also completely stopped decrease of peptide concentration (Section 5-4-2). This indicates both that peptide uptake is strictly dependent on an exogenous energy source, and that extracellular peptidase action is not occurring at detectable levels.

4.5.3.6 Strain Differences in Peptide Uptake

The assay of homo-alanyl peptide uptake by three strains of Strep faecalis showed distinct differences in relative rates of uptake (Fig. 4-5). This inter-strain variation for features of peptide uptake has been noted for Sacc. cerevisiae (Section 1-2-2-4) and E.coli (Payne and Bell, 1979; W.J. Lloyd, personal communication), and is clearly of relevance if the design of antimicrobial agents of broad specificity is to be successful. In the present studies, peptide transport in two of the above strains (ATCC 9790 and OG-FS5) was studied further.

4-5-4 Results and Discussion - Strain ATCC 9790 and Derivatives

4-5-4-1 The Uptake of Peptides by Strain ATCC 9790

The uptake of peptides by strain ATCC 9790 was followed by the automated fluorescamine procedure (Table 4-9). Dipeptides were generally transported more rapidly than tripeptides, and the uptake of tetra- and pentanalanine was indetectable. Similar findings were obtained from the assay of ^{14}C labelled-peptide uptake (Table 4-10). The uptake of Ala-Ala(P) and Ala-Ala-Ala(P) is slow compared to their 'parent' peptides; the more rapid uptake of Ala-Ala(P) than Ala-Ala-Ala(P) or Ala-Ala-Ala-Ala(P) is consistent with its greater growth inhibitory effect in this strain (Table 4-3).

4-5-4-2 Affinity of Ala-Ala Uptake in Strain ATCC 9790

The rate of Ala-Ala uptake by strain ATCC 9790 at different initial peptide concentrations was studied using the automated fluorescamine procedure. At low initial peptide concentrations low cell densities were used to ensure that only a small proportion of the added peptide was taken up. A Lineweaver-Burk plot of the data (Fig. 4-6) gave an apparent K_m of 8×10^{-5} M and a V_{max} of

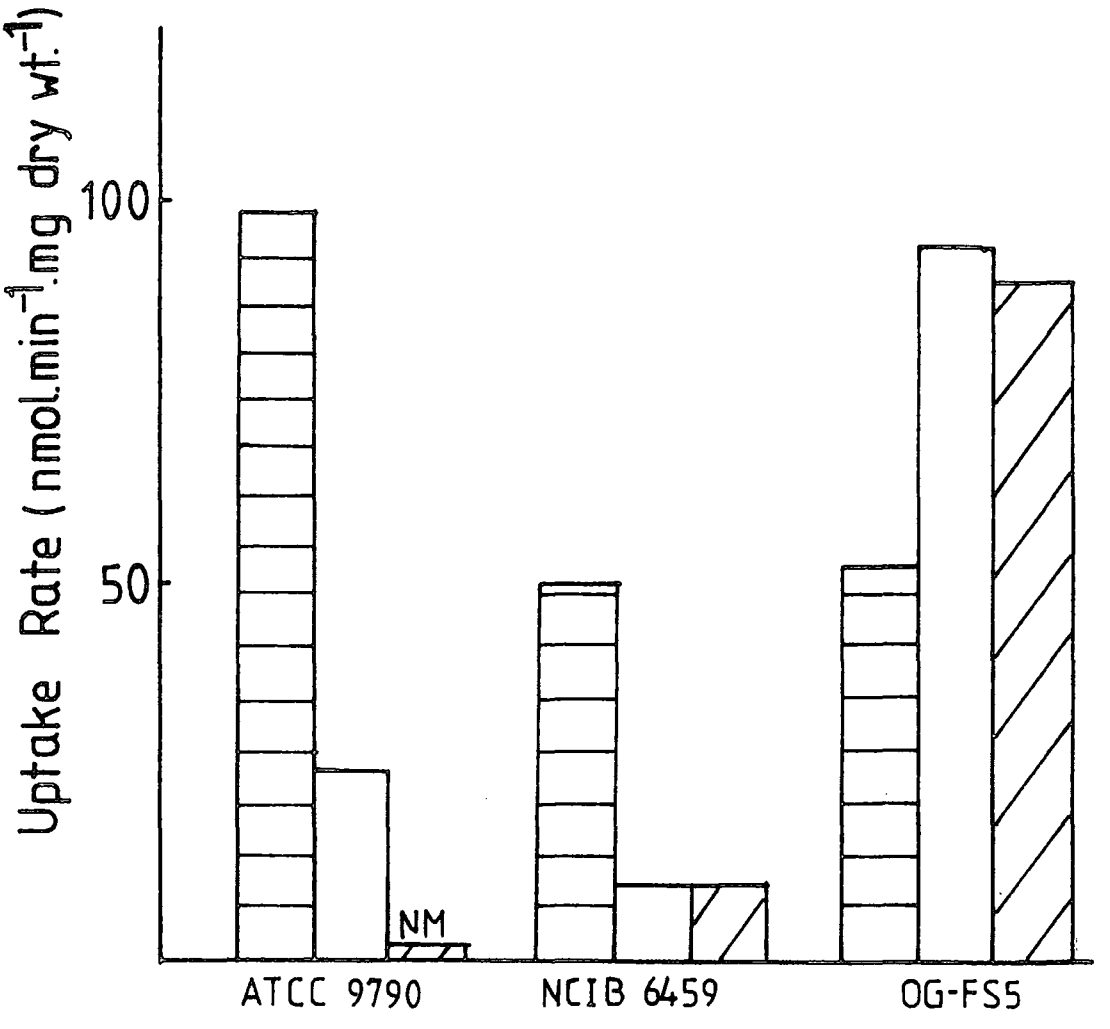


Fig 4-5 Strain Differences in Peptide Uptake

The initial rate of uptake of label from:
Ala- [^{14}C] Ala; Ala-Ala- [^{14}C] Ala;
Ala-Ala-Ala- [^{14}C] Ala, from phosphate buffer (pH 6.9, 50 mM) with glucose (0.2%) was determined in three strains of Strep. faecalis by the standard radiotracer method.
(NM = not measurable)

Table 4-9 The Uptake of Peptides by Strain ATCC 9790

Peptide	Rate of Uptake ($nmol\ min^{-1}\ mg\ dry\ wt^{-1}$)
Ala-Ala	92 - 103 (5)
Leu-Leu	58 - 66 (2)
Lys-Lys	13 - 20 (4)
Ala-Ala-Ala	21 - 29 (5)
Met-Gly-Met	21 - 25 (3)
Lys-Ala-Ala	7 (1)
Val-Val-Val	ND (2)
Ala-Ala-Ala-Ala	ND (1)
Ala-Ala-Ala-Ala-Ala	ND (1)

Uptake was assayed by the automated fluorescamine procedure. Organisms were resuspended to a density of 0.1 - 0.3 mg dry wt ml^{-1}. Peptide was supplied at 0.1 mM. Uptake rates are the range for the number of determinations in parentheses. ND: not detectable

Table 4-10 The Uptake of Labelled Peptides by Strain ATCC 9790

Peptide	Initial Rate of Uptake nmol min^{-1} mg dry wt^{-1}
Ala-[^{14}C]Ala	98 - 100 (3)
Ala-Ala-[^{14}C]Ala	23 - 27 (2)
Ala-[^{14}C] Ala(P)	2.6 (1)
Ala-Ala-[^{14}C] Ala(P)	1.0 (1)
Ala-Ala-Ala-[^{14}C]Ala(P)	1.0 (1)

Uptake was measured by the standard radiotracer procedure. Organisms were suspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v) to a density of 0.12 - 0.24 mg dry wt ml^{-1}. Peptide was supplied at 0.1 mM. Samples were taken every 8s for natural peptides and every 2 - 5 min for phosphonopeptides. Initial rates are the range for the number of determinations in parentheses.

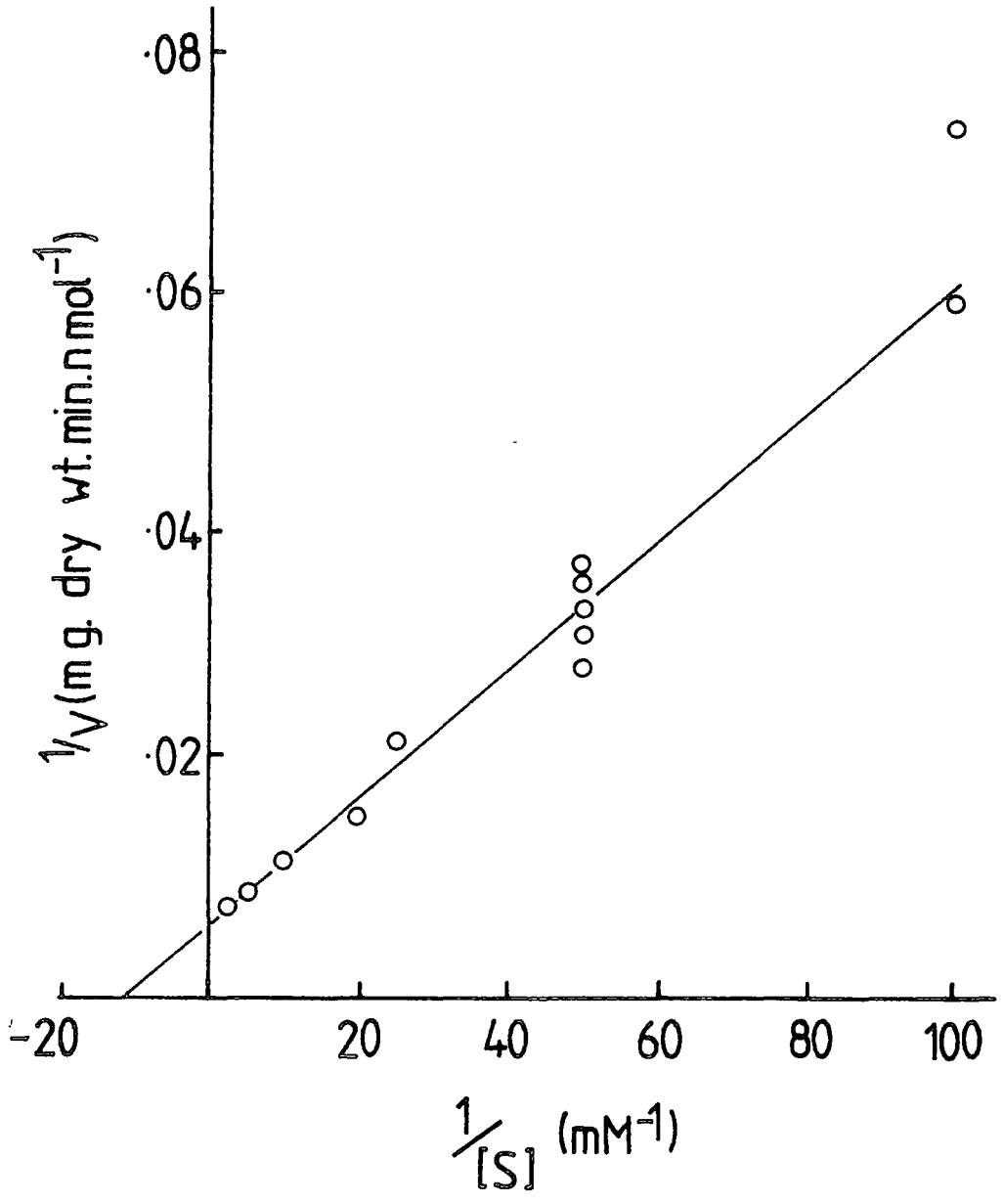


Fig 4-6 Affinity of Ala-Ala for Uptake in Strain ATCC 9790

Rates of Ala-Ala uptake were determined using the automated fluorescamine procedure. Cell density 0.06 - 0.26 mg dry wt ml^{-1}.

150 nmol. min^{-1} mg dry wt^{-1}.

4-5-4-3 Competition for Peptide Uptake in Strain ATCC 9790

Potential competition for uptake was investigated for several peptides in strain ATCC 9790 (Table 4-11). Gly-Sar accumulation was inhibited by dipeptides, but weakly or not at all by oligopeptides. Ala-Ala was a good inhibitor of Ala-Ala-Ala uptake, but not of Ala-Ala-Ala(P) uptake, suggesting that the phosphonopeptide and its 'parent' peptide have a different major mode of uptake. Taken as a whole, the data in Table 4-11 indicate the presence of two peptide permeases, one which will handle dipeptides and to a lesser extent tripeptides, and a second system with a higher affinity for oligopeptides than dipeptides. These two systems will henceforth be referred to as the dipeptide and oligopeptide permeases, respectively. The oligopeptide permease apparently operates at a very low rate; the uptake of peptides via this route is indetectable using the standard fluorescamine method (e.g. Ala-Ala-Ala-Ala and Ala-Ala-Ala-Ala-Ala, Table 4-9); only for those labelled peptides for which the labelled moiety does not undergo exodus (e.g. Ala-Ala-[^{14}C]Ala(P), Table 4-10) can label uptake be detected.

The above proposal that two permeases are present is supported by the analysis of peptide uptake in the mutant strains TN 97902 and TN 979021 described below.

4-5-4-4 Peptide Uptake in Strains TN 97902 and TN 979021

The uptake of peptides in the two aminoxypeptide-resistant strains TN 97902 and TN 979021 (Table 4-5) was studied (Table 4-12).

Strain TN 97902 apparently has the same uptake rate as the parent strain ATCC 9790 for natural di- and tri-peptides and Ala-Ala(P), but

Table 4-11 Competition for Peptide Uptake in Strain ATCC 9790

Substrate	Conc (mM)	Competitor	Conc (mM)	% Inhibition of Uptake
Ala- [^{14}C] Ala	0.1	Ala_3	0.1	-8
			1.0	17
		Ala	1.0	15
Ala-Ala- [^{14}C] Ala	0.1	Ala_2	0.1	74
Ala-Ala- [^{14}C] Ala(P)	0.1	Ala_2	0.1	26
			1.0	89
		Ala_3	0.1	72
			1.0	96
		Ala_4	0.1	78
		Ala_5	0.1	69
		Ala	1.0	0
Gly-Sar	1.0	Ala_2	1.0	50
		Leu_2	1.0	66
		Ala_3	1.0	16
		Leu_3	1.0	0
		Ala_4	1.0	0

Organisms were suspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v), or (for incubation with Gly-Sar) in SF 35 medium, to a density of 0.10 - 0.21 mg dry wt ml^{-1}. Uptake of label from Ala- [^{14}C] Ala and Ala-Ala- [^{14}C] Ala was measured every 8s for 48s, and that from Ala-Ala- [^{14}C] Ala(P) every 2 min for 8 min. Gly-Sar uptake was measured by dansylation of cell extracts taken after 30 min incubation. Results are expressed as % inhibition of uptake in the presence of competitor relative to a control in its absence.

Table 4-12 Peptide Uptake in Transport-Deficient Strains Derived from ATCC 9790

Peptide	Assay	Rate of Uptake (nmol min^{-1} mg dry wt^{-1}) Strain ATCC 9790	Strain TN 97902	Strain TN 979021
Ala-Ala	F	92-103(5)	95-106(2)	ND(1)
	R	98-100(3)	98(1)	1(1)
Lys-Lys	F	13-20(4)	18(1)	ND(1)
Ala-Ala-Ala	F	21-29(5)	22(1)	ND(1)
	R	23-27(2)	22-28(2)	ND(1)
Met-Gly-Met	F	21-25(3)	20(1)	ND(1)
Ala-Ala(P)	R	3(1)	1-5(2)	ND(1)
Ala-Ala-Ala(P)	R	1(1)	ND(2)	ND(1)
Ala-Ala-Ala-Ala(P)	R	1(1)	ND(1)	ND(1)

Uptake of peptide (0.1 mM) by organisms (0.1 - 0.3 mg dry wt ml^{-1}) in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v) was assayed by the automated fluorescamine (F), or radiotracer (R) methods. Uptake rates are the range for the number of determinations in parentheses. ND: not detectable

is deficient in Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P) uptake. These results are consistent with the presence of a (low rate) system for the uptake of AX5, Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P), the loss of which gives rise to resistance to AX5. Competition studies (Section 4-5-4-3) suggest that Ala-Ala-Ala, Ala-Ala-Ala-Ala and Ala-Ala-Ala-Ala-Ala also use this system, though their uptake by this mode is too slow to be detectable directly by the assays used.

Strain TN 979021 is defective in the uptake of all peptides tested. This is consistent with the loss of a (high rate) transport system for the uptake of di- and, to a lesser extent, tripeptides and is again in agreement with the competition studies performed (Section 4-5-4-3).

4-5-4-5 L-Aminoethylphosphonic Acid is Impermeant in Strain ATCC 9790

Cells of strain ATCC 9790 were resuspended in RST 2 medium to a density of 0.3 mg dry wt ml^{-1}. Uptake of label from [^{14}C] Ala(P) (0.1 mM) was assayed periodically for 40 min. No uptake of label was detectable, suggesting that the free Ala(P) moiety is impermeant.

4-5-5 Results and Discussion - Strain OG-FS5 and Derivatives

4-5-5-1 Uptake of Peptides by Strain OG-FS5

The uptake of peptides by strain OG-FS5 was followed by the automated fluorescamine procedure and by the use of labelled peptides (Table 4-13). In contrast to the findings with strain ATCC 9790, uptake of oligopeptides was more rapid than that of dipeptides, and phosphonopeptides were rapidly transported.

