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UTILIZATION OF LEUCINE AND LYSINE PEPTIDES BY AN AUXOTROPH OF *SACCHAROMYCES CEREVISIAE*

by

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Abstract

UTILIZATION OF LEUCINE AND LYSINE PEPTIDES BY AN AUXOTRPOH OF SACCHAROMYCES CEREVISIAE

by

Robert Marder

Advisor: Dr. Fred Naider

Saccharomyces cerevisiae Z1-2D possesses a functional peptide transport system. This lysine/leucine auxotroph utilized a variety of leucine peptides and several lysine peptides as sources of these amino acids required for growth. Evidence suggests that these peptides were not cleaved extracellularly but enter the cell intact. Other peptides containing leucine and lysine were not growth-supporting but were hydrolyzed by the cell extract. These results indicate that the growth assay indirectly measures the ability of a peptide to be transported to the site of peptidase activity inside the cell.

Competition experiments determined that peptides were transported into Z1-2D by a system independent from the one used to transport amino acids. Both dipeptides and tripeptides compete for the same transport system. Hydrophobic peptides have a higher affinity for transport than acidic or basic peptides. The sequence of amino acids in a peptide may determine its affinity for transport. Several imino acid dipeptides were growth substrates. No acylated peptides were growth-supporting, indicating a requirement for a protonatable amine

terminal residue. A free carboxyl terminus is not required for transport. Peptides with D-residues have a reduced affinity for uptake. No peptides with more than three amino acid residues containing leucine or lysine were growth substrates. However, if a size limit exists at the tetrapeptide level, it is unrelated to the molecular weight of the peptide.

A peptide transport-deficient strain, Z1-2D Eta^r, was isolated from Z1-2D on the basis of its resistance to the toxic peptide ethionylalanine. This mutant simultaneously lost the ability to utilize dipeptides and tripeptides that were growth substrates for the parent strain as a result of the one step selection process. Peptidase activity in the mutant strain is present against these dipeptides and tripeptides. Transport assays with Z1-2D and Z1-2D Eta^r confirm that the mutant is deficient in a component of the peptide transport system.

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I. INTRODUCTION

A. Transport and Membrane Structure

The chemistry and biology of small peptides has been the subject of intense study over the last decade. Many small peptides such as TRF, somatostatin, and growth hormone have distinct biological activities. Other oligopeptides have been demonstrated to have a nutritional role in delivering to the cell amino acids for protein synthesis. Several drugs and toxic agents show an enhanced activity when given to the test organism conjugated to a peptide.

Recent technological advances have stimulated investigations into these areas. The identification and sequencing of peptides with a particular biological function has given rise to studies of structure-activity relationships. In addition, advances in synthetic techniques have made a variety of peptides available for biological study. To explain the activity of the oligopeptides, as peptide-hormones, peptide-drugs or as peptide-nutrients it is necessary to consider other factors beyond that of the peptide sequence. In particular, it is essential to understand the interaction of these substrates with cells.

It is necessary for normal biological function that the cell be able to regulate the manner in which nutrients and other metabolites are transported. The general category of mechanisms describing the way these solutes pass from one phase (the cell exterior) to the second phase (the cytoplasm) is known as transport[17], and the cell membrane is the barrier separating the two distinct environments. The composition and structure of the cell membrane has been studied

extensively. For example, in the yeast Saccharomyces cerevisiae[14] the plasma membrane is composed of 46 - 47.5% protein, and 37.8 - 45.6% lipids. Davson and Danielli[24] showed that the phospholipids were oriented in a double layer where the polar end groups were located on the inner and outer surfaces with the hydrocarbon portion extended in between. It is generally accepted[27] that the lipid bilayer is common to most biological membranes. The arrangement of proteins in this bilayer is less clear. Danielli[23] indicated that the proteins could line the inner and outer surfaces of the membrane, or extend through the membrane creating hydrophilic channels. Singer and Nicholson[70] formulated another model emphasizing that the lateral movement of membrane proteins can occur. The fact that several membrane proteins have been isolated with a globular structure[44] indicates that the proteins in the bilayer may be less unifirmly aligned than originally postulated. In addition, membrane proteins have been classified into two categories, those that are shock sensitive (periplasmic proteins) and those that are shock resistant (membrane-bound, remain firmly associated with the cell after shocking). Both types of membrane protein can display lateral mobility and asymmetric distributions in the plane of the membrane.[79] Therefore Singer's fluid mosaic model reflects the distribution, shape and mobility of most membrane proteins.