4-5-5-2 Competition for Peptide Uptake

Inhibition of uptake of Ala-Ala by Ala-Ala-Ala and vice-versa

Table 4-13 The Uptake of Peptides by Strains OG-FS5 and TN FS51

Peptide	Assay	Rate of Uptake (nmol min^{-1} mg dry wt^{-1}) OG FS5	TN FS51
Ala-Ala	R	43-45(2)	6-14(2)
	F	52(1)	-
Ala-Ala-Ala	R	94(1)	0.8-1.6(3)
	F	82(1)	-
Ala-Ala-Ala-Ala	R	89(1)	11(1)
	F	110(1)	-
Ala-Ala-Ala-Ala-Ala	F	107(1)	-
Ala-Ala(P)	R	12(1)	1.7-2.1(2)
Ala-Ala-Ala(P)	R	80(1)	1.4(1)
Ala-Ala-Ala-Ala(P)	R	80(1)	2.7(1)

Organisms were resuspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v) to a density of 0.11 - 0.27 mg dry wt ml^{-1}. Uptake was assayed by the standard automated fluorescamine assay (F) or radiotracer assay (R). Peptide was supplied at 0.1 mM. Rates of uptake are the range for the number of determinations in parentheses (-: not determined)

could not be detected in strain OG-FS5 using 0.1 mM labelled substrate and 1 mM unlabelled inhibitor (data not shown). This observation is consistent with two situations: the presence of separate major permeases for the two peptides; the presence of a common single permease of low affinity. More detailed competition studies, which would resolve this point, were not performed.

4-5-5-3 The Uptake of Peptides by Strain TN FS51

For all peptides tested, uptake in strain TN FS51 (Ala-Ala(P)-resistant) is severely reduced relative to the parent strain (Table 4-13). This observation has two possible explanations:

a) There is one major permease for peptides in strain OG-FS5, and resistance to Ala-Ala(P) is confered by the loss or severe rate reduction of this permease. The residual uptake of peptides in strain TN FS51 is explained by the presence of one or more other (low rate) peptide permeases.

b) The reduction of peptide uptake in the Ala-Ala(P)-resistant strain is due to the loss of a common component (e.g. an energy coupling factor) of more than one peptide permease.

Without performing extensive competition studies between the peptide substrates used, or extending the range of substrates tested in the hope of finding one whose uptake is unaffected by the mutation in strain TN FS51, it is not possible to distinguish between these two explanations.

4-6 Peptidase Deficient Strains of Strep. faecalis

4-6-1 Introduction

For certain of the peptide-analogue-resistant strains of Strep. faecalis isolated (Section 4-4), initial studies showed that the

rate of uptake of the peptide analogue used in the isolation is unaltered relative to the parent strain. The observed resistance could therefore be caused by either inability to cleave the toxic moiety from the peptide or by an alteration in the site of action of the 'warhead'. Analysis of cell extracts during uptake shows that cleavage is the step altered in these strains.

4-6-2 Methods

Organisms were grown and harvested as in Section 4-5-2-1, uptake of labelled peptides was assayed as in Section 4-5-2-4, and the intracellular fate of label from phosphonopeptides was monitored as in Section 4-5-2-6.

4-6-3 Results and Discussion

4-6-3-1 Uptake of Ala-Ala(P) in Strain TN 9790l

The uptake rates of Ala-Ala, Ala-Ala-Ala and Ala-Ala(P) from 0.1 mM solutions are indistinguishable in strain ATCC 9790 and its Ala-Ala(P) resistant derivative TN 9790l (Table 4-14), which suggests that the resistance is not due to defective uptake. In a further experiment, cultures of the two strains were harvested, resuspended in fresh RST 2 medium containing 0.2 mM Ala- [^{14}C] Ala(P) and uptake of label and growth were monitored (Fig. 4-7). For about 30 min. the uptake rate is similar for both strains, after which growth and uptake cease in the parent, presumably because of the toxic effect of Ala-Ala (P).

4-6-3-2 Strain TN 9790l Is Deficient in Intracellular Ala-Ala(P) Cleavage

Cell extracts were prepared from strains ATCC 9790 and TN 9790l during incubation with 0.1 mM Ala- [^{14}C] Ala(P) and the peptide

Table 4-14 Uptake of Peptides by the Peptidase-Deficient Strain TN 97901

Peptide	Uptake Rate (nmol min^{-1} mg dry wt^{-1})	
	Strain ATCC 9790	Strain TN 97901
Ala-Ala	98-100(3)	99(1)
Ala-Ala-Ala	23-27(2)	26(1)
Ala-Ala(P)	3.0(1)	2.4-2.6(2)

Uptake of peptide (0.1 mM) was determined by the standard radiotracer method. Organisms were resuspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/w) to a density of 0.12 - 0.24 mg dry wt ml^{-1}. Rates are the range for the number of determinations in parentheses.

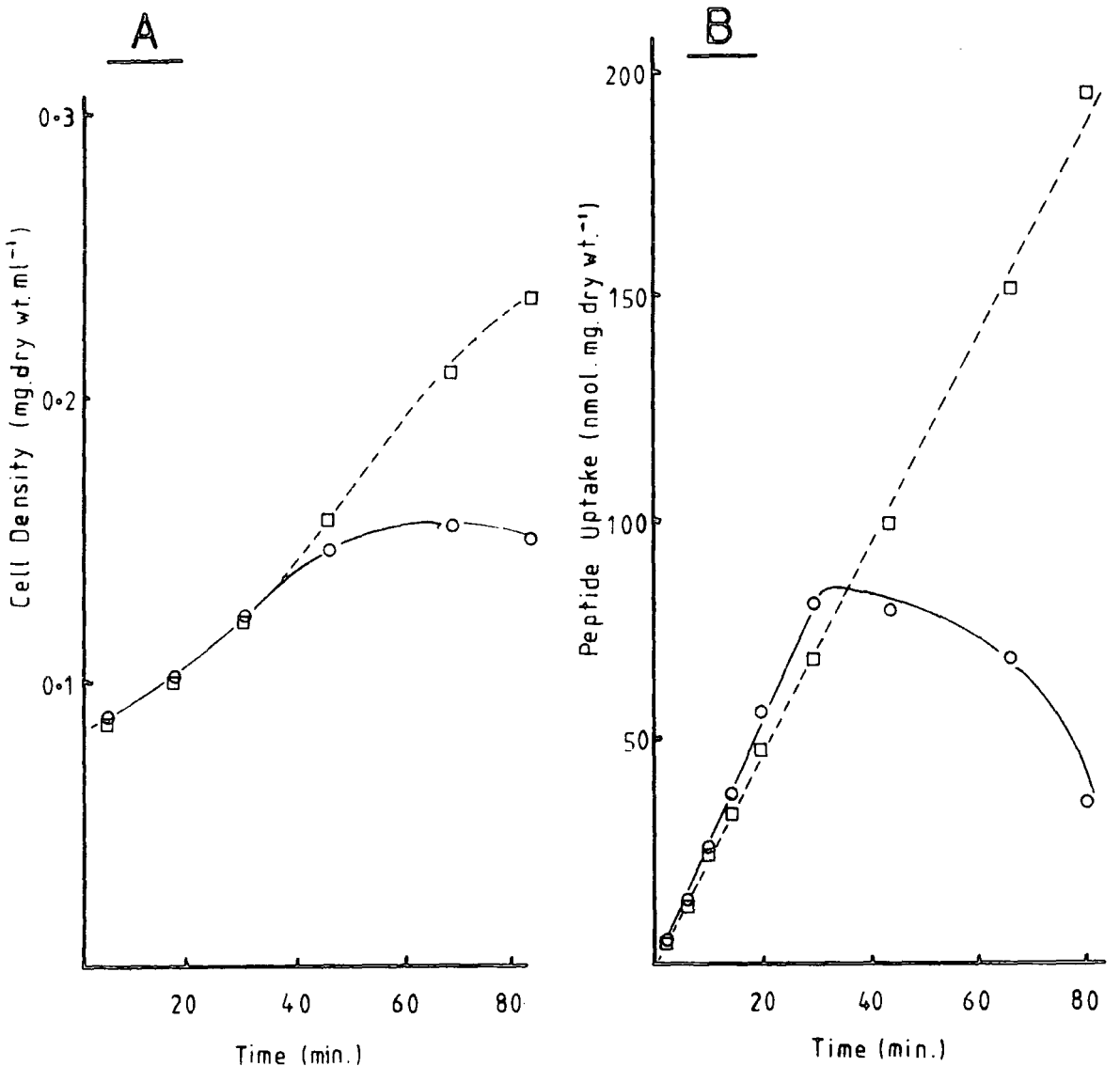


Fig 4-7 Uptake of Ala-Ala(P) by Strains ATCC 9790 and TN 97901

Organisms were resuspended in RST 2 medium to a density of 0.08 mg dry wt ml^{-1}. Ala-[^{14}C] Ala(P) (0.2 mM, 0.2μCi ml^{-1}) was added, and periodically, A) cell density, and B) label accumulation, were monitored. (o) strain ATCC 9790, (□) strain TN 97901.

and free [^{14}C] Ala(P) therein separated using high-voltage paper electrophoresis. The initial rate of label accumulation was similar in both strains, but in strain TN 97901 much less free Ala(P) was present (Fig. 4-8), suggesting that this strain is deficient in the cleavage of Ala-Ala(P). The smaller amounts of label present in the parent strain at later times (Fig. 4-8) are consistent with the toxic effect of Ala-Ala(P) leading to a cessation of uptake. The intracellular concentration of Ala-Ala(P) at 30 min in strain TN 97901 is 37 mM (being a 420 fold concentration gradient), but there is no evidence that the peptide per se is inhibitory (see also Fig. 4-7).

4-6-3-3 Uptake of Peptides by Strains TN FS511 and TN FS12

Uptake rates of peptides are similar in strain TN FS51 (resistant to Ala-Ala(P)), and in strains TN FS511 and TN FS512 (also resistant to Ala-Ala-Ala(P) and to Ala-Ala-Ala-Ala(P), Table 4-7) (Table 4-15), which indicates that the resistance to the higher homologues in the latter strains is not due to defective uptake.

4-6-3-4 Intracelular Release of Ala(P) from Phosphonopeptides in Strains TN FS51 and TN FS512

The amount of labelled Ala(P) and phosphonopeptide present in cell extracts of strains TN FS51 and TN FS512 was monitored during uptake of Ala-[^{14}C] Ala(P) (Fig. 4-9) and Ala-Ala-[^{14}C] Ala(P) (Fig. 4-10). In both cases, much less Ala(P) was released intracellularly in strain TN FS512, implying a peptidase deficiency in this strain.

Interestingly, although strain TN FS51 is resistant to Ala-Ala(P) and sensitive to Ala-Ala-Ala(P), the rates of uptake of these peptides (Table 4-15) and the amounts of Ala(P) released

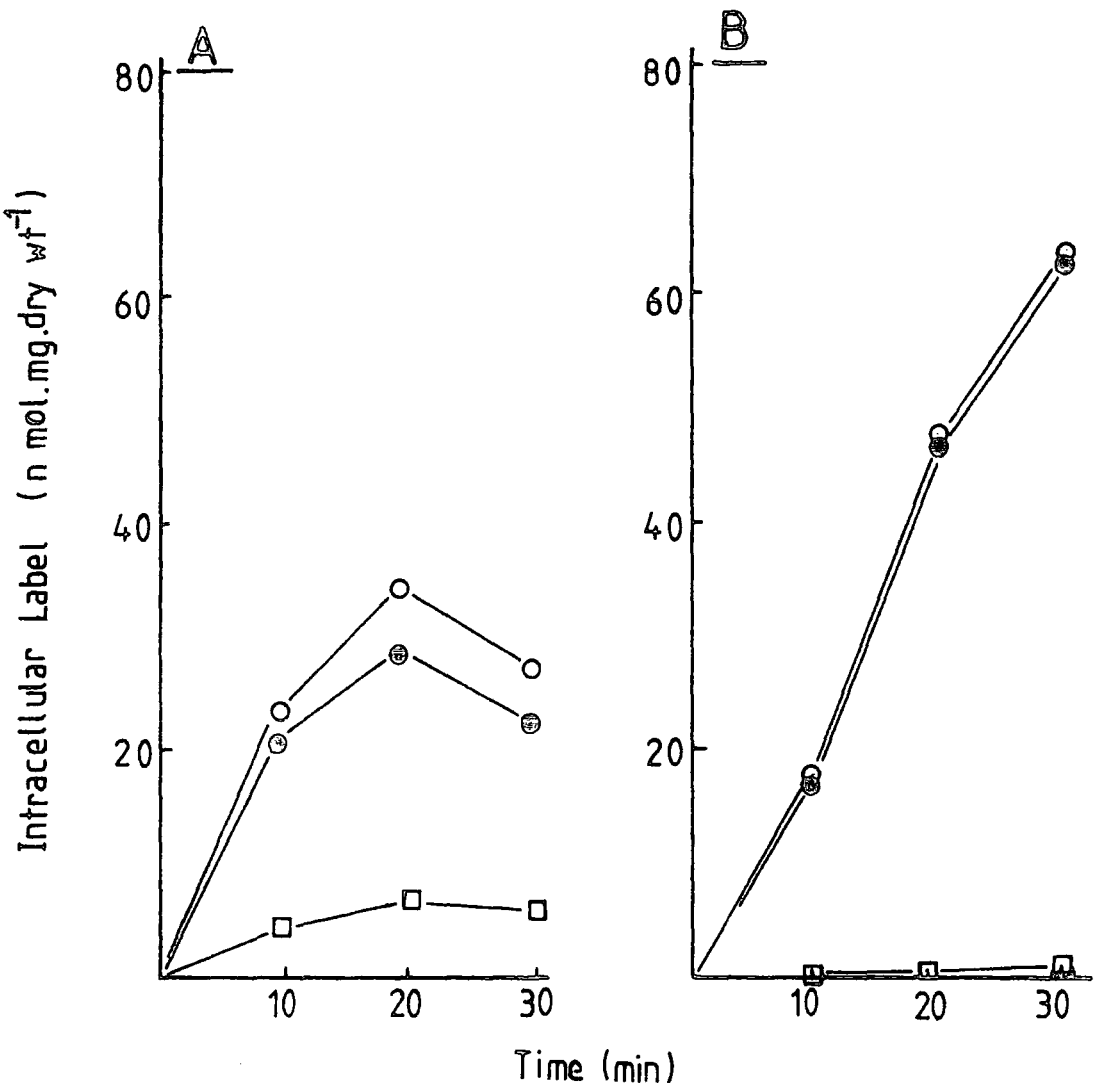


Fig 4-8 Intracellular Cleaveage of Ala-Ala(P)

Cell extracts of A) strain ATCC 9790 (0.11 mg dry wt ml^{-1}), and B) strain TN 97901 (0.19 mg dry wt ml^{-1}) were prepared during incubation of organisms with Ala-[^{14}C] Ala(P) (0.1 mM, 0.19 μCi ml^{-1}) and analysed by the standard method. (□) [^{14}C] Ala(P); (⊙) Ala-[^{14}C] Ala(P); (○) Total label present.

Table 4-15 Uptake of Peptides in Peptidase-Deficient Strains TN FS511 and TN FS512

Peptide	Uptake Rate (nmol min^{-1} mg dry wt^{-1})		
	Strain TN FS51	Strain TN FS511	Strain TN FS512
Ala-Ala	6-14(2)	6(1)	6(1)
Ala-Ala-Ala	0.8-1.6(3)	-	1.2(1)
Ala-Ala-Ala-Ala	11(1)	11(1)	12(1)
Ala-Ala(P)	1.7-2.1(2)	-	0.9-1.2(2)
Ala-Ala-Ala(P)	1.4(1)	-	1.4(1)
Ala-Ala-Ala-Ala(P)	2.7(1)	-	2.6-3.1(2)

Uptake of peptide (0.1 mM) was determined by the standard radiotracer method. Organisms were resuspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2% w/v) to a density of 0.12 - 0.24 mg dry wt ml^{-1}. Rates are the ranges for the number of determinations in parentheses. (-: not determined)

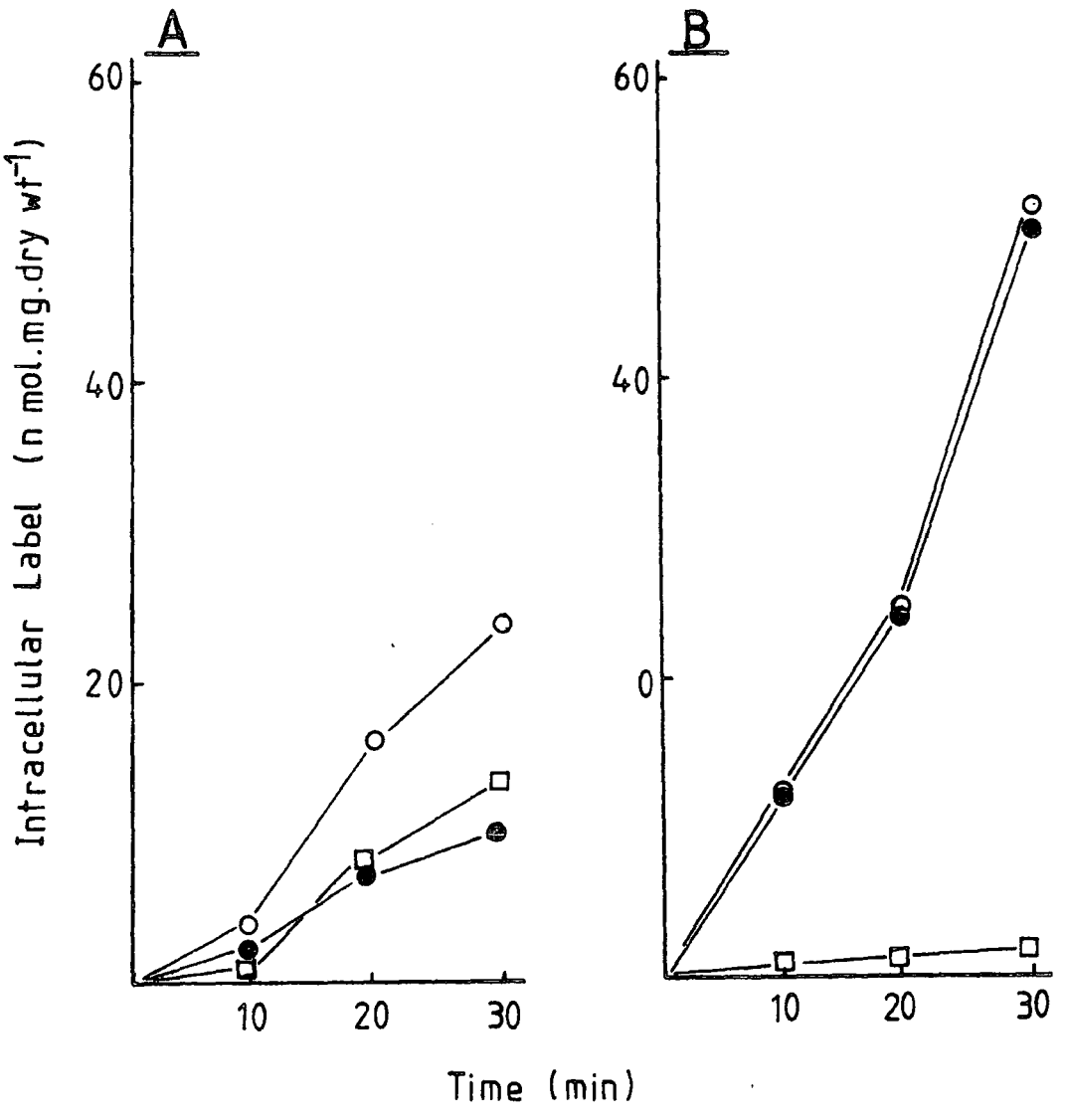


Fig 4-9 Intracellular Cleavage of Ala-Ala(P)

Cell extracts of A) strain TN FS51 and B) strain TN FS 512 (both 0.15 mg dry wt. ml^{-1}) were prepared during incubation of organisms with Ala-[^{14}C] Ala(P) (0.1 mM, 0.19 μCi ml^{-1}) and analysed by the standard method. (□) [^{14}C] Ala(P); (●) Ala- [^{14}C] Ala(P); (○) Total label present .

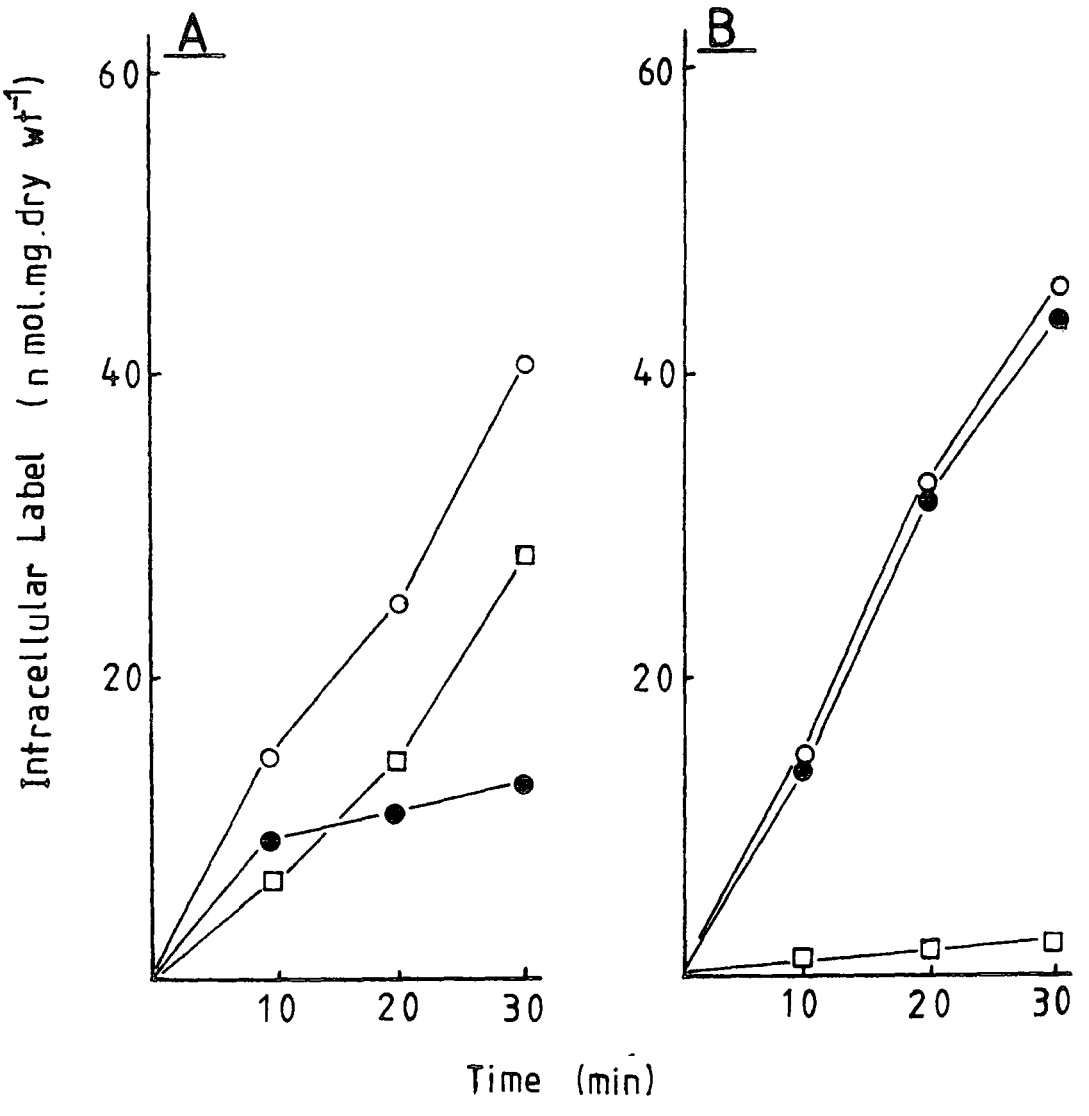


Fig 4-10 Intracellular Cleavage of Ala-Ala-Ala(P)

Cell extracts of A) strain TN FS51 (0.14 mg dry wt ml^{-1}) and B) strain TN FS512 (0.15 mg dry wt ml^{-1}) were prepared during incubation of organisms with Ala-Ala-[^{14}C] Ala(P) (0.1 mM, 0.09 μCi ml^{-1}) and analysed by the standard method. (□) [^{14}C] Ala(P), (●) Ala-Ala- [^{14}C] Ala(P) and Ala- [^{14}C] Ala(P), (○) Total label present.

intracellularly (Figs. 4-9, 4-10) are comparable. One possible explanation for this observation is that the rate of Ala-Ala$^{(P)}$ uptake is lower than that of Ala-Ala-Ala (P) under the conditions used for the assay of growth inhibition (i.e. in complex RST 2 medium). This situation could be explained by the presence of distinct major uptake systems for Ala-Ala (P) and Ala-Ala-Ala(P) in these strains; differences in the effects of medium components on the activity of the two permeases would account for the observed effects. However, the alternative explanation of differential peptidase activity towards the two substrates in different media cannot be discounted.

4-7 Concluding Discussion

4-7-1 Extracellular Cleavage Does not Play a Signficant Role in Peptide Uptake

The results presented in this chapter provide conclusive evidence for the uptake of peptides as such in Strep. faecalis: the lack of inhibition of Ala-Ala and Ala-Ala-Ala(P) uptake by free alanine (Table 4-11), although not kinetically rigorous, is circumstantial evidence for the separation of amino acid and peptide transport; the lack of any reduction in external peptide concentration when cells are incubated without an energy source (Section 4-5-3-5) indicates that extracellular peptidase activity is not significant; intact uptake of Gly-Sar and phosphonopeptides has been demonstrated (Table 4-8).

Perhaps the best evidence, however, comes from studies of uptake of phosphonopeptides. As the Ala$^{(P)}$ moiety is itself impermeant (Section 4-5-4-5), uptake of labelled Ala(P) from phosphonopeptides must be in the peptide form. Ala-Ala-Ala(P)

uptake is sensitive to inhibition by other peptides (Table 4-11) and loss of Ala-Ala(P) uptake in strain TN 979021 is associated with loss of uptake of di- and tripeptides (Table 4-12). That the uptake observed in these experiments is of peptide per se, and not of amino acids subsequent to extracellular cleavage, is therefore not in doubt.

4-7-2 Strain ATCC 9790 Has Two Peptide Permeases

The results of competition studies in strain ATCC 9790 (Table 4-11) and peptide uptake assays in the mutant strains TN 97902 and TN 979021 indicate that there are two peptide transport systems in strain ATCC 9790. The major system handles di- and, to a lesser extent, tripeptides, whereas the second, low-rate system handles oligopeptides preferentially to dipeptides.

The evidence for this can be summarised as follows:
a) Ala-Ala-Ala(P) uptake, which is slow in strain ATCC 9790, and subject to inhibition by Ala-Ala-Ala, Ala-Ala-Ala-Ala, Ala-Ala-Ala-Ala-Ala, and to a lesser extent by Ala-Ala, is abolished in strain TN 97902, which is resistant to the tripeptide-mimetic AX5. Uptake of natural peptides via this system is of low rate; thus Ala-Ala-Ala-Ala and Ala-Ala-Ala-Ala-Ala uptake is indetectable in strain ATCC 9790, and uptake of Ala-Ala and Ala-Ala-Ala is apparently unaltered in strain TN 97902 relative to the parent strain.
b) Uptake of natural di- and tripeptides and Ala-Ala(P) is lost in strain TN 979021, which is resistant to the dipeptide-mimetic AX1. Competition studies show that Ala-Ala and Ala-Ala-Ala use this system.

The presence of two peptide permeases is the only logical conclusion from the data; other explanations display inconsistencies:

a) Uptake via either permease cannot be explained in terms of extracellular cleavage and uptake of released amino acids (see Section 4-7-1).
b) Uptake of oligopeptides cannot be by cleavage extracellularly to di- and tripeptides which are taken up via the major permease. If this were the case, mutants resistant to AX1 should be as easy to isolate from strain ATCC 9790 as from strain TN 97902. The inability to obtain such mutants directly from strain ATCC 9790 is good evidence for the existence of two distinct modes of uptake of AX1, i.e. two distinct peptide permeases.

4-7-3 Specificity of Uptake

Only limited information as to the specificity of uptake with respect to side chain, C-terminus, and peptide bond was obtained. Although a systematic study of side chain specificity of the type performed for Sacc. cerevisiae (Section 3-5-4-1) was not carried out, the major permease of strain ATCC 9790 was shown to transport peptides containing diverse amino acid residues.

The lack of strict C-terminal specificity for uptake is illustrated by the transport of phosphonopeptides. In strain ATCC 9790 the major permease apparently has a rigorous requirement for a terminal α-carboxyl group, as Ala-Ala and Ala-Ala-Ala were transported rapidly, but Ala-Ala(P) was a poor substrate, and Ala-Ala-Ala(P) uptake was indetectable (uptake in strain TN 97902, Table 4-12). In contrast, the low-rate, oligopeptide system in this strain apparently handles phosphonopeptides at comparable rates to the 'parent' peptides. Rates of uptake via this system are too low to detect by the standard fluorescamine assay used, and assay of labelled oligopeptide (e.g. Ala-Ala-Ala- [^{14}C] Ala) uptake is

impracticable at these low rates because of the complications of amino acid exodus. Only for peptides in which the labelled moiety does not undergo exodus subsequent to cleavage (e.g. phosphonopeptides) can these low rates of uptake be quantitated. However, if the rates of uptake of natural oligopeptides were appreciably higher than those of phosphonopeptides via this system, they would be detectable by the assays used. In strain OG-FS5, a similar C-terminal specificity to that of strain ATCC 9790 was observed: Ala-Ala-Ala, Ala-Ala-Ala-Ala, Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P) were transported at similar rates, but Ala-Ala(P) was a much poorer substrate than Ala-Ala.

Both permeases in strain ATCC 9790 have some tolerance of peptide bond modifications, as both sarcosine peptides and aminoxy-peptides are taken up. However, as the rates of uptake of these substrates are not compared to the 'parent' peptides in the present studies, the effects of these modifications on kinetic parameters of transport cannot be assessed.

4-7-4 Strain Differences in Peptide Uptake

There are clear differences in the rates of uptake of peptides in the different parental strains studied here (see for example Fig. 4-5). Whereas in strain ATCC 9790 the presence of two permeases has been demonstrated, in strains OG-FS5 and NCIB 6459 the number and specificity of the permease(s) is not known. The differences in uptake rates in the three strains could result from either, a) the presence of permeases of the same substrate specificity but different rates in each strain, or b) a different array of permeases in each strain. Although in the absence of firm evidence any discussion must be speculative, the circumstantial evidence available will be

considered below.

The data from the uptake of homo- alanyl peptides (Fig. 4-5) can in each strain be explained by the existence of two permeases, one handling Ala-Ala and to a lesser extent Ala-Ala-Ala, and a second handling Ala-Ala-Ala, Ala-Ala-Ala-Ala, and to a lesser extent Ala-Ala, operating at differing relative rates. In strain OG-FS5, Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P) are transported at similar rates to Ala-Ala-Ala and Ala-Ala-Ala-Ala (a property of the oligopeptide permease in strain ATCC 9790; Section 4-7-3), whereas Ala-Ala(P) is transported much more slowly than Ala-Ala (a property of the dipeptide permease in strain ATCC 9790); this is consistent with the presence of two permeases in strain OG-FS5 of similar specificity to those characterised in strain ATCC 9790. Additionally, in strain TN FS51, the apparent discrepancy between the similarity in the rates of uptake of Ala-Ala(P) and Ala-Ala-Ala(P) from phosphate buffer, and their differing anti-bacterial activity in RST 2 medium (Section 4-6-3-4) could be explained in terms of separate permeases for the two substrates, although the alternative explanation of differential peptidase activity in the two media cannot be discounted on the basis of experimental evidence. If two permeases are present, the mutation giving rise to Ala-Ala(P) resistance in strain TN FS51 must be in some factor common to both permeases, as uptake of all tested peptides was affected by it. Such a mutation could, for example, be in an energy-coupling component, or some factor, such as a cell-wall component, which limits accessibility to the permease itself. Investigations on the nature of the mutation in strain TN FS51, to assess whether it affects peptide transport specifically, or transport processes more

generally, are indicated.

In summary, the observed features of peptide uptake in the strains studied are compatable with the presence of two permeases of similar specificity to those in strain ATCC 9790. The isolation of transport-deficient mutants from, and more exhaustive competition studies in, strains other than ATCC 9790 should elucidate this point.

4-7-5 Exodus of Amino Acids

The exodus of constituent amino acids of transported peptides (Section 4-5-3-2) is a similar finding to that reported in other organisms such as E.coli, Salmonella typhimurium, and Staph. aureus (Section 1-1-3). The present studies do, however, present firm evidence that the exodus here occurs via amino acid permease(s), as the presence of unlabelled amino acid in the medium accelerates exodus of peptide-derived [^{14}C] Ala (Fig. 4-3).

It has been reported (Harold and Spitz, 1975) that, in Strep. faecalis, accumulated Asp and Glu do not undergo exchange for the corresponding amino acids in the medium. In view of this, it would be of interest to study the uptake of say, Glu-Ala and to see if, a) Glu exodus, and b) trans-stimulation of Glu exodus, occurred.

4-7-6 Peptide Transport in Peptidase-Deficient Strains

It is logical to assume that, if peptide is accumulated to high levels by cells, and it is not cleaved intracellularly, that the net rate of uptake will decrease, due either to inhibition of uptake by the accumulated substrate, or enhanced exodus of previously accumulated substrate. In the present studies on peptide uptake in Strep. faecalis, however, very large accumulation ratios of peptides

were detected with no apparent slowing of net uptake rate. For example, the uptake of Ala-Ala(P) in the peptidase-deficient strain TN 97901 (Fig. 4-7) led to an intracellular concentration of approximately 115 mM (a 750 fold gradient) with no apparent reduction in net uptake rate. Presumably, even larger internal concentrations are necessary to slow the net rate of uptake. In order to achieve such gradients, a rapidly transported, non-cleavable substrate is needed. Further investigation of the peptidase activity in strain TN 97901 may reveal such a substrate. However, in any such study, possible general inhibitory effects of high intracellular substrate must always be considered.

CHAPTER 5

THE ENERGETICS OF PEPTIDE TRANSPORT IN *SACCHAROMYCES CEREVISIAE* AND *STREPTOCOCCUS FAECALIS*

5-1 Introduction - Action of Metabolic Inhibitors

The literature information on energy coupling of peptide uptake is sparse, the only firm information being on E.coli (Section 1-4-4-2). For both Sacc. cerevisiae (Section 3-5-5-2), and Strep. faecalis (Section 4-5-3-1), intact peptide accumulation, and thus active peptide transport, has been demonstrated in the present work. In this chapter, the effects of metabolic inhibitors on the uptake of peptides in these two organisms are investigated and, for Strep. faecalis, some preliminary studies of the energy consumption of uptake are made.

Metabolic inhibitors are generally considered to be inhibitors of ATP production or of the maintenance of a proton-motive force. However, they generally have more than one site of action, and so care must be taken in interpreting their effects. A brief survey of the known effects of the inhibitors used in these studies is therefore relevant.