B. Amino Acid Transport

The connection between membrane structure and biological transport has been made through a series of distinct observations. Many studies measuring the transport of amino acids illustrate this relationship. First, the transport of amino acids appears to be specific. In E. coli for example, the branched amino acids valine, leucine and isoleucine

share one uptake mechanism.[18] Other studies[60] demonstrated specific transport systems for glycine, arginine, proline, lysine and other amino acids in this organism. Nearly 20 amino acid transport systems have been classified.[62] The specificity excluded D-isomeric analogues, as well as those amino acids derivatized at the amine or carboxyl terminus. In the yeast S. cerevisiae[21,35] amino acids are transported by a general system and a number of specific systems. Clearly the presence of transport systems for amino acids of a defined structure indicates the involvement of specific uptake mechanisms rather than a passive diffusion process. Amino acids were the first class of nutrients whose transport was demonstrated to exhibit a concentrative effect with regard to the final amino acid distribution between the outside and inside of the cell.[78]

The genetic control of amino acid transport in yeast[34] and Neurospora crassa[65] has been established by isolating mutants deficient in one or more amino acid transport systems. For example, in N. crassa[65] a mutant was isolated that was deficient in the transport of neutral and basic amino acids. However this mutant retained the ability to transport phenylalanine and arginine, and subsequent analysis confirmed a single genetic locus control for the general amino acid uptake system.

In addition, the demonstration of an energy requirement for amino acid transport was made in E. coli.[18] Metabolic inhibitors such as 2,4-dinitrophenol and sodium azide inhibited amino acid uptake, and a carbon source (e.g. glucose) was necessary for uptake in this microorganism.

Finally, the isolation of membrane components implicated in the transport of specific amino acids has been demonstrated. Ames[4]

isolated a peripheral membrane protein responsible for the binding of L-histidine in S. typhimurium, under the control of a specific gene (his J). The relationship between binding and transport is direct; osmotically shocked cells, or mutant cells lacking the his J gene, lost the binding protein with a simultaneous loss of uptake of the amino acids. Ames[6] later isolated a membrane bound protein of the histidine transport system and postulated (as Singer[71] did) that this component functions to create a non-specific pore for the transport of the amino acid across the cell membrane. The binding (peripheral) protein would dictate the specificity of substrates that have access to this pore.

These studies on the uptake of amino acids in a variety of organisms have demonstrated the following:

(1) substrate specificity

(2) uptake against a concentration gradient

(3) energy requirements

(4) transport under specific genetic regulation

(5) isolation of specific membrane components responsible for transport

These criteria have been instrumental in constructing models to explain transport. By definition, uptake characterized by the first 3 criteria is called "active transport," that is specific uptake against a thermodynamic gradient where metabolic energy is required. The proposed model of the cell membrane would involve recognition and/or binding of the substrate to a specific site on the outer surface of the membrane, followed by translocation of the substrate across the cell membrane. Clearly, in the absence of the isolation and characterization of specific membrane components, conclusions regarding the final picture

of the transport process are tentative. For example, does a binding protein also translocate the substrate to the interior of the cell, or is there another specific protein carrier (permease) responsible for this function? Transport models for peptides will be discussed later in this introduction.

C. Peptide Transport

The classic description of the digestive process showed all protein completely degraded to its constituent amino acids, prior to absorption in the intestine.[13] However this hypothesis was proven to be incorrect. Newey and Smyth[55,56] demonstrated that when peptides are incubated with epithelial cells in the intestine, a greater amount of hydrolytic activity occurred than when these peptides are subject to hydrolysis by the fluid containing extracellular peptidases. Using the peptide glycylglycine they argued that the peptide had to gain entry to the cell to be subject to the higher level of cytoplasmic hydrolysis. Therefore the rate of transport was the rate-limiting step in the hydrolysis of the peptide. Their work with glycylglycine led other investigators to determine[51] in the human intestine that absorption of glycine from diglycine and triglycine was faster than glycine. This absorption of peptides could complement the amino acid absorption process to provide a more efficient usage of partially digested protein fragments in the gut.

The earliest evidence of peptide utilization in microorganisms is found in studies with Lactobacilli. Wooley and coworkers[74,75,84-86], in measuring the growth stimulatory effect of peptones and other protein hydrolysates on these organisms believed the effect to be due to a specific factor called strepogenin. Proteins that were hydrolyzed

by trypsin, resulting in a variety of peptides of molecular weight from 300-500, as well as a variety of synthetic and natural peptides all showed this stimulation of growth. Other investigations[41] showed the enhancement of growth was not a property of any peptide, but reflected the ability of a specific peptide, or group of peptides, to efficiently supply the organism (in this case L. casei) with a growth essential amino acid. The authors theorized that these peptides represented a faster way to transport the required amino acids into the Lactobacilli than from mixtures of the free amino acids.

Most studies with microorganisms made use of amino acid auxotrophs to assay for peptide transport. Without complicated techniques, expensive or unavailable radioactively labeled peptides, one could measure transport indirectly by measuring the growth response of the organism to a peptide containing the required amino acid.