5-1-1 Azide

Sodium azide is an inhibitor of cytochrome oxidase and membrane bound ATP-ase, and acts as a proton-conducting ionophore (Harold, 1972). In E.coli, at low concentrations (1 mM) azide acts as an ATPase inhibitor, but at higher concentration (10 mM) acts as an uncoupler of oxidative phosphorylation (Kobayashi et al., 1977). In organisms that rely on oxidative phosphorylation for ATP synthesis, azide causes severe depletion of ATP levels (Ridgway, 1977; Reber et al., 1977). In Sacc. cerevisiae, glycolytic ATP production is unaffected by azide, but ion transport is severely inhibited (Riemersma, 1968), presumably as a result of dissipation

of the proton gradient (Riemersma and Alsbach, 1974). Apart from effects on energy metabolism, azide also affects other metabolic processes, for example cysteine biosynthesis in Salmonella typhimurium. In Neurospora crassa, the mitochondrial ATPase is sensitive to azide, but the plasmalemma ATPase is not (Bowman et al., 1978).

5-1-2 2-4, Dinitrophenol

2-4, dinitrophenol (DNP) is a specific proton conducting ionophore in both biological and artificial membranes, and thus acts as an uncoupler of oxidative phosphorylation (Harold, 1972). However, in organisms such as Sacc. cerevisiae and Strep. faecalis, which rely on a membrane ATPase to maintain a proton motive force (p.m.f) across the cell membrane, uncouplers may lead to an increase in ATPase activity in an attempt to maintain the p.m.f., and so to a decrease in ATP levels in the cell. For example, in a respiratory deficient strain of Sacc. cerevisiae, DNP caused a 35% fall in cellular ATP level, although fermentation rate was not affected (Serrano, 1980). Similarly, in Strep. faecalis (Bakker and Harold, 1980), DNP caused a 45% fall in ATP level during a 50 min incubation. This effect seems likely to be general for all uncouplers in these organisms.

5-1-3 Carbonylcyanide m-chlorophenylhydrazone

Carbonylcyanide m-chlorophenylhydrazone (CCCP) is, like DNP, recognised as a proton-translocating uncoupling agent (Harold, 1972). It has been shown, for example, to facilitate diffusion of protons across the membranes of mitochondria (Mitchell and Moyle, 1967), Strep. faecalis (Harold and Baarda, 1968), and Klebsiella

aerogenes (Reber et al., 1977). A lowering of cellular ATP levels in the presence of CCCP was reported not to occur in Strep. faecalis (Harold and Baarda, 1968), but does occur in a respiration-deficient strain of Sacc. cerevisiae (Serrano, 1980), presumably as a result of increased plasma-membrane ATPase activity.

CCCP also acts as a sulfhydryl reagent, and in E.coli directly inhibits some permeases that possess an essential -SH group (Kaback et al., 1974). The half-life of such inhibition has been found to be 20 min, whereas the uncoupling action is complete within 1 min (Cecchini and Koch, 1975). CCCP also acts as an inhibitor of the Ca^{2+}-stimulated ATPase in erythrocyte ghosts (Hayashi et al., 1975) and of respiration in the gliding bacterium Flexibacter polymorphus (Ridgway, 1977), although in both cases higher concentrations than those needed for uncoupling are required. In the latter study, 4×10^{-6} M CCCP caused a transient (3 min) increase in respiration rate, consistent with uncoupling, followed by a decrease in respiration rate, consistent with inhibition of a component of the respiratory chain.

5-1-4 N-N' Dicyclohexylcarbodiimide

N-N' dicyclohexylcarbodiimide (DCCD) is a potent inhibitor of ATPases from various sources: mitochondrial ATPases from, for example, mammals (Beechey et al., 1967) and Neurospora crassa (Bowman et al., 1978); plasmalemma ATPases from, for example, Sacc. cerevisiae (Serrano, 1980) and Neurospora crassa (Bowman et al., 1978); bacterial ATPases from, for example, Strep. faecalis (Harold et al., 1969), E.coli (Evans, 1970), and Klebsiella aerogenes (Reber et al., 1977). In Strep. faecalis,

DCCD does not inhibit glycolysis and, in energy starved cells, lowers the rate of depletion of the ATP pool (Harold et al., 1969). Similarly, in a respiration deficient strain of Sacc. cerevisiae, DCCD inhibits the ATPase and proton pumping, but does not affect ATP levels (Serrano, 1980). Studies using Strep. faecalis have shown that DCCD interacts with part of the ATPase complex that is closely associated with the membrane (Abrams et al., 1972). This is likely to be analogous to the low molecular weight, hydrophobic, DCCD reacting protein that is part of the beef heart mitochondrial ATPase complex (Tzagoloff, 1976). As DCCD is highly reactive towards carboxyl groups, other less specific interactions, for example with components of permeases, cannot be entirely ruled out.

5-1-5 Arsenate

Arsenate is an antagonist of phosphate in phosphorylation reactions and thus interferes with both glycolytic and respiratory ATP production. Arsenate inhibits oxidative phosphorylation in rat liver cells (Crane and Lipmann, 1953), and drastically reduces ATP levels in both aerobic and anaerobic E.coli (Klein and Boyer, 1972).

5-2 Materials

N-N' Dicyclohexylcarbodiimide, carbonylcyanide m-chlorophenylhydrazone, N-ethylmorpholine, and dimethylglutaric acid were from Sigma (London Ltd). Sodium azide, potassium arsenate and 2,4 dinitrophenol were from BDH Ltd. Reagents for the assay of peptide uptake were as given previously (Section 2-2).

5-3 Methods

5-3-1 Harvesting of Organisms

Cultures of Sacc. cerevisiae were grown as previously described (Section 3-3-2), harvested by membrane filtration, and resuspended in citrate phosphate buffer (pH 4.5, 25 mM phosphate) with glucose (1% w/v) to a density of 1 mg dry wt ml^{-1}.

Cultures of Strep. faecalis were grown in RST 2 medium, as previously described (Section 4-3-2), harvested by membrane filtration, and resuspended in K_2HPO_4/KH_2PO_4 buffer (pH 6.9, 50 mM phosphate) with glucose (0.2% w/v) to a density of 0.07-0.17 mg dry wt ml^{-1}.

In studies on the effect of starvation for an energy source, organisms were resuspended in the above buffers, but without glucose. In incubations with arsenate, phosphate-free buffers were used, as arsenate action is antagonised by phosphate. Details of these buffers are given in the results sections.

5-3-2 Monitoring Peptide Uptake in the Presence of Inhibitors

Resuspended organisms were preincubated at 28°C (for Sacc. cerevisiae) or 37°C (for Strep. faecalis) in the presence of a metabolic inhibitor. Stock solutions of DNP, CCCP, and DCCD were in ethanol, but the final concentration of ethanol in incubations never exceeded 1% (v/v). In all cases where ethanol was introduced, peptide transport rate was determined relative to a control containing ethanol, but no inhibitor. Similarly, in experiments with arsenate, uptake rate was determined relative to a control in the same incubation buffer. After pre-incubation (10 min), peptide (0.1 mM) was added, and uptake assayed by the standard dansylation (Section 2-3-2), automated fluorescamine (Section 2-5-2), or radiotracer (Section 2-6-2) methods.

5-3-3 Peptide Uptake in Response to Glucose Addition to Starved Strep. faecalis

Strep. faecalis cells were resuspended to a density of 0.11 mg dry wt ml^{-1} in the absence of glucose. Organisms were pre-incubated for 10 min at 37°C (after which time they are not capable of peptide transport - Section 5-4-3), peptide (routinely 0.1 mM) was added, and sampling by the automated fluorescamine method (pump speed 5, 0.45 ml min^{-1}) was commenced. As no peptide uptake occurred, a plateau corresponding to the peptide concentration added was obtained on the chart recorder. After 5 min 40s (2.5 ml of incubation medium pumped) glucose (routinely 0.1 mM) was added, and pumping was continued for another 11.5 min (5 ml of incubation medium). Addition of glucose gave rise to peptide uptake which continued until the glucose was all utilized (Fig. 5-1).

5-4 Results and Discussion

5-4-1 Effect of Metabolic Inhibitors on Peptide Uptake in Sacc. cerevisiae

The effect of various metabolic inhibitors, or anaerobiosis, on Ala-Ala uptake in Sacc. cerevisiae was assayed by using the automated fluorescamine method (Table 5-1). As fluorescamine reacts with amino acids (although only to a small extent under the assay conditions used, Section 2-4-3-4), extensive generalised amino acid exodus in response to an inhibitor could mask peptide uptake from detection. Potential loss of cellular amino acid was checked by monitoring cell extracts and samples of the medium by using dansylation before and after addition of peptide, in the presence of metabolic inhibitors. In no instance was leakage of dansyl-reactive material detectable (data not shown). In no case did the

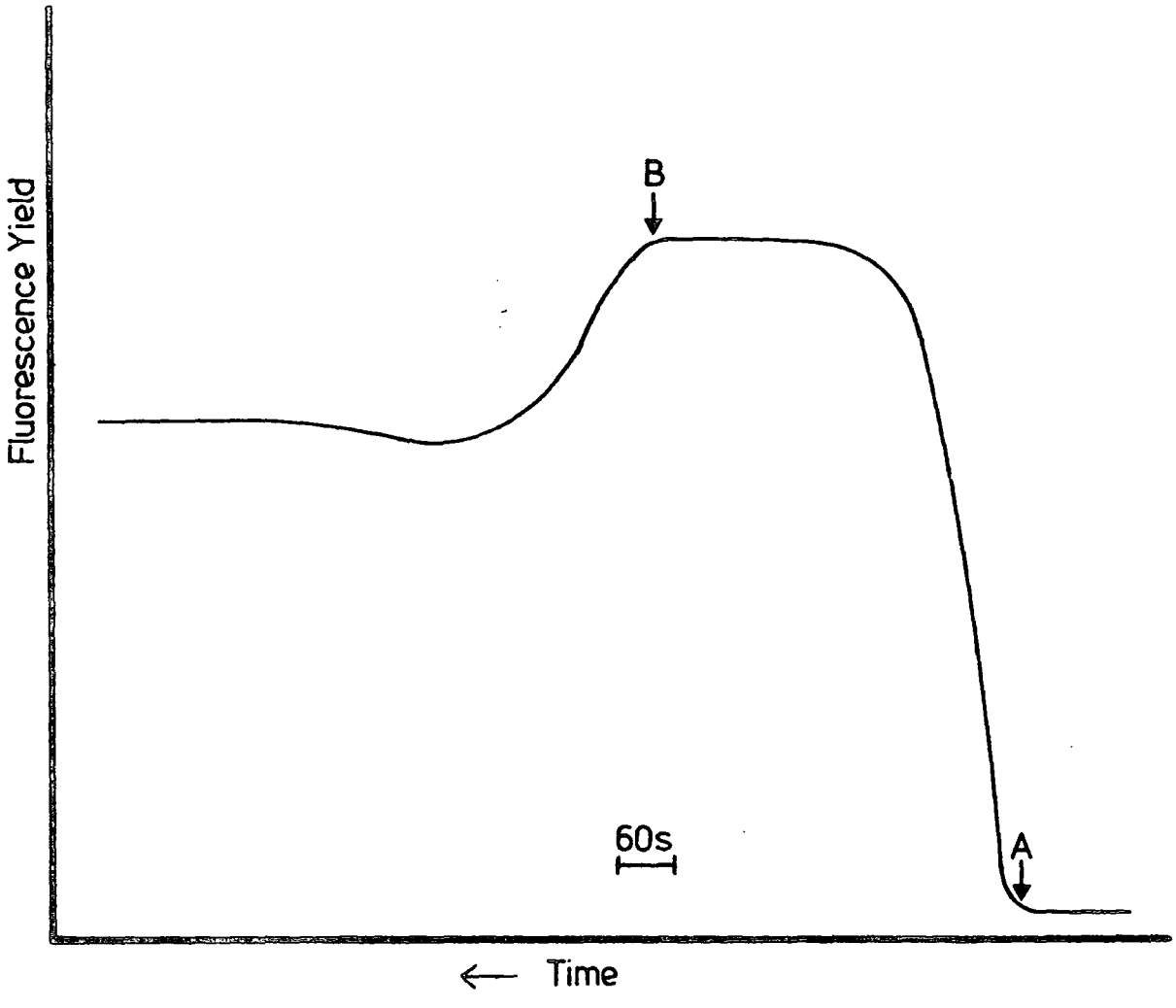


Fig 5-1 The Uptake of Ala-Ala in Response to Glucose in Starved Strep. faecalis

Strep. faecalis (faecium) strain ATCC 9790 was harvested and resuspended in $KH_2PO_4/K_2H\ PO_4$ (50 mM, pH 6.9) to a density of 0.11 mg dry wt ml^{-1}. After pre-incubation (10 min), Ala-Ala (0.1 mM) was added (A). After a further 5.75 min glucose (0.1 mM) was added (B). Ala-Ala concentration was monitored using the automated fluorescamine assay.

Table 5-1 Effect of metabolic inhibitors on Ala-Ala uptake in Saccharomyces cerevisiae Strain Σ1278b

Inhibitor	Conc.	% Inhibition of Ala_2 Uptake
azide	10 mM	100
arsenate	30 mM	100
DCCD	1 mM	100
CCCP	20 μM	65
	100 μM	100
Under N_2	-	10

Organisms were prepared as in Section 5-3-1 to a density of 1 mg dry wt ml^{-1}, preincubated for 10 min with inhibitor at 28°C, and uptake of Ala-Ala (100 μM) assayed by the automated fluorescamine procedure. Incubations with arsenate were carried out in dimethylglutaric acid/KOH (25 mM, pH 4.5). Inhibition is expressed relative to a control without inhibitor.

pH of the incubation medium change more than 0.1 unit after addition of inhibitor.

In the discussion of the results in Table 5-1 that follows, it is assumed that the active transport step being monitored is at the plasmalemma, although the alternative model of facilitated diffusion across the plasmalemma and active accumulation into a subcellular compartment (Fig. 1-1d) cannot be discounted on the basis of the experimental evidence.

The inhibition of Ala-Ala uptake by arsenate is to be expected regardless of the energy linkage of peptide uptake, as in Sacc. cerevisiae the plasmamembrane potential is generated by an ATPase (Section 1-4-3-2). The report (Becker and Naider, 1977) that arsenate was a poor inhibitor of peptide uptake in Sacc. cerevisiae is not useful, as incubations were performed in phosphate buffer.

Anaerobiosis did not significantly inhibit Ala-Ala uptake, indicating that mitochondrial respiration is not a necessity for peptide uptake to occur. As Sacc. cerevisiae cells grown in the high (2% w/v) concentrations of glucose used here possess few mitochondria, and rely largely on glycolysis for ATP production (Chandrasekaran et al., 1978) this result is to be expected.

The inhibitory action of the uncoupler CCCP indicates that peptide uptake is dependent on the maintenance of a p.m.f.. However, as cells were preincubated with CCCP for 10 min, secondary effects (Section 5-1-3) cannot be discounted. In experiments in which CCCP was added during Ala-Ala uptake by Sacc. cerevisiae (Payne and Nisbet, 1980c), uptake was abolished within 30s, giving strong

evidence that CCCP is acting as an uncoupler in this case (Section 5-1-3).

Preincubation with the ATPase inhibitor DCCD also totally inhibited uptake, which is, like the action of uncouplers, evidence for a role of the p.m.f.. Action of DCCD on the mitochondrial ATPase can be excluded here, as results from anaerobic cells (see above) rule out an obligate role for aerobic respiration. Inhibition of Ala-Ala uptake by diethylstilbestrol (Payne and Nisbet, 1980c) is additional evidence for the involvement of the plasmamembrane p.m.f.,(see Serrano, 1980), as in Neurospora crassa it is a specific inhibitor of the plasmalemma ATPase but not the mitochondrial ATPase (Bowman et al., 1978).

In summary, the uptake of Ala-Ala in Sacc. cerevisiae strain Σ1278b depends on the presence of a proton-motive-force across the plasmalemma. However, it should not necessarily be deduced that this is the primary driving force for uptake.

5-4-2 Effect of Metabolic Inhibitors on Peptide Uptake in Strep. faecalis.

The effects of metabolic inhibitors on peptide uptake by Strep. faecalis (faecium) strain ATCC 9790 are summarised in Table 5-2. Ala-Ala and Ala-Ala-Ala(P) were chosen for study, as these are representative substrates of the two peptide permeases in this strain (Section 4-7-2). Preliminary studies with DNP, DCCD, CCCP, and arsenate were carried out using dansylation of medium samples, to see whether or not there occurs any generalised amino acid exodus in response to the inhibitors. In no case did the addition of inhibitor give rise to exodus, the only detectable amino acids in the medium

Table 5-2 Effect of metabolic inhibitors on peptide uptake in S. faecalis (faecium) strain ATCC 9790

Inhibitor	Conc.	% Inhibition of Uptake			
		Ala_2 DNS	Ala_2 F	Ala_2 RT	Ala_3(P) RT
DNP	1 mM	75	-	-	35
	6 mM	100	-	-	95
Arsenate	10 mM	100	-	-	100
	40 mM	100	-	-	100
DCCD	1 mM	-	100	-	-
Under N_2	-	0	-	-	-
No Glucose	-	-	100	100	100
CCCP	20 μM	-	95	-	-

Organisms were prepared as in Section 5-3-1 to a density of 0.07 - 0.17 mg dry wt ml^{-1}, preincubated for 10 min with inhibitor at 37°C, and uptake of peptide (100 μM) assayed as in Methods. (DNS: dansyl chloride assay; F: automated fluorescamine assay; RT: radiotracer assay). Incubations with arsenate were carried out in N-ethylmorpholine-HCl (0.2M, pH 6.9). Inhibition of uptake is expressed relative to a control without inhibitor.

being those normally found in low amounts for cells resuspended in phosphate buffer (see e.g. Fig. 4-2, A).

Anaerobiosis did not affect the rate of Ala-Ala uptake in strain 9790. As Strep. faecalis lacks respiration, and relies on glycolysis for ATP production, this result is entirely predictable.

Inhibition of peptide uptake by arsenate is also predictable, as in Strep. faecalis the p.m.f. is produced by a membrane ATPase, and so inhibition of ATP production will affect transport whether it is linked directly to phosphate bond energy or to the p.m.f.

When cells were preincubated with the uncouplers DNP or CCCP, peptide uptake was strongly inhibited, indicating a role of the p.m.f. As for Sacc. cerevisiae (Section 5-4-1), addition of CCCP to Strep. faecalis (faecium) strain ATCC 9790 accumulating Ala-Ala led to a very rapid inhibition of transport (Payne and Nisbet, 1980c), which argues for the primary action of CCCP here being as a protonophore and not as a sulfhydryl reagent. The addition of CCCP to strain ATCC 9790 during Ala-Ala uptake caused extensive exodus of peptide from the cells (Payne and Nisbet, 1980c), which indicates that Ala-Ala transport in this strain is occurring against a large concentration gradient.

The ATPase inhibitor DCCD gave complete inhibition of Ala-Ala uptake, which, like the action of uncouplers, implicates the p.m.f. in peptide transport. Therefore, as with Sacc. cerevisiae, the maintenance of a p.m.f. seems necessary but, as is always the case with studies of this type with metabolic inhibitors, it cannot be defined as the driving-force for transport.

5-4-3 Glucose-Stimulated Peptide Uptake in Starved Strep. faecalis

5-4-3-1 Introduction

When strain ATCC 9790 is resuspended in phosphate buffer and preincubated for 10 min at 37°C, peptide uptake is indetectable when assayed either by disappearance of Ala-Ala from the medium, or by uptake of radiolabel from Ala-Ala or Ala-Ala-Ala(P) (Table 5-2). Similar results were obtained with strain OG-FS5 (data not shown). The energy reserves of Strep. faecalis (faecium) ATCC 9790 are small and readily depleted (e.g. Harold and Spitz, 1975; Bakker and Harold, 1980) and it appears that a 10 min glucose starvation is sufficient to reduce them to a level where peptide transport cannot be supported.

The addition of glucose to a suspension of starved cells, in the presence of peptide, leads to rapid (< 15s delay) energisation of peptide uptake, which continues until the supplied glucose is consumed (see for example Fig. 5-1). From the amount of peptide taken up, an apparent stoichiometry of uptake (peptide: glucose) can be derived. In some cases a slight increase in medium peptide concentration was noted after depletion of glucose. This is consistent with exodus of pre-accumulated peptide when the energy supply for transport is exhausted, and is analagous to the effects of CCCP and DCCD added to cells transporting peptide (Section 5-4-2; Payne and Nisbet, 1980c).

5-4-3-2 Apparent Stoichiometry of Peptide Uptake

The apparent stoichiometry and initial rate of uptake of a range of di- and tripeptides in strain ATCC 9790 was assayed by

using the automated fluorescamine procedure (Section 5-3-3). There is a correlation between apparent stoichiometry and initial rate of uptake (Fig 5-2). Studies of the uptake of di- to penta-peptides in strain OG-FS5 show a similar correlation (Fig. 5-3). These correlations between rate of uptake and apparent stoichiometry are consistent with competition for available energy between peptide transport and other metabolic processes, more rapidly transported substrates obtaining a greater proportion of the available energy.

5-4-3-3 Peptide Transport Rate Depends on Glucose Concentration

The effect of the amount of glucose supplied on the initial rate of peptide uptake by starved cells of strain ATCC 9790 was investigated (Fig. 5-4). The increase in rate of uptake with increasing glucose concentration is consistent with the rate of peptide transport being limited by the energy supply under the conditions of the assay.

5-4-3-4 Endogenous Rate of Glucose Utilization

As it appears likely from the above that there is competition for available energy between peptide uptake and other metabolic events, the endogenous rate of glucose utilization in the absence of peptide transport was measured: glucose was added to starved cells of strain ATCC 9790, and Ala-Ala added at various times afterwards. In each case the amount of peptide transported, relative to a control where the peptide was added before the glucose, was established and taken as an indication of the amount of glucose remaining unmetabolised (Fig. 5-5). For both 100 μM and 200 μM glucose, the endogenous rate of utilization was the same

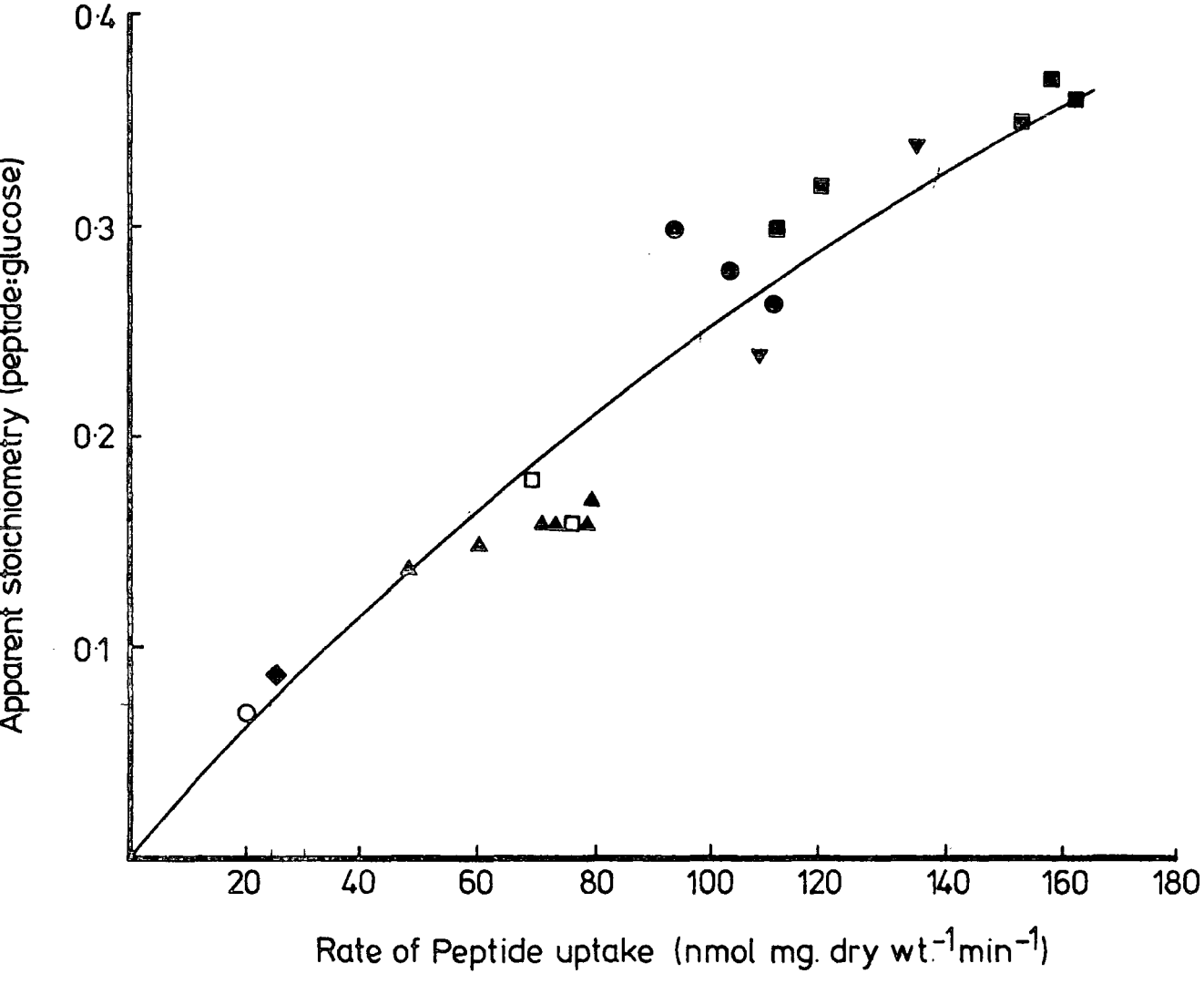


Fig 5-2 Relationship Between Uptake Rate and Apparent Stoichiometry for Peptides in Strain ATCC 9790

Uptake of peptides in glucose-pulsed, starved cells of strain ATCC 9790 was monitored by the standard method (Section 5-3-3). Maximum rate of uptake and apparent stoichiometry were measured.

(■) Ala-Ala, (▼) Ala-Ser, (●) Lys-Ala, (▲) Leu-Leu,
(□) Ala-Leu, (◆) Ala-Ala-Ala, (○) Met-Gly-Met

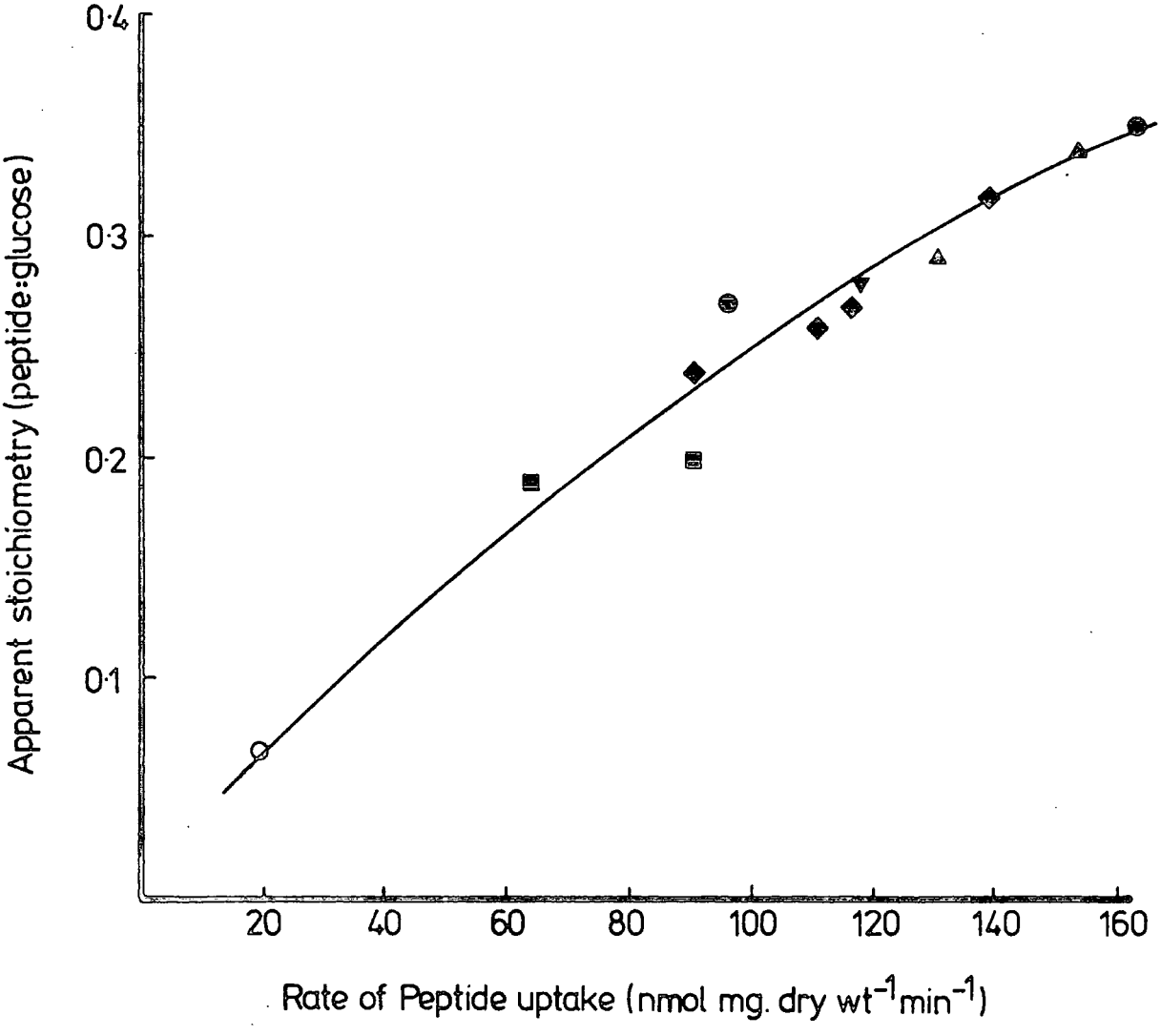


Fig 5-3 Relationship Between Uptake Rate and Apparent Stoichiometry for Peptides in Strain OG-FS5

Uptake of peptides in glucose-pulsed, starved cells of strain OG-FS5 was monitored by the standard method (Section 5-3-3). Maximum rate of uptake and apparent stoichiometry were measured. (■) Ala-Ala, (◆) Ala-Ala-Ala, (●) Ala-Ala-Ala-Ala, (▲) Ala-Ala-Ala-Ala-Ala, (○) Ala-Ala(P), (▽) Ala-Ala-Ala(P).

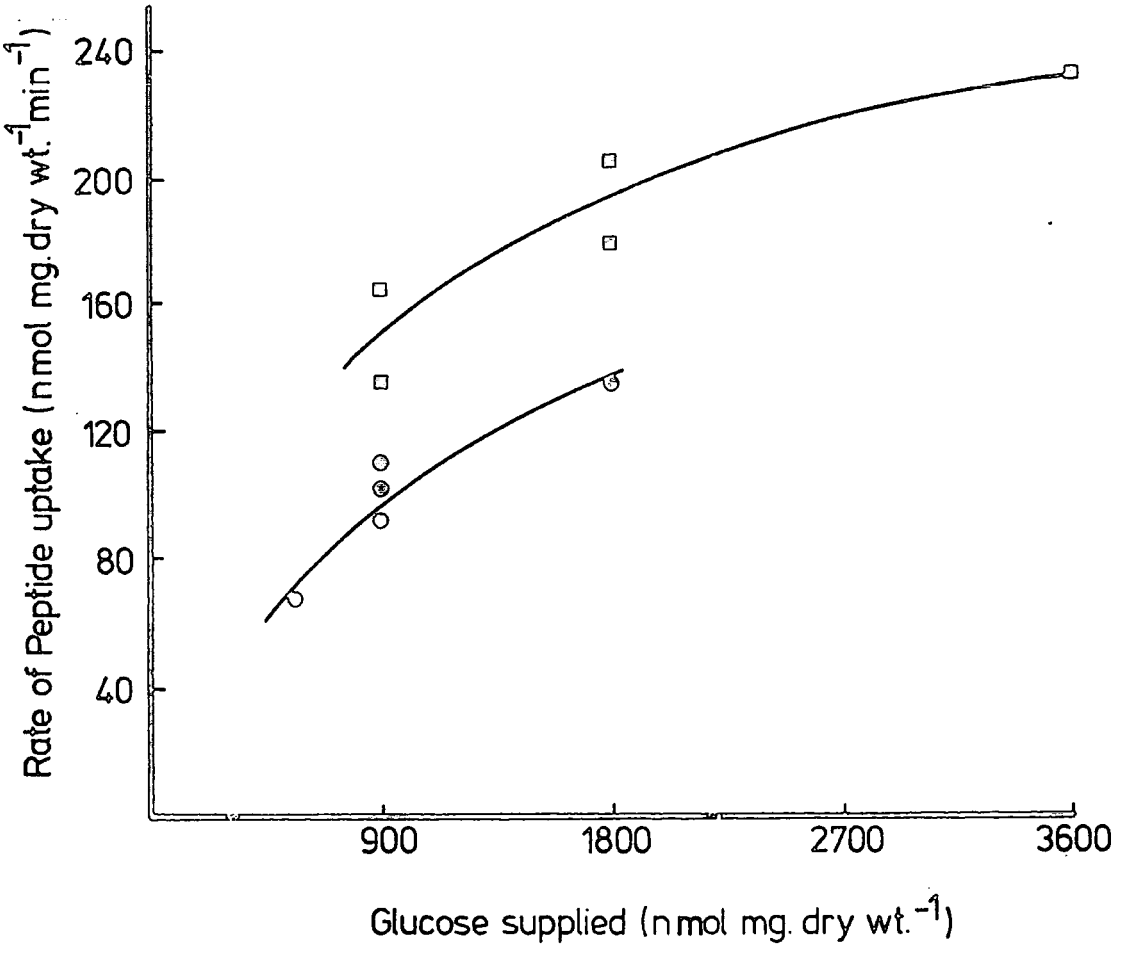


Fig 5-4 The Effect of Glucose Concentration on the Rate of Peptide Uptake in Starved Cells of Strain ATCC 9790

Uptake of 0.2 mM Ala-Ala (□) or 0.1 mM Lys-Ala (o) by starved cells of strain ATCC 9790 was assayed by the standard method, except that the amount of glucose added was varied. Initial rate of peptide uptake was measured.