An important criteria for concluding a positive growth response as evidence of transport is that there be no extracellular peptidase activity capable of cleaving the peptide substrate external to the cell membrane. Conclusions based on a negative growth are less clear: 1) the peptide may not be transported, 2) the peptide may be transported but the cell may lack the cytoplasmic peptidase to liberate the required amino acid, 3) the peptide may be transported across the outer (periplasmic) membrane but not be hydrolyzed because the peptidase is sequestered inside a subcellular organelle, impervious to the peptide. Competition experiments of a growth essential peptide with other peptides are a way to measure the affinity of these substrates for the same transport system. In these indirect growth assays the competition results in an inhibition of the growth rate, or an increase in the lag

time for the organism under investigation. Again, these experiments require careful analysis to obtain accurate conclusions from the information obtained. The competition must be at the site of transport, and not for the intracellular peptidases. The competing peptide must not inhibit the metabolism of the required amino acid once inside the cell.

The use of radioactively labeled peptides enabled researchers to measure transport directly. Uptake of the label did not depend on cellular peptidases, and competition studies were powerful tools to conclude whether or not the 'hot' and 'cold' peptide shared a common uptake mechanism. Studies with radioactive peptides have also been valuable to measure the kinetic parameters of peptide transport. In S. cerevisiae,[12] Met-Met-[1-^{14}C]Met was determined to exhibit an apparent Km = 7.7 x 10^{-5}M. In N. crassa[82] Gly-D,L-Leu-L-[^{3}H]Tyr was accumulated by a system with a Km = 3.4 x 10^{-5}M. While these studies led to many important observations, the demonstration of an active transport process for peptide uptake was not definitive since the labeled peptides that are transported are almost completely hydrolyzed to the component amino acids. Therefore these peptide growth substrates were not accumulated against a concentration gradient. Only in work with cells lacking a particular peptidase,[40,87] or with peptides containing unnatural amino acid residues,[36] has the accumulation of intact peptide been demonstrated.

Since there is much evidence linking the transport of peptides to an energy coupled process[9,61] (as described earlier for amino acids), this additional criteria that intact peptides are accumulated is prima facie evidence of an active transport process. There are two significant

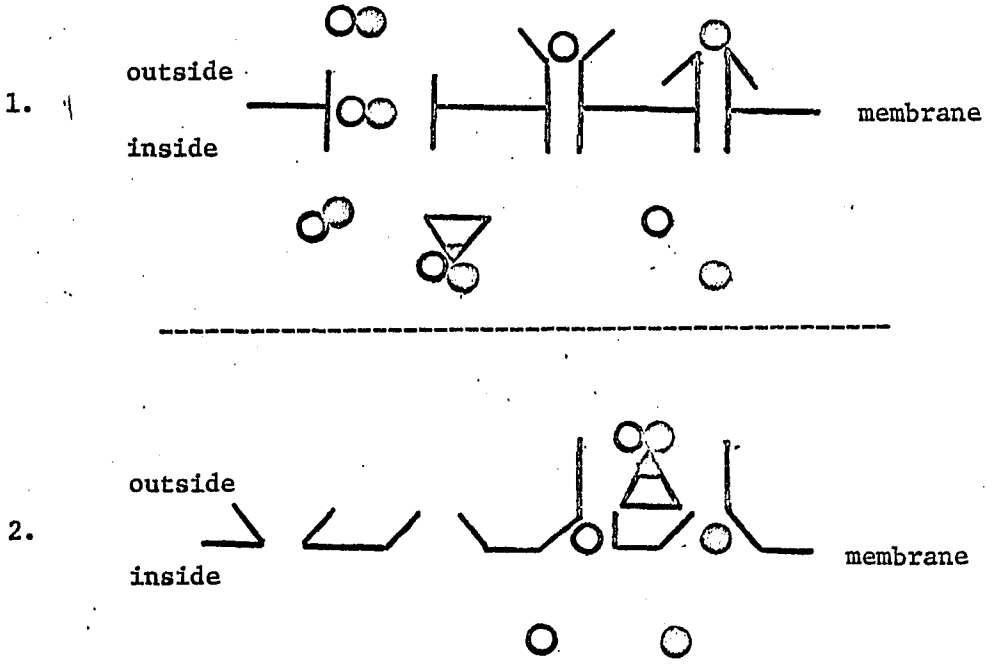
cases: (1) In a glycine auxotroph of E. coli lacking peptidase activity to hydrolyze diglycine, Kessel and Lubin[40] demonstrated intact accumulation of the peptide. (2) In another peptidase deficient mutant of S. typhimurium, glycyl-L-proline was transported to a concentration gradient of more than 1000:1, and revealed a Km = 5.3×10^{-7}.

Cowell has demonstrated[20] that osmotic shock inhibits the uptake of [^{14}C]glycylglycine, implying the requirement of a binding protein for transport. Other preliminary studies[9] show a reduction of [^{14}C]glycylglycylglycine uptake in shocked cells and no uptake in membrane vesicles of E. coli W M-123. However in none of the above cases was a binding protein isolated. While it is tempting to apply Mitchell's[52] chemiosmotic hypothesis to couple peptide uptake and trans-membrane proton movements,[12] the paucity of evidence at present makes conclusions tentative at best. In addition, it has recently been suggested[64] that perhaps the uptake of a peptide is coupled to a simultaneous exodus of specific amino acids from the cell, but these observations are also of a preliminary nature.

The general characteristics of peptide transport described above are representative criteria elucidated from studying this process in a variety of organisms. The specific requirements for transport depends on the particular organism, as will be described in another section of this introduction. However, one can use the general criteria to construct simple membrane models for the transport of peptides in many organisms without accounting for the specificity of any one particular organism. Three possible models are illustrated in Figure 1. The first model is the uptake of intact peptide by a specific transport site, followed by cytoplasmic hydrolysis. This model characterizes those

Figure 1

Models of Peptide Transport *



*adapted from reference 62.

systems where identification of intact peptide inside the cell has been made. The second model postulates a surface recognition of the peptide and membrane hydrolysis followed by uptake of the cleaved amino acids at sites accessible only to those amino acids released from the peptide. A third mechanism would have the membrane function simultaneously to transport and hydrolyze peptides. The absence of additional information about the membrane structure and location of peptidases would not distinguish between models 2 and 3. Of course evidence for the accumulation of an intact peptide would be conclusive evidence for model 1. This model, representing active, carrier-mediated uptake, bears a significant resemblance to the models proposed for the uptake of amino acids. However the distinction of the two uptake systems is clear from studies in several organisms. For example, in E. coli,[18] peptides containing valine did not inhibit the entry of free valine into the organism, but free amino acids were strongly competitive. Other studies showed that organisms lacking the ability to transport a particular amino acid could absorb the amino acid in peptide form. For example in S. typhimurium,[3] the toxic amino acid analogue, L-ethionine, can't enter the cell as the free amino acid, but when L-ethionyl-L-alanine is supplied in the growth medium, the peptide can enter the cell and release L-ethionine in the cytoplasm.

The distinction of amino acid and peptide transport systems is just one example of the substrate specificity demonstrated by peptide transport systems in the many organisms studied. To understand completely the role of peptide transport in each organism it is necessary to have a complete picture of the specific criteria that permit the uptake of certain, but not all peptides. The differences that exist in peptide

transport between different organisms can reflect changes of an evolutionary nature. For example, how did cells of higher organisms of a greater complexity derive transport systems consistent with the more specialized intercellular biochemical processes that characterize such organisms? Or, are differences in the characteristics of peptide transport systems related to the organism's adaptation to its particular environment? In either case, characterizing the peptide transport system of different organisms can be very useful in adding to our knowledge of the specific uptake systems in each organism.

The important characteristics of peptide transport in E. coli and S. cerevisiae will be reviewed here. Except for data to be presented on S. cerevisiae later in this thesis, the other criteria reflect the most current developments, including results published after this thesis problem was commenced. Since most studies on peptide transport have been carried out on E. coli, the gram negative procaryote, it is useful to compare the characteristics from that organism with S. cerevisiae. Not only will the differences between the two microorganisms be emphasized, but the need for specific transport studies to complete the picture of peptide utilization in yeast will be apparent. In Table 1 several criteria for transport in these two organisms are illustrated. The distinction of the peptide and amino acid uptake systems in both the bacterium and yeast is clear. However that is essentially the only criterion common to both microorganisms. For example in E. coli a methionine auxotroph[53] can't use pentamethionine as growth substrate, but a S. cerevisiae methionine auxotroph G1333 does utilize this substrate. The size limit to transport in E. coli was related to the hydrodynamic volume of the peptide[31,59] by either a

Table 1

Characteristics of Peptide Transport in Escherichia coli and Saccharomyces cerevisiae

	E. coli	S. cerevisiae
1. Separate amino acid and peptide transport systems	+	+
2. Separate dipeptide and oligopeptide transport systems	+	ND
3. Peptide sequence affects transport	-	+
4. Side chain specificity affects transport		+
a. dipeptides	-/+	
b. Opp(general oligopeptide permease	-	
c. Opr. I(specific oligopeptide permease)	+	
5. a. Acylated peptides transported	-	+
b. Monoalkylated peptides transported	+	ND
6. Alpha peptide bond required for transport	+	ND
7. Peptide esters transported		
a. dipeptides	-	+
b. oligopeptides	+	+

(continued)