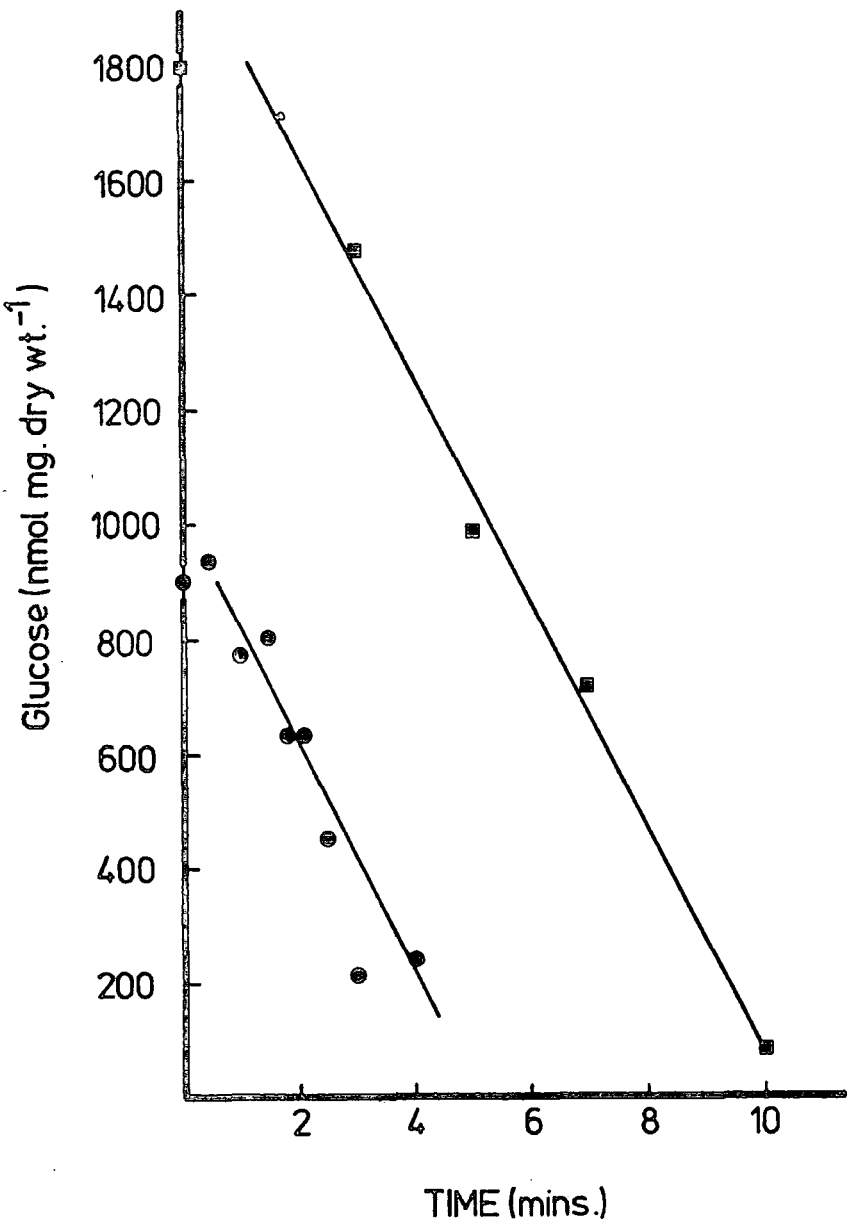


Fig 5-5 Endogenous Rate of Glucose Utilization by Strain ATCC 9790

Tne endogenous rate of utilization of 0.1 mM (⊙) or 0.2 mM (⊡) glucose by starved cells of Strep. faecalis (faecium) strain ATCC 9790 (0.11 mg dry wt ml^{-1}) was determined as described in Section 5-4-3-4.

(approx. 180 nmol min^{-1} mg dry wt^{-1}).

5-4-3-5 Peptide Uptake Consumes Energy

The intact accumulation of glycyl-sarcosine and Ala-Ala(P) by Strep. faecalis has been directly demonstrated (Section 4-5-3-1) and that of Ala-Ala indirectly demonstrated by CCCP-induced exodus (Payne and Nisbet, 1980c). The intact uptake of Ala-Ala by OG-FS5 was similarly shown under the assay conditions used here (Fig. 5-6), addition of CCCP causing peptide exodus. Although concentration gradients during uptake will presumably vary from peptide to peptide depending on the intracellular peptidase specificity, it seems reasonable to assume that uptake is concentrative, and hence active, for most substrates.

Evidence for the consumption of energy by peptide uptake comes from studies in which the time taken to utilize a given amount of glucose is measured. Whereas in energy-starved cells of strain ATCC 9790 the rate of glucose consumption from a 100 μM solution is 180 nmol min^{-1} mg dry wt^{-1} (Fig. 5-5) in the absence of peptide uptake, utilization in the presence of peptide uptake is quicker (approx. 200-250 nmol min^{-1} mg dry wt^{-1}), as derived from many uptake experiments, Section 5-4-3-2). However, because of the lag in response of the automated fluorescamine assay system, it is not yet possible to precisely measure the time that uptake is energised by a pulse of glucose. A rapidly transported peptide (Ala-Ala) did, however, consistently utilize the available glucose more quickly than a slowly transported peptide (Ala-Ala-Ala) in strain ATCC 9790.

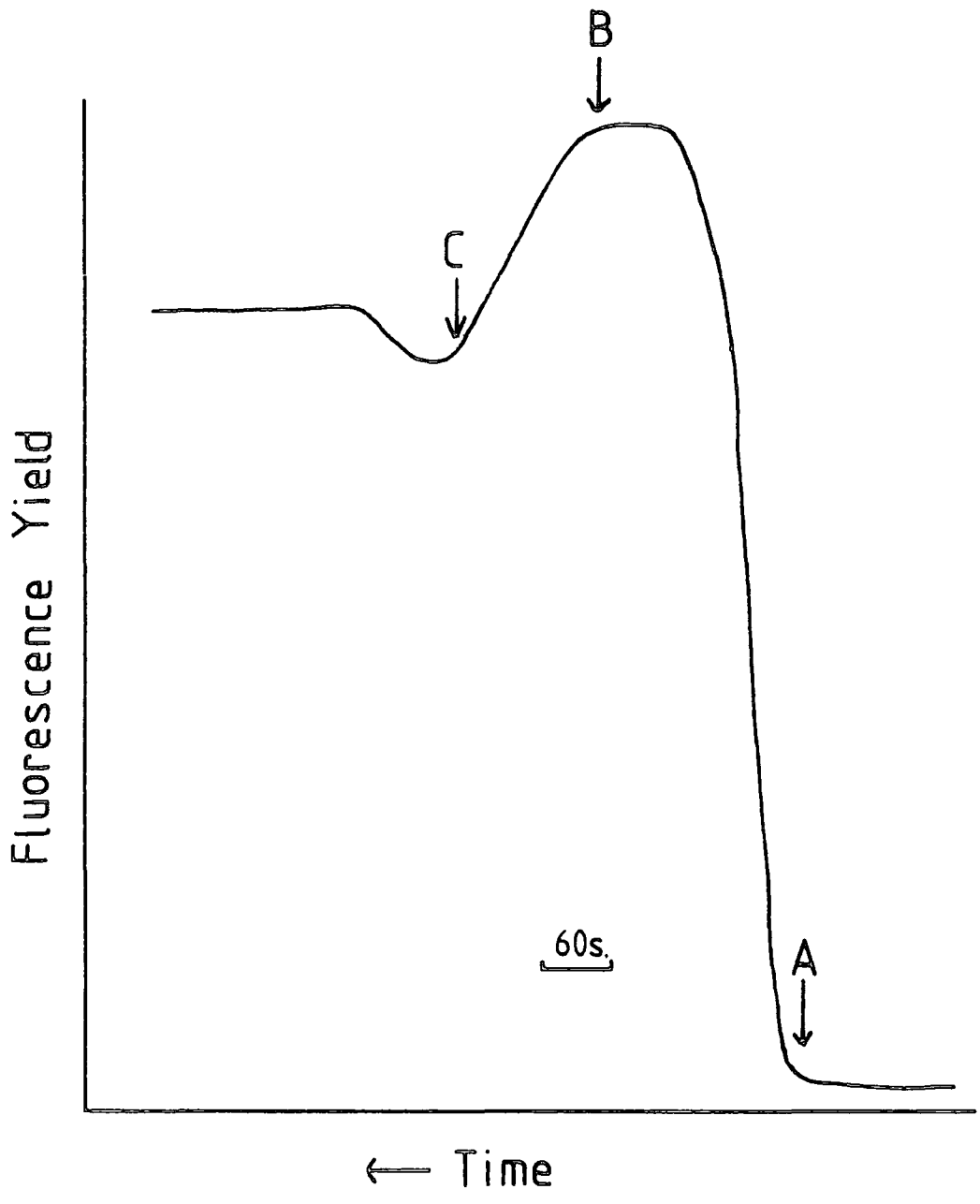


Fig 5-6 CCCP-Induced Peptide Exodus from Strep. faecalis Strain OG-FS5

Strain OG-FS5 was suspended in K_2HPO_4/KH_2PO_4 (50 mM, pH 6.9) to a density of 0.11 mg ml^{-1}, pre-incubated for 10 min and uptake of Ala-Ala followed by the automated fluorescamine method. A) Ala-Ala (0.1 mM) added, B) Glucose (0.1 mM) added, C) CCCP (0.1 mM) added. Addition of CCCP leads to exodus of pre-accumulated Ala-Ala

5-4-3-6 Rates of Peptide Uptake in Starved and Non-Starved Cells

The initial rates of uptake of peptides in glucose-pulsed, energy-starved cells are generally greater than those in non-starved cells (Table 5-3). This effect could arise in a number of ways, for example, the removal of an inhibitory control of uptake in starved cells, or an 'overshoot' above the normal p.m.f. of unstarved cells when glucose is supplied to starved cells.

5-4-3-7 Discussion and Comparison of Stoichiometry of Different Peptides

The utilization of glucose by Strep. faecalis to energise peptide transport and other metabolic processes can be modelled as in Figure 5-7. The intermediate 'X' may be the p.m.f. or some "high-energy" coumpound. In the absence of peptide transport, increasing the glucose concentration does not lead to a greater rate of utilization (Section 5-4-3-4), which indicates that the rate of use of ATP by endogenous sinks (C1 and C2 in Fig. 5-7) is the limiting step. In the presence of a rapidly-transportable peptide, however, rate of glucose metabolism (A) appears limiting as increasing the glucose concentration leads to more rapid transport (Section 5-4-3-3). The presence of potentially transportable peptide when 100 μM glucose is added to a cell suspension thus appears to make the rate of supply of energy from glucose limiting, whereas in the absence of peptide the rate of use, not supply, is the limiting factor.

The rate of endogenous glucose utilization can be simply measured (Section 5-4-3-4). If this rate of use could be assumed

Table 5-3 Rates of Peptide Uptake in Energy-Starved and Non-Energy-Starved Cells of Strep. faecalis

Strain	Peptide	Uptake Rate (nmol min^{-1} mg dry wt^{-1})	
		Energy Starved	Non-Energy-Starved
ATCC 9790	Ala-Ala	110-160(5)	92-103(5)
	Leu-Leu	50-80(3)	58-66(2)
	Ala-Ala-Ala	25(1)	21-29(5)
	Met-Gly-Met	20(1)	21-25(3)
OG FS5	Ala-Ala	65-90(2)	52(1)
	Ala-Ala-Ala	90-140(4)	82(1)
	Ala-Ala-Ala-Ala	100-160(2)	110(1)
	Ala-Ala-Ala-Ala-Ala	130-155(2)	107(1)

Uptake rates for starved cells are obtained from Figs 5-2 and 5-3, and those for non-starved cells from Tables 4-9 and 4-13. Rates given are the range of the number of determinations in parentheses.

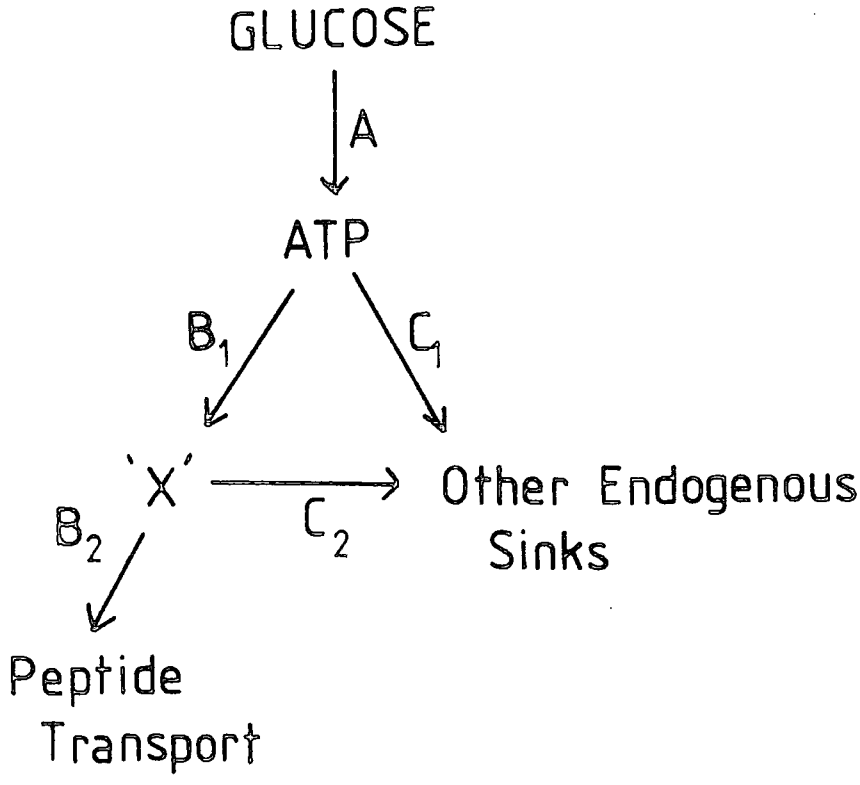


Fig 5-7 Routes of Energy Flow for Transport and Metabolism in Strep. faecalis.

"X" is the direct energy source for transport.

to apply in the presence of peptide uptake, and the time taken for peptide uptake accurately measured, real stoichiometries of peptide uptake could be derived. However, as there is competition for energy between other sinks and peptide uptake, the rate of utilization of energy by these endogenous processes is likely to be less in the presence of peptide transport, and so a model in which the endogenous rate is fixed is unlikely to be valid.

In the absence of information on the rate of utilization of energy by endogenous sinks in the presence of peptide transport (it not being obvious how such measurements could be made), it is not possible to derive real stoichiometries for peptide uptake. However, if there were to be differences in real stoichiometry, it is pertinent to consider whether they would be detectable as differences in apparent stoichiometry in the assays performed:

The maximum apparent stoichiometry obtained was 0.36 peptide: glucose (Figs. 5-2 and 5-3). This is clearly not near to the real stoichiometry, as then all available energy would go to peptide transport, and peptides transported at different rates would give the same stoichiometry. The observed increase in apparent stoichiometry with rate of transport implies a stoichiometry of at least one peptide per glucose.

The utilization of one molecule of glucose by Strep. faecalis can yield 2 molecules of ATP, and these can power the extrusion of 4 protons. Therefore, assuming the uptake of only one proton per peptide molecule, the maximum theoretical stoichiometry of uptake is 4 peptide per glucose. An uptake of 0.4 peptide per glucose would then represent the use of 10% of the energy for peptide uptake.

So, let us consider two peptides of different true stoichiometries with the same rate of uptake, say 160 nmol min^{-1} mg dry wt^{-1} in strain OG-FS5, and allocate one of them a true stoichiometry of 4 peptide per glucose (i.e. one proton per peptide). Now, from the experimentally observed uptake (Fig. 5-3) of 0.36 peptide per glucose, uptake of this peptide would use 9% of the available energy. If the second peptide is allocated a stoichiometry of 2 peptide per glucose (i.e. 2 protons per peptide) it would need to use 18% of the available energy to give the same apparent stoichiometry.

Now, it must be considered how peptide transport is liable to compete for energy. If one assumes a simple competition, based on the demand of different processes for energy, one can obtain a value for the 'demand' of other metabolic sinks.

For the first peptide, the 'demand' is 9% of the energy, so the 'demand' of other sinks is 91%. For the second peptide, the 'demand' is 18%, and assuming the 'demand' of other sinks is unaltered, energy will be distributed in the ratio 18:91. Thus, the transport process for the second peptide would only receive 16.5% of the energy, which would yield an apparent stoichiometry of 0.33.

Therefore, two peptides that are transported at the same potential (high) rate would yield different apparent stoichiometries in the assay used if their real stoichiometries were different, even if one assumes that energy coupling is at its theoretically most efficient. Now, Ala-Ala-Ala, Ala-Ala-Ala-Ala, and Ala-Ala-Ala-Ala-Ala all give the same relationship between rate and apparent stoichiometry in strain OG-FS5 (Fig. 5-3), suggesting

that they have the same real stoichiometry for transport in this strain. For more slowly transported peptides, which use a smaller proportion of the supplied energy, a similar argument is not useful, as differences in real stoichiometry would not lead to significant differences in apparent stoichiometry. The above argument rests on there being simple competition between peptide transport and other sinks, and a given substrate competing for available energy in proportion to its rate of use of energy. An opposite situation, in which the uptake of peptides gets 'priority' use of available energy under the assay conditions used, and proceeds as quickly as substrate can be transported, cannot be discounted, and would lead to the observed relationships between apparent stoichiometry and rate regardless of real stoichiometry, unless the majority of the available energy were to be used for transport. In studies of the energy cost of galactoside transport in E.coli (Purdy and Koch, 1976) it was found that, at low rates of energy supply, transport was the priority sink for the energy, which, if the argument is extended to Strep. faecalis, would be against the model proposed above. However, the harsh and protracted starvation procedures used in E.coli (40 mM azide for 1 hr) make such comparisons of dubious worth.

In summary, the results in this section are not presented as firm evidence for identical stoichiometries for the uptake of different peptides, but rather as preliminary evidence that this may be so. Certain refinements of the techniques used (Section 5-5-2) should allow firmer statements to be made in the future.

Another factor not given rigorous consideration in these studies is the possible energetic contribution of amino acid exodus subsequent to intracellular peptide hydrolysis (Section 4-5-3-2). This could conceivably contribute to the membrane potential, be passive, or require energy. Some information on the possible role of amino acid exodus was obtained from the use of phosphonopeptides (Fig. 5-3). These peptides are cleaved more slowly than their 'parent' peptides in Strep. faecalis, but obey the same relationship between uptake rate and apparent stoichiometry, which implies that amino acid exodus is not energetically significant in these studies.