Table 1 (continued)

	E. coli	S. cerevisiae
8. Peptides with D residues transported		
a. dipeptides	-	+
b. oligopeptides	-/+	-/+
9. Size limit for transport	+	ND
10. Nitrogen source affects transport	ND	+
11. Dinitrophenol inhibits transport	+	+
12. Concentrative uptake demonstrated	+	ND
13. Peptide transport-deficient mutants isolated	+	ND
14. Peptidase-deficient mutants isolated	+	+

[a] A plus symbol(+) means that characteristic has been shown to exist, a minus sign(-) indicates a lack of that particular trait. A -/+ figure in the table indicates that the criteria are not consistent for all substrates examined in that particular category. ND means the criterion was not determined.

sieving effect of the cell wall, or the lipopolysaccharide membrane[19] limiting the peptides access to the protein carrier. No size limit to transport has been measured in S. cerevisiae. Another difference in the two organisms is that E. coli does not transport acylated peptides but S. cerevisiae is able to utilize several N-acetyl and N-butyloxy-carbonyl peptides as growth substrates.[53] The most significant information about peptide transport in E. coli centers around the discovery of distinct dipeptide and oligopeptide uptake systems. This work,[57] based on the isolation of a mutant of E. coli resistant to a toxic tripeptide, triornithine $(Orn)_3$, was instrumental in establishing several fundamental criteria of peptide transport in this organism: (1) The transport system that $(Orn)_3$ used to enter E. coli was a general one (called (Opp), and many other oligopeptides that shared this system were simultaneously excluded from the mutant resistant to $(Orn)_3$. (2) Dipeptides utilized a separate transport system (Dpp), as evidenced by their ability to enter the $(Orn)_3$ resistant (Opp^-) mutant. A dipeptide[25] transport deficient (Dpp^-) mutant was also isolated, and both Opp^- and Dpp^- genetic defects have been shown to map to separate loci,[7,25] demonstrating the distinction between the two systems. (3) In addition, a specific oligopeptide transport system (Opr I) has been identified,[8,54] which can transport certain peptides that also have access to the general (Opp) system. The ability to isolate these transport-deficient mutants was crucial to the success in characterizing these separate uptake systems. For example, the ability of dipeptides with carboxyl blocked residues such as lysylcadaverine,[57] and certain dipeptide amides[38] to enter E. coli is apparently contradictory with the requirement of dipeptides for a free carboxyl group. However these

substrates do not enter an Opp$^-$ mutant, as other unblocked dipeptides do, indicating that they use the oligopeptide transport system. In E. coli there is no direct evidence for multiple dipeptide transport systems. However the relative ability of certain peptides to compete with the uptake of glycylglycine in E. coli[39] is an indication that different substrates may have different affinities for the transport system. Similarly[58] lysine and ornithine oligopeptides appear to have a higher affinity for transport by the Opp system than glycine or tyrosine peptides. In all the peptide transport-deficient mutants, the hydrolytic activity towards the peptides is unaffected, as is the growth response towards amino acids. Other studies in E. coli showed that D-residues could not be accommodated at either position in the dipeptide,[39,45] but that D-residues at the amine or carboxyl terminus of an oligopeptide, would be recognized by the general (Opp) system.[10] The isolation of a general (Opp) and specific (Opr I) peptide transport system in E. coli is somewhat analagous to the existance of similar systems for the transport of amino acids in this organism. The studies with S. cerevisiae G1333[53] have not indicated specific transport systems for dipeptides and oligopeptides. While recent evidence[12] indicating that the uptake of radioactively labeled $(Met)_3$ in S. cerevisiae 139 is only slightly inhibited by $(Met)_2$, as compared to the greater inhibition exhibited by tripeptides, a thorough assay measuring competition for peptide uptake by dipeptides and tripeptides has not been done. In the above yeast strain, Ac-$(Met)_3$ was demonstrated to enter the cell, but did not compete with radioactively labeled $(Met)_3$ for entry. What is most apparent about peptide utilization in S. cerevisiae is that the sequence of amino acid residues in a peptide may affect the peptides'

affinity for transport. In strain G1333,[53] a methionine auxotroph, peptides containing a carboxyl terminal glycine residue are much poorer growth substrates than similar peptides with a carboxyl terminal methionine residue. For example, Gly-Met-Gly and Met-Met-Met-Gly were not growth substrates, but Gly-Gly-Met and Met-Met-Gly-Met were utilized as methionine sources. Three other strains of S. cerevisiae auxotrophic for lysine[11] did not utilize any lysine peptides as growth substrates. The ability of these lysine strains to transport other peptides was not examined. Data from the study showing the lack of competition for uptake with $(Met)_3$ in S. cerevisiae 139 by $(Lys)_3$ also seemed to indicate that these lysine peptides were not substrates for the peptide transport system in this organism.

D. Proposal for Thesis Research

It is obvious from table 1 and the above discussion that much more is known about peptide transport in E. coli than S. cerevisiae. An obvious limitation of the intensive studies with E. coli is that, as a procaryotic organism containing little subcellular organization, few conclusions can be made about the transport properties of the cells of higher organisms. Yeast is a eucaryote, having a membrane-bound nucleus and other intracellular organelles typical of mammalian cells. It has a sexual cycle and can readily be studied by genetic analysis in contrast to the more complex mammalian systems.[66] The uptake of amino acids has been thoroughly characterized, as noted earlier. The availability of amino acid auxotrophs makes assays of transport based on the utilization of peptides as growth substrates straightforward. The absence of extracellular peptidase activity towards free peptides in a strain of S. cerevisiae[11] as measured by osmotic shock indicates that a positive

growth response is good evidence for transport. It was therefore decided to examine the utilization of peptides in another strain of S. cerevisiae. This strain, Z1-2D, is auxotrophic for leucine and lysine. Like methionine, leucine is a hydrophobic amino acid, and comparisons between the two S. cerevisiae strains were useful in establishing whether generalizations can be made regarding the utilization of these neutral peptides. The demonstration of the transport of leucine peptides allowed the study of the uptake of lysine peptides in an organism with a functional oligopeptide transport system. The utilization of leucine and lysine peptides was measured using growth experiments with peptides containing one of the required amino acids. The use of a variety of peptides specified the general characteristics of peptide utilization with regard to those enumerated for the previously studied S. cerevisiae G1333. Competition studies between the leucine and lysine peptides, and other peptides determined the affinity of different peptides for the transport system. Criteria such as size limit, stereospecificity and the effect of the sequence of amino acids in a peptide were measured. Peptides of interest that were not commercially available were synthesized by standard solution procedures.

Using a peptide containing a toxic amino acid, a peptide transport-deficient mutant was isolated and characterized. Such a mutant, as was documented above for E. coli, was invaluable in studying whether separate dipeptide and tripeptide uptake systems exist in yeast. The mutant served to corroborate data from competition studies on the parent auxotroph. Another obvious importance of isolating a peptide transport-deficient mutant is that the genetic characteristics

of the strain could be used to help isolate the particular steps of the transport process. Finally, studies with radioactively labeled peptides in the mutant and parent strains provided direct evidence for much of the data gathered from growth experiments.

The studies discussed in the body of this thesis have resulted in two publications:

1. Marder,R.,J.M.Becker and F.Naider (1977) J. Bacteriol. 131: 906-916.

2. Marder,R.,B.Rose,J.M.Becker and F.Naider (1978) J. Bacteriol. 136: 1174-1177.

II. EXPERIMENTAL

A. Yeast Strain and Growth Conditions

Strain Z1-2D, a lysine/leucine auxotroph of S. cerevisiae was received from Nasim Khan, Brooklyn College, City University of New York. This strain was derived from a cross between strains KC-372 (R. Mortimer, University of California, Berkeley) and 1323-1B (D.C. Hawthorne, University of Washington, Seattle). The strain was maintained on YEPD slants composed of 1% yeast extract, 2% peptone, 2% dextrose and 2% agar. Fresh slants were prepared regularly, allowed to incubate 2 days at 30°C and stored at 4°C. The minimal medium used for all growth experiments was yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.). This medium (SM) was prepared in a ten-times-concentrated form (6.7 g of yeast nitrogen base plus 5 g dextrose in 100 ml of H_2O) and the solution was filter sterilized (Nalge, 0.22 μm) and refrigerated. All amino acid and peptide solutions were individually filter sterilized and stored in a similar manner.

Starter cultures for growth studies were prepared in the following manner. A Klett tube with 4.5 ml distilled H_2O was autoclaved. Leucine and lysine were added aseptically to final concentrations of 60 μg/ml and 20 μg/ml respectively. A 0.5 μl portion of the sterile concentrated yeast nitrogen base was added to make the final volume 5 ml. The starter culture was inoculated from a refrigerated slant, and then allowed to grow with agitation for approximately 24 hours to 160-190 Klett units. Either an American Optical Instrument Company thermostated water shaker, or a New Brunswick Scientific G24 (Air) Environmental Incubator Shaker was used for all growth experiments. Growth was

determined by the increase in turbidity at 400 to 420 nm (blue filter) with a Klett-Summerson photoelectric colorimeter.