5-5 Concluding Discussion

5-5-1 Energy Coupling

In organisms such as E.coli, which produce a p.m.f. by respiration, it is possible, particularly by using uncoupled mutants, to distinguish whether transport systems are coupled to phosphate bond energy or to the p.m.f. (Section 1-4-3-1). However, in organisms which, like Strep. faecalis and Sacc. cerevisiae, rely on a membrane ATPase to maintain the p.m.f., such definitive experiments are not possible. The possible interpretation of the results obtained in the present work, and suggestions for further experiments, are considered below.

In Strep. faecalis, uncouplers and the ATP-ase inhibitor DCCD abolish peptide transport. The simplest explanation of this is that peptide transport is linked to the p.m.f. rather than phosphate bond energy; the transport of arsenate, phosphate, aspartate and glutamate (proposed as ATP-linked) has previously been shown to be

insensitive to DCCD and uncouplers (Harold and Spitz, 1975). In addition, the exodus of pre-accumulated peptide upon the addition of CCCP or DCCD is more a feature of a p.m.f-linked system than an ATP-linked one.

Based on the accepted primary actions of a range of metabolic inhibitors, therefore, the uptake of peptides by Strep. faecalis can be said to require the maintenance of a p.m.f.. Because of the time-courses of the inhibitions by uncouplers, their secondary roles such as lowering ATP pools (Section 5-1-2) can reasonably be discounted. However, a requirement for the p.m.f. alone, or the p.m.f. and "ATP" (as for potassium uptake; Bakker and Harold, 1980) cannot be distinguished on the basis of such experiments.

The role of the proton-motive-force in peptide transport could be investigated further by experiments designed to produce accumulation in response to an artificially imposed p.m.f. in starved cells (such as those performed for neutral amino acid uptake in Strep. faecalis; Asghar et al., 1973). Such experiments would provide strong evidence as to whether or not the p.m.f. is the sole driving force, or whether there is an additional requirement for 'ATP'.

Much of the argument presented above for Strep. faecalis applies equally to Sacc. cerevisiae. Thus, although the data obtained are strongly suggestive of linkage to the p.m.f. it cannot be identified unambiguously as the driving force. Experiments such as those performed for amino acid and sugar uptake in Sacc. cerevisiae (Eddy, 1980) in which proton symport in energy-starved cells is monitored with an ion-sensitive electrode,

would prove technically difficult for peptide uptake in Sacc. cerevisiae because of the relatively low rate of transport (P. Earnshaw, UMIST, personal communication).

5-5-2 Energy Consumption of Peptide Transport in Strep. faecalis - Improvements in Experimentation

The experiments performed here into the energy consumption of peptide transport in Strep. faecalis are preliminary in nature, and possible improvements which may yield more conclusive results are considered below:

a) If it were possible to use peptide substrates which are more rapidly transported than those used here, a greater proportion of the energy supply would presumably be diverted to peptide transport. Thus, from the relationship between apparent stoichiometry and rate, a better idea of the real stoichiometry could be obtained. However, although an exhaustive survey of possible substrates has not been carried out, variations in size and charge have been investigated, and it seems unlikely that substrates with an appreciably quicker transport rate will be found.

b) If it were possible to slow down the rate of endogenous energy consumption in starved cells, the proportion of energy available for peptide transport would again be greater. How such a situation could be achieved is not obvious. As, in the conditions of the assay, cells are nitrogen starved, protein synthesis should not be occuring. It seems likely that much of the energy supply is needed to replace and maintain gradients of various molecules across the membrane, and as such is an 'obligate' drain on the energy supply. Experiments with different resuspension buffers may, however, lead to a lessening of energy expenditure in this way.

(c) The most obvious way in which the precision of the experiments could be improved is by refining the apparatus. The proposed incorporation of a microprocessor into the output of the fluorimeter (J.W. Payne, personal communication) should allow the time that uptake is energised by a given pulse of glucose to be accurately determined. Analysis of the rate of uptake, extent of uptake, and time that uptake is energised for different substrates should allow more complex modelling of the process, and better estimates of the real stoichiometry of the process should be possible.

5-5-3 The Role of Amino Acid Exodus in Strep. faecalis

The possible energetic contribution to peptide uptake of amino acid exodus has not been investigated directly. However, if parallel experiments to those performed here with strain ATCC 9790 (Section 5-4-3-2) were to be performed with a peptidase-deficient strain, such a contribution could perhaps be identified.

Strain TN 97901 is deficient in the cleavage of Ala-Ala(P) (Section 4-6-3-2), and therefore seems a good candidate to assay for deficient cleavage of rapidly transported peptides such as Ala-Ala. If such substrates could be identified, comparison with the parent strain should give information on the contribution of amino acid exodus.

5-5-4 Accumulation Ratios

Large accumulation ratios for peptides (up to 850 fold) have been demonstrated in the present work with Strep. faecalis. However, these are not the maximum attainable, and a rapidly transported, non-cleavable peptide substrate would be needed

measure such a maximum ratio (Section 4-7-6). Strain TN 97901 is an obvious candidate for such studies. Further, if a strain defective in say, Glu-Ala or Lys-Ala cleavage could be obtained (possibly as resistant to Glu-Ala(P) or Lys-Ala(P), respectively), it would be of interest to compare the maximum accumulation ratios of peptides bearing neutral, negative or positive net charge.

CHAPTER 6

CONCLUDING REMARKS

6-1 Methods for the Assay of Peptide Transport

The available methods for the assay of peptide transport have been discussed in Chapter 2, and three direct assays of uptake (using fluorescamine, dansyl chloride and radiolabelled peptides) have been used in the present studies. It is pertinent to reiterate the advantages and disadvantages of these assay methods.

The assay of peptide uptake using radiolabelled substrates has the advantage of being very sensitive, and of unambiguously demonstrating uptake (as opposed to solely extracellular cleavage). In addition, the labour involved in the assay is relatively small, and so much experimentation can be performed in a short time. However, the lack of commercially available substrates dictates the time-consuming synthesis of many labelled peptides if studies of substrate specificity are to be undertaken. Major problems can also arise from intracellular metabolism of substrate. Firstly, there is the problem, general to all labelled, metabolisable substrates, that label may be lost to the gas-phase. Although it is possible to recognise this effect, it is hard to quantify it in an experimentally simple way. Secondly, for peptides, the exodus of amino acids subsequent to intracellular cleavage can lead to incorrect estimates of the rate and extent of label accumulation. This second problem can, to some extent, be overcome by very rapid sampling, which apparently yields rates unaffected by exodus.

Until recently, chemical methods for the assay of peptide uptake have been both insensitive and cumbersome. However, the two fluorescence techniques described here have overcome these problems. Both methods rely on the monitoring of disappearance of substrate

from the medium rather than accumulation by cells, an approach that has both advantages and disadvantages. The most obvious advantage is that metabolism of peptide subsequent to uptake does not lead to errors in the estimate of uptake rates. However, it is not possible to discriminate directly between intracellular and extracellular events, and so it is essential that control experiments to exclude or define any extracellular peptidase or deaminase action are performed. Additionally, the detection of low uptake rates from high external concentrations is not possible.

The dansyl chloride technique has the specific advantages of allowing the simultaneous monitoring of peptide uptake and amino acid exodus, and also the monitoring of uptake of more than one peptide at a time. The disadvantages are primarily lack of precision, and the amount of labour involved in the technique.

The fluorescamine technique, when automated, is very fast, and has the added advantage of allowing continuous monitoring of uptake. However, competition for uptake between peptides cannot be monitored in this way.

In summary, the fluorescence techniques allow the use of a wide range of commercially available unlabelled peptides and, used in combination, allow the characterisation of effects such as amino acid exodus, and the gathering of much uptake data in a short time. Use of radiolabelled substrates is not as useful, except where effects of amino acid exodus and metabolism are well defined; in these cases, the sensitivity of the assay is an advantage in detecting very low uptake rates.

6-2 A Model Strategy for the Characterization of Peptide Uptake in a Microorganism

In this section it will be attempted to outline a 'model' strategy for the investigation of peptide transport in an organism in which this has not previously been studied. Particular attention will be paid to features relevant to the design of 'smugglin' type molecules. The series suggested below should lead to a knowledge of the broad characteristics of peptide transport in the study organism.

a) Initial experiments to detect peptide uptake are clearly speculative. Alanyl peptides seem most suitable as representative substrates, as these are commonly among the most rapidly transported substrates. In contrast, glycyl peptides, often used in early studies because of their availability, generally prove to be poor substrates. It is of interest to note that alanyl residues commonly occur in the carrier portion of natural 'warhead delivery' peptide antibiotics (Ringrose, 1980). As an initial experiment, the uptake of di- to pentaalanine would be assayed using, from experience with other organisms, 0.1 mM substrate and designed to detect an uptake rate of 5-50 nmol min^{-1} mg dry wt^{-1}. If such an experiment were performed by using dansylation of medium samples, any concurrent amino acid exodus could be detected. If no uptake was detected, experiments designed to detect slower rates should then be performed.

b) Once peptide uptake has been detected, optimum medium conditions (such as pH) should be determined, and it should be established whether the system is inducible and/or regulated by

nitrogen source.

c) It should be established whether extracellular cleavage plays a role in the uptake mechanism, and to this end experiments to see whether free amino acids inhibit peptide uptake, and to investigate the influence of metabolic inhibitors on uptake should be performed.

d) Experiments to assess the permease specificity with respect to amino acid side chains and N- and C- termini of the substrate should be carried out. Experience has shown that peptide permeases are generally less strict in their requirements for amino acid side chains and C- terminal α-carboxyl group than for other parameters, and so these portions of the peptide substrate are of most potential for derivatization in the design of 'smugglin' molecules.

A group of substrates (say 25-30 to provide sufficient information but to keep the labour involved in the assays to manageable proportions) that vary systematically in features such as amino acid sequence, charge, and hydrophobicity should allow broad outlines of the specificity of the permease to be deduced. Peptides containing, for example, Glu, Lys, Gly or Phe residues are obvious candidates. The uptake of C- terminal derivatives can be monitored directly by using the fluorescamine method, but N-terminal derivatives would need to be synthesised in labelled form for their uptake to be assayed directly, although some preliminary evidence could be obtained from competition studies.

e) It is clear that observations on the substrate specificity of peptide uptake may be clouded if more than one permease is

present. It is therefore of interest to investigate this point. Again, there are practical applications to the design of antimicrobial compounds, as resistance arising from transport defects is less likely to occur if a compound exploits more than one permease. The most economical approach to deriving the number of permeases present is to study transport in mutants of the parent strain that are resistant to toxic peptide analogues. Such work can be reinforced by judiciously chosen competition studies.

f) If one is interested in applying the results of studies on peptide transport to the rational design of antimicrobial compounds, it is necessary to study more than one strain of a species, as strain to strain differences occur. In particular, experiments with clinical isolates of organisms, in addition to type cultures, are indicated.

If the work suggested in the above, brief outlines were to be carried out for a given organism, it should allow enough of the broad characteristics of peptide transport to be deduced to allow the rational design of peptide analogues that would be good substrates for uptake.

If peptide is taken up by an organism and not cleaved, the net uptake will decline and then stop (Section 4-7-6). Therefore, experiments in which uptake is followed over relatively long periods, using fluorescence techniques such as those employed in the present studies, will provide information not only about the uptake of peptide, but about its intracellular cleavage. For a discussion of the uptake kinetics likely to be observed with

peptides varying in their relative rates of uptake and cleavage, see Payne (1980a).

If one considers the action of a typical 'warhead-delivery' peptide analogue, there are three steps affecting its activity: rate of transport; rate of cleavage; toxicity of the warhead amino acid analogue. In a typical industrial development programme, after the discovery of a class of peptide antimicrobial compounds, many analogues differing in the 'carrier' portion of the molecule are synthesised, and screened for antimicrobial activity. If one possessed a more detailed knowledge of the transport and cleavage specificities of the target organism, the number of analogues that would need to be synthesised would be reduced considerably, as many could be dismissed on 'theoretical' grounds as likely poor transport and/or cleavage substrates. Such 'basic' studies as those proposed here can thus be justified not only on scientific, but on practical grounds also.

6-3 Are Microbial Peptide Permeases of Only a Few Basic Types?

Literature evidence indicates that E.coli possesses a dipeptide permease and two oligopeptide permeases (Section 1-1-5), the oligopeptide permease(s) displaying tolerance of C- terminal modifications, and the dipeptide permease having a strict requirement for a C-terminal α-carboxyl group. However, recent evidence (Alves and Payne, 1980) suggests that the dipeptide permease will also handle tripeptides. These features are closely parallelled in Strep. faecalis (faecium) strain ATCC 9790 (Section 4-7-2), which also possesses a 'dipeptide' permease, with

a strict C-terminal requirement, which handles di- and tripeptides, and an oligopeptide permease which has a less strict C- terminal requirement.

As E.coli and Strep. faecalis are diverse bacterial species, it is perhaps not unreasonable to propose the existence of two basic types of bacterial peptide permease, displaying the characteristics of the "di-" and oligopeptide systems of these two organisms. The available literature information does not provide evidence contrary to this proposal, and there are some isolated pieces of evidence (e.g. the large inhibition of Gly-Leu uptake by Gly-Gly-Gly in Strep. lactis; Rice et al., 1978) which support the proposal that tripeptides may use "dipeptide" permeases. Work in other bacteria, designed specifically to test the proposal, is indicated.

The peptide permeases of eukaryotic organisms seem to be more diverse than those of bacteria, and attempts to categorise them in the same way run into difficulties. In Sacc. cerevisiae two strains have been characterised as having a single permease which handles di- and tripeptides only (Chapter 3; Marder et al., 1978), but other strains can apparently handle larger peptides. The uptake of these larger peptides, and C-terminal methyl esters (Naider et al., 1974) could be made to fit the 'bacterial' model if there was a second, oligopeptide permease in these strains, but there is no experimental evidence for this. Results with Neurospora crassa (Wolfinbarger and Marzluf, 1974, 1975a) can be explained by the presence of an oligopeptide transport system only. However, in

Candida albicans, tetra- and pentapeptides are handled, and yet there is evidence for a strict C-terminal requirement (Lichliter et al., 1976), which is not explicable in terms of the 'two permease' proposal above. The extension of the proposal from bacteria to eukaryotic microorganisms is thus not justified on the basis of the available evidence.

6-4 The Subcellular Location of Transport Components

In these studies, peptide transport systems have been treated as sited in the cytoplasmic membrane. Although there must exist a component to mediate uptake across this membrane, there may be other components situated in, for example, the outer membrane of Gram-negative bacteria, the cell wall, and in intracellular organelles of eukaryotes. The nature of such components is considered below.

In Gram-negative bacteria, the external layer is the outer membrane, under which lies the peptidoglycan component of the cell wall. Between this and the inner (cytoplasmic) membrane is the periplasmic space. A substrate for uptake must clearly cross these areas before reaching a cytoplasmic membrane transport system. In E.coli and Salmonella typhimurium evidence has been presented that the outer membrane possesses many pores, formed of 'porin' protein, which allow the passage of various hydrophilic molecules (Di Masi et al., 1973). There are reports of specific outer membrane penetration systems (e.g. for nucleosides, maltose and maltodextrins, and vitamin B_{12}) (Kadner and Bassford, 1978); however, there is no evidence to suggest that small peptides do not cross the outer membrane simply via 'general' pores. It is not yet entirely clear

whether such pores extend only through the outer membrane or also through the underlying peptidoglycan layer. The size limit for peptide transport in E.coli (Section 1-1-6) is consistent with the demonstrated sieving properties of the pores and/or the cell wall.

As peptide transport in E.coli is ATP-linked (Section 1-4-4-2), the involvement of periplasmic binding proteins might be expected. Such proteins have not been demonstrated directly, a fact which perhaps reflects the lack of suitable techniques. The use of a photoaffinity-labelled peptide (Staros and Knowles, 1978) seems a likely way to detect such components. There is, however, indirect evidence for the involvement of binding proteins: osmotic shock severely inhibits uptake (Cowell, 1974; Payne and Gilvarg, 1978); uptake has been reported as indetectable in membrane vesicles (Cowell, 1974), although there are other reports of uptake in vesicles (Payne and Gilvarg, 1978) and spheroplasts (Ringrose and Lloyd, 1979).

The cell envelope of Gram-positive bacteria is very different from that of Gram-negative bacteria. The former lacks an outer membrane, and has a much thicker cell wall. In addition, Gram-positive bacteria do not possess a periplasmic space, although an analogous region has been described (Giesbrecht et al., 1977). Some studies have indicated that the cell wall exerts sieving properties; if correct, this may affect the transport of substrates of the size of small peptides (Scherrer and Gerhardt, 1971; Scherrer et al., 1971), although there is some doubt as to whether the exclusion limits proposed are applicable to whole cells (Scherrer et al., 1977). There is no evidence for the existence in the cell wall of

Gram-positive bacteria of specific components (e.g. pores) with a role in peptide transport, but the possibility cannot be excluded.

The cell wall of yeast has been reported as displaying an exclusion limit (Scherrer et al., 1974), similarly to Gram-positive bacteria. This has been considered in relation to peptide transport (Section 1-2-2-4). Again there is no firm evidence for cell-wall components with a specific role in peptide transport, although the recent report (Naider et al., 1980) that a tripeptide bound to poly-(ethylene glycol) competitively inhibited trimethionine uptake in Sacc. cerevisiae, would seem to imply either the presence of some specific transport component near the periphery of the cell or a much larger exclusion volume for the cell wall than that previously reported.