The cells from the starter culture were centrifuged, washed twice with sterile distilled water, and resuspended in one-half of the original volume of sterile water. A 0.1 ml portion of this cell suspension (1-2 x 10^7 cell/ml) was inoculated into 25 ml of single-strength minimal medium with the desired amino acids or peptides. This complete medium containing an initial cell concentration of 5-6 x 10^5 cells/ml, was contained in either a 125 ml or 250 ml Erlenmeyer flask equipped with a side arm (Klett flask) and shaken at 100-200 rpm at 30°C. Klett readings were taken periodically to construct a growth curve for each flask. From such plots the lag time and generation times were determined. All Klett values were normalized to an initial reading of 2 to correct for slight differences in the transmittance of the glass of the side arm of the Klett flasks used in the growth experiments. All growth experiments were terminated at 40 hours, except when a growth flask was in the early or mid-log phase of growth at that time. In those cases, Klett readings were taken until the cultures were in stationary phase. Every growth experiment assayed the starter culture's requirement for leucine and lysine by inoculating control growth flasks containing minimal medium (SM) and lysine (no leucine), and minimal medium (SM) and lysine (no leucine). No growth in either control flask verified the requirements of Z1-2D for lysine and leucine. A growth flask containing the complete media (+ amino acids) but no inoculum, always served as a check on the sterility of the media and growth conditions.

Growing cells were checked for revertants by plating out portions from growth flasks onto several synthetic minimal (SM) plates: SM plates contained 0.66% Difco yeast nitrogen base without amino acids, 2% dextrose and 2% agar; SM + Leu plates contained, in addition, 30 µg of leucine per ml; SM + Lys plates contained, in addition, 30 µg (or 15 µg) of lysine per ml; SM + Lys + Leu plates contained, in addition, 30 µg amounts of leucine and 30 (or 15) µg amounts of lysine per ml. YEPD plates were prepared for viable counting and contamination checks. Each series of plates described above was prepared by dissolving the appropriate media components in 600 ml of distilled water, autoclaving at 121°C for 15 minutes at 15 psi, and then adding the sterilized amino acids as specified to the freshly autoclaved solutions. After cooling for 1 hour the plates were poured, allowed to cool and stored at room temperature. The absence of growth on SM, SM + Leu, and SM + Lys plates, and a positive growth response on an SM + Lys + Leu plate after incubation at 30°C for a minimum of 48 hours indicated maintenance of the organism's requirement for both leucine and lysine. Plates were also prepared in a similar manner containing peptides and/or toxic amino acid analogues. In each case the peptide of toxic analogue was supplied in the media at the equivalent concentration of leucine (0.23mM). The plate designations and compositions are: SM + $(Leu)_2$, lysine + dileucine; SM + $(Leu)_3$, lysine + trileucine; SM + Eth, lysine + leucine + ethionine; SM + Eth-Ala, lysine + leucine + ethionylalanine.

B. Chemicals

All amino acids and amino acid residues are of the L-configuration, unless otherwise specified. The abbreviations used in this

thesis for amino acid residues in peptides are: Leu = leucine, Pro = proline, Met = methionine, Eth = ethionine, Gly = glycine, Tyr = tyrosine, Phe = phenylalanine, D,L-p-F-Phe = DL-p-fluorophenylalanine, Ala = alanine, Lys = lysine, and Val = valine. For amine and carboxyl terminal groups, Boc = t-butyloxycarbonyl, Ac = acetyl, OMe = methyl ester, OBzl = benzyl ester, and OSu = N-hydroxysuccinimide.

The peptides Pro-Pro-Leu, Met-Met-Leu and Ac-$(Met)_3$ were prepared by previously described procedures.[53] The peptides HCl$(Leu)_2$-ε-amino-caproic acid, HCl$(Leu)_2$OMe, HCl$(Leu)_2$OMe, Pro-Pro-D,L-F-Phe, and Met-Met-Eth were a gift of Dr. F. Naider. The peptide Met-Met-[1-^{14}C] Met was prepared by F. Naider.[12] The peptides HCl$(Leu)_2$-OBzl, Ac-$(Leu)_3$ and Leu-Leu-Eth were prepared as described below.

[1-^{14}C]Gly-Leu(20 μCi/μmole), [1-^{14}C]methionine(50 μCi/μmole), [U-^{14}C]leucine(351 μCi/μmole) and Aqueous Counting Scintillant were purchased from Amersham/Searle, Arlington Leights, Ill.

The peptides Lys-Leu acetate, Gly-Leu-Gly-Leu, Gly-Leu-Gly-Gly, Gly-Leu-Leu-Gly, $(Leu)_2$, $(Leu)_3$, $(Gly)_2$, $(Gly)_3$, D-Leu-L-Leu, L-Leu-D-Leu, $(L\text{-}Ala)_2$, $(L\text{-}Ala)_3$, $(D\text{-}Ala)_3$, L-Ala-L-Ala-D-Ala, Leu-Leu-Gly, Gly-Gly-Leu, Gly-Leu-Gly, Pro-Leu, Leu-Pro trifluoroacetate, Ac-Gly-Leu, $(Gly)_3$, $(Glu)_2$ and Lys-Gly-Gly acetate were purchased from Bachem, Marina Del Rey, California. The peptides $(Lys)_4 \cdot 4$ HCl and $(Lys)_5 \cdot 6$ HCl were a gift from Arieh Yaron, Weizmann Institute, Israel.