Experiments to evaluate whether there are 'cell-envelope' components of peptide transport systems are not straightforward. If it is attempted to assess the role of the cell wall by using spheroplasts, the results are open to misinterpretation because certain loosely-bound components of the cytoplasmic membrane transport system may also be lost. Likewise, the loss of peptide transport activity in osmotically shocked-cells of E.coli does not unambiguously demonstrate the involvement of specific binding proteins.

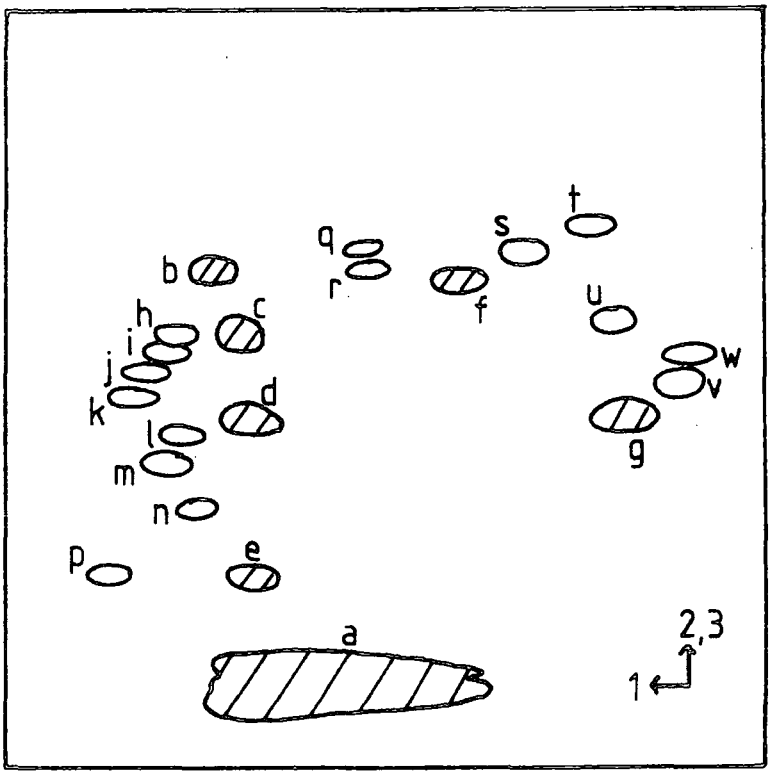
In eukaryotic organisms such as yeast, the presence of intracellular compartmentation is a further complicating factor in the study of transport. As stated at various points in this work, the active step in peptide transport in Sacc. cerevisiae cannot be

conclusively identified as being at the plasmamembrane. There are peptidases capable of cleaving small peptides in both the cytoplasm and the vacuole (Section 1-2-3-3), and so the major site of cleavage may be in either. If the active step was accumulation into the vacuole, and uptake into the cytoplasm was merely passive, then it would be anticipated that the small amounts of uptake in cells in which active transport was abolished by metabolic inhibitors would not have been detected in the assays used in these studies. One way to discover whether peptide is accumulated by the cell as a whole or merely by the vacuole would be to incubate whole cells with glycyl-sarcosine, sequentially prepare spheroplasts and vacuoles (Wiemken and Durr, 1974), and analyse the glycyl-sarcosine content of each.

It has not been the aim of the present studies to investigate the structural components and sub-cellular location of peptide transport systems, but rather to characterise their physiological features. However, it should be appreciated that values such as apparent affinity for substrate may be influenced by factors (e.g. outer membrane receptors) other than the cytoplasmic membrane permease, and so such values should be treated with caution. Studies on peptide transport systems have not reached the level at which structural components have been recognised, although this has been done for some amino acid and sugar transport systems. Work such as that presented in this thesis, apart from its relevance to understanding the physiology of microorganisms, and to the design of antimicrobial agents, is necessary as a basis if the molecular functioning of the peptide transport process is to be investigated.

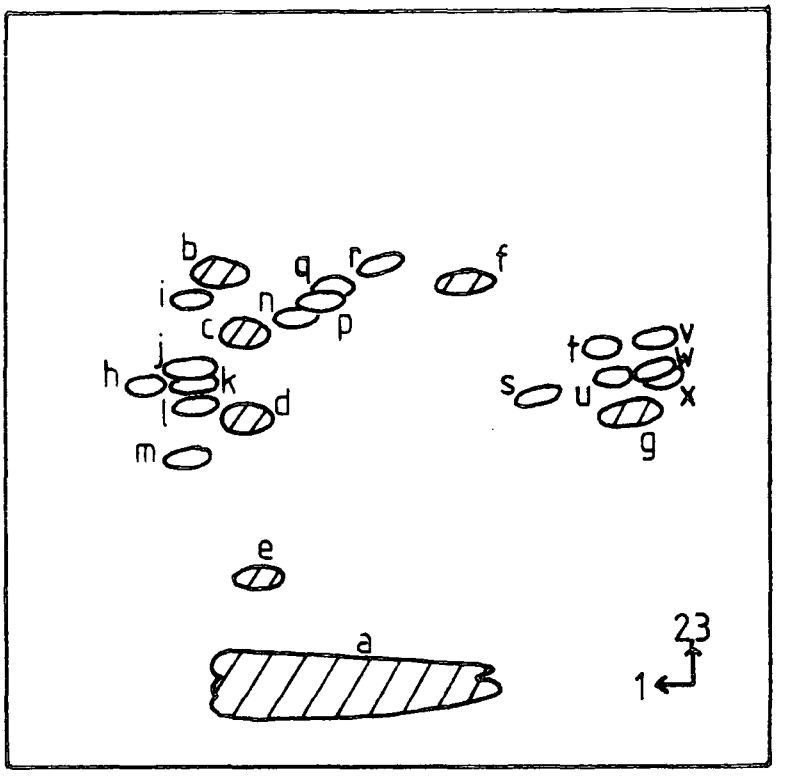
Appendix 1 Diagrams Illustrating the Positions of Some Chromatographed Dansyl Peptide Derivatives.

A) Homo- Di- to Pentapeptides



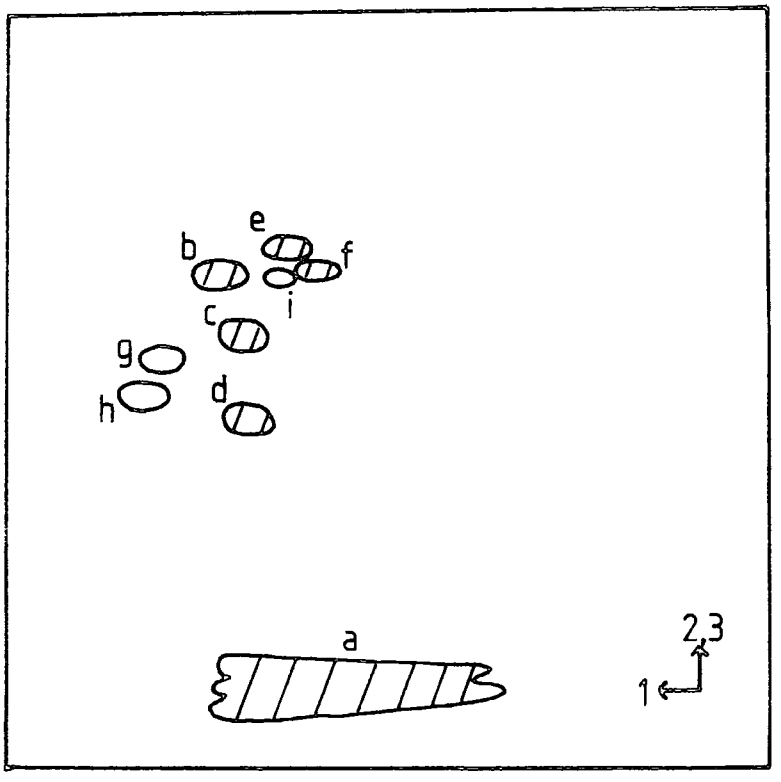
a) dansyl hydroxide, b) dansyl ammonia, c) Ala, d) Gly, e) Asp, f) Leu, g) Lys, h) Ala-Ala, i) Ala-Ala-Ala, j) Ala-Ala-Ala-Ala, k) Ala-Ala-Ala-Ala-Ala, l) Gly-Gly, m) Gly-Gly-Gly, n) Glu-Glu, p) Arg-Arg, q) Val-Val, r) Val-Val-Val, s) Leu-Leu, t) Leu-Leu-Leu, u) His-His, v) Lys-Lys, w) Lys-Lys-Lys

B) Ala-X and X-Ala Peptides



a) dansyl hydroxide, b) dansyl ammonia, c) Ala, d) Gly, e) Asp, f) Leu, g) Lys, h) Ala-Ser, i) Pro-Ala, j) Gly-Ala, k) Ala-Gly, l) Glu-Ala, m) Ala-Glu, n) Ala-Met, p) Met-Ala, q) Ala-Pro, Leu-Ala, r) Phe-Ala, s) Ala-Phe, t) Ala-His, u) Lys-Ala, v) Ala-Tyr, w) Ala-Trp, x) Ala-Lys

C) Sarcosine Peptides



a) dansyl hydroxide, b) dansyl ammonia, c) Ala, d) Gly,

e) Pro, f) Val, g) Gly-Sar, h) Gly-Sar-Sar, i) Sar

Appendix 2 Intracellular Volume of Sacc. cerevisiae

The value for the intracellular volume of yeast used in the present studies is derived from literature information: Arnold (1973) found a packed cell volume of 0.189 cm^3 ml^{-1} for a suspension of 1.94×10^9 cells ml^{-1}. Arnold and Lacy (1977) found a volume for the protoplasm of 0.66 ml (ml of pellet)$^{-1}$. Combination of these two values gives an intracellular volume of:

$$\frac{0.66 \times 0.189}{1.94 \times 10^9} \quad \text{ml cell}^{-1}$$

Now, taking a cell count of 2.3×10^7 cells mg dry wt^{-1} (Section 3-3) we get an intracellular volume of:

$$\frac{0.66 \times 0.189 \times 2.3 \times 10^7 \times 10^3}{1.94 \times 10^9} \quad \mu l.\,mg.\,dry\ wt^{-1}$$

$$= 1.5\ \mu l\ mg\ dry\ wt^{-1}$$

Although this figure is an approximation, it is likely to be of the right order of magnitude, and so is unlikely to invalidate the conclusions drawn with regard to peptide accumulation.

Appendix 3 Application of Inui-Christensen Plots

Inui and Christensen (1966) proposed a transformation of Michaelis-Menten kinetics that allowed the determination of the portion of the uptake of a given substrate that was subject to inhibition by a second substance: A plot of $\frac{1}{1 - \frac{V_i}{V_o}}$ against $1/[I]$ (where V_i is the velocity of substrate uptake in the presence of inhibitor, V_o is the uninhibited rate, and [I] is the inhibitor concentration) yields a straight line with intercept 1.0 if all uptake is subject to competitive inhibition. In the present work, it was of interest to determine if, in Sacc. cerevisiae, the uptake of sarcosine peptides was completely subject to competitive inhibition by natural peptides, or whether a second, minor mode of uptake was present (Chapter 3). However, variability of the data obtained meant that an intercept in a plot of $\frac{1}{1 - \frac{Vi}{V_o}}$ against $\frac{1}{[I]}$ could not be estimated within sufficiently close limits to reach a firm conclusion.

In an attempt to evaluate the type of data needed for a clear conclusion to be drawn on this point, a model system, where there are two modes of substrate uptake, only one of which is subject to inhibition by a second substance, was derived as below, and values for kinetic constants were substituted.

Let there be two systems,

$$V_o = \frac{V_{max}[S]}{K_m + [S]} + \frac{V'_{max}[S]}{K'_m + [S]}$$

$$V_i = \frac{V_{max}[S]}{\frac{K_m}{K_i}[I] + K_m + [S]} + \frac{V'_{max}[S]}{K_m' + [S]}$$

If model values are substituted in these equations, plots of $\frac{1}{1 - \frac{V_i}{V_o}}$ against [I] can be made.

Case 1

Let $K_m = 2 \times 10^{-4}$ M

$K_i = 2 \times 10^{-5}$ M

$[S] = 5 \times 10^{-3}$ M

$V_{max} = V'_{max}$

(these values are feasable ones for the inhibition of Gly-Sar uptake by natural peptides (Section 3-5-5).

If K'_m and [I] are varied, the plots in Fig A3-1 are obtained

Case 2

Let $K_m = 2 \times 10^{-4}$ M

$K'_m = 10^{-3}$ M

$K_i = 2 \times 10^{-5}$ M

$[S] = 5 \times 10^{-3}$ M

$V_{max} =$ 5 units

Table A3-1 gives values of the intercept of $\frac{1}{1 - \frac{V_i}{V_o}}$ vs $1/[I]$

for different values of V'_{max}

Discussion

The two cases considered above show that, if the two systems postulated have kinetic characteristics of the same order, then their presence should be easily demonstrable by an Inui-Christensen plot, even with data of large variability. However, if the second, non-inhibitable system has an affinity about two orders of magnitude less than the first, or a V_{max} one order of magnitude less, such a plot is useful only for very reproducible data.

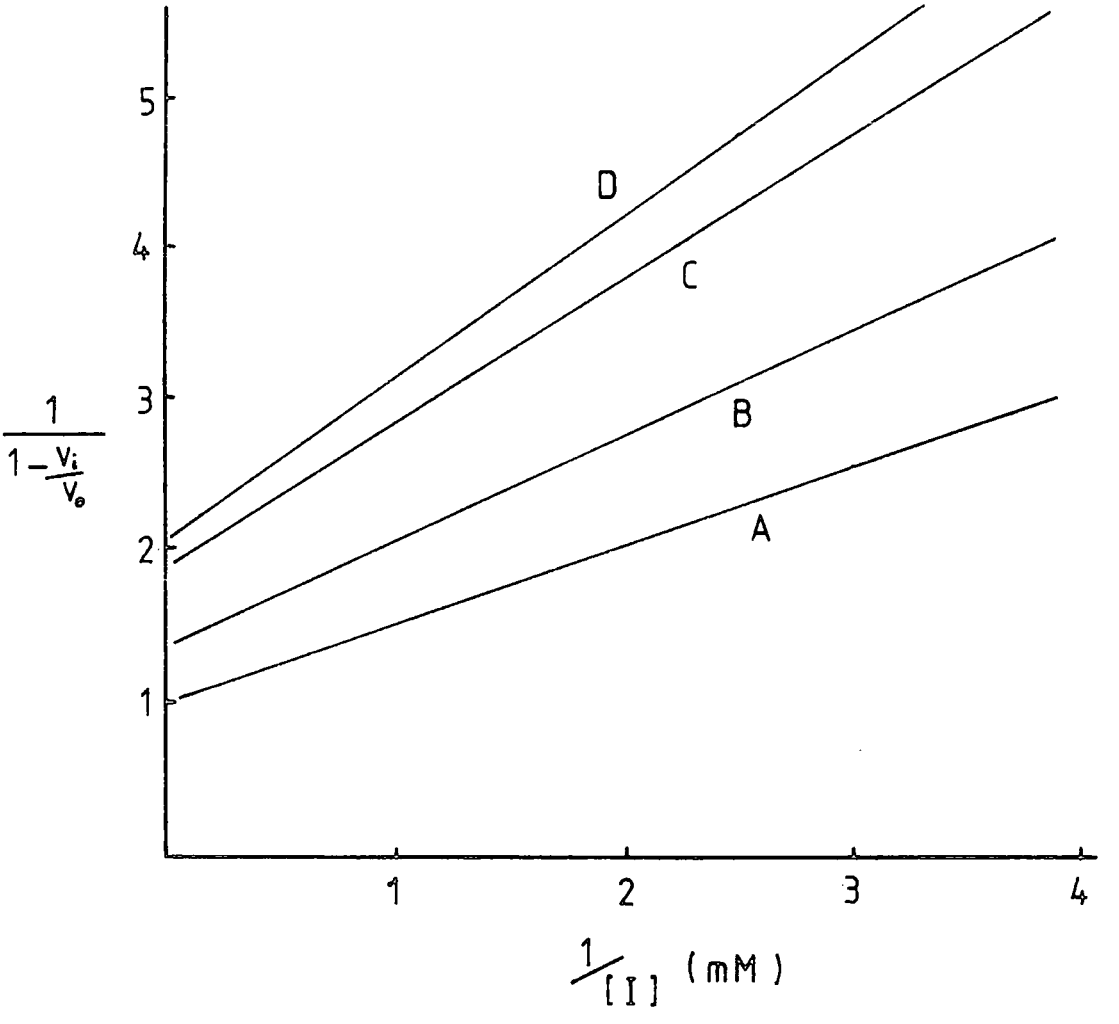


Fig A3-1 Inui-Christensen Plots for Model Data

Inui-Christensen plots for two uptake systems (see text). $V_{max} = V'_{max}$, $K_m = 2 \times 10^{-4}$ M, $K_i = 2 \times 10^{-5}$ M, $[S] = 5 \times 10^{-3}$ M
$K'_m = 1$ M (A), 10^{-2} M (B), 10^{-3} M (C), 10^{-6} M (D)

Table A3-1 Model Data for Two Permeases

V'_{max}	$\frac{V'_{max}}{V_{max}}$	$\frac{1}{1-\frac{V_i}{V_o}}$ at $[I] = \infty$
10	2	2.72
5	1	1.86
2.5	0.5	1.43
0.5	0.1	1.085

The influence of V'_{max} on $\frac{1}{1-\frac{V_i}{V_o}}$ at $[I] = \infty$ for two uptake systems (see text). $K_m = 2 \times 10^{-4}$ M, $K'_m = 10^{-3}$ M, $K_i = 2 \times 10^{-5}$ M, $[S] = 5 \times 10^{-3}$ M, $V_{max} = 5$ units

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