The peptides Val-Leu, Leu-Gly, Leu-Val, $(Lys)_2 \cdot 2$ HCl, $(Lys)_3 \cdot 3$ HCl, $(Lys)_4 \cdot 4$ HCl, Lys-Gly·2 HBr, Gly-Lys·HCl were purchased from Miles Labs., Kankakee, Illinois.

The peptide Met-Leu was purchased from Mann Research Labs., New York, New York.

The peptide Leu-Gly-Leu was purchased from Research Plus Labs., Dennville, New Jersey.

The peptides Leu-Ala, Gly-Leu, Ala-Leu, $(Leu)_3$ and the amino acid ethionine were purchased from Sigma Chemical Co., St. Louis, Missouri.

The peptide Pro-Leu-Gly-$NH_2 \cdot \frac{1}{2} H_2O$ was purchased from Chemalog, Plainfield, New Jersey.

[^{14}C]Toluene (4.24×10^5 dpm/ml) was obtained from New England Nuclear, Boston, Mass. All other amino acids and reagents were reagent grade or the purest commercially available.

Peptides were prepared for use in growth experiments by dissolving in distilled water to a concentration of approximately 2 mg/ml. For Ac-$(Met)_3$, one equivalent of sodium bicarbonate per mole of peptide was used to aid in the dissolution of the substrate. Every peptide in solution was demonstrated, as described in a later section, to be homogenous on a high voltage paper electrophoresis chromatogram when $\geq$ 100 µg of peptide and its constituent amino acids were applied to the paper.

Leu-Gly-Leu showed a very minor leucine impurity. The impurity was judged to be less than 10% leucine by a qualitative visual comparison of the impurity spot to that given by a known amount of leucine. The peptide Met-Met-[1-^{14}C]Met was observed to undergo radiolysis if left in solution for several weeks at 4°C. A strip count (Packard Radiochromatogram Scanner) of a high voltage paper electrophoresis chromatogram of 27.5 µg of an applied Met-Met-[1-^{14}C]Met solution showed the appearance of appreciable quantities of [1-^{14}C] methionine. Therefore a fresh solution of the peptide was prepared (0.44 mg/ml) and immediately stored in small aliquots (150 µl) at -30°C until the uptake

assays were to be performed. A similar electrophoresis and strip count on one of the frozen aliquots of the fresh sample (30.8 μg) revealed no trace of [1-^{14}C]methionine. For both samples, cold methionine and $(Met)_3$ were electrophoresed along with the labeled peptides and developed separately with ninhydrin. The movement of Met-Met-[1-^{14}C]Met and the labeled impurity in the old sample as revealed by the strip count was identical with that of cold $(Met)_3$ and methionine, as shown by the respective ninhydrin-positive markers for each substrate.

C. Peptide Synthesis

For the synthesized compounds described in this section, uncorrected melting points are determined on a Buchi Types apparatus, Switzerland. NMR spectra were run on a JEOL Co. Model JNM-MH-100 Hz spectrometer.

1) Preparation of Ac-$(Leu)_3$

Trileucine, (100 mg, 0.28 mmole) and sodium bicarbonate (47 mg, 0.56 mmol) were dissolved in 15 ml of distilled water. The stirred solution was cooled in ice, and 25 μl of acetic anhydride (0.26 mmole) was added. After 15 minutes the solution was acidified to pH 2 with concentrated HCl and stirred in an ice bath for 15 minutes. The precipitate that formed was filtered through a fritted glass funnel of medium porosity and washed once with distilled water. The yield was 25 mg (22%). Thin-layer chromatography (MeOH-$CHCl_3$ 2:1) revealed one iodine-positive, ninhydrin negative spot (Rf = 0.61).

2) Preparation of HCl-$(Leu)_2$-OBzl

Boc-$(Leu)_2$-OBzl was prepared from Boc-Leu-OH and Leu-OBzl by the procedure of D'Alagni, et al.[22] The yield was 85%. The product

was recrystallized from hexane: mp = 89.2 to 90°C. $[\alpha]_D^{25°}$ = -42.54 (C 1.06, $CHCl_3$), Rf = 0.66 ($CHCl_3$-MeOH, 95:5); literature: $[\alpha]_D^{25}$ = -41.65 (C 1.06, $CHCl_3$), mp = 92-92.5°C.[22] The structure was confirmed using NMR: δ 0.80-1.06(12 H,m, 4 x δ-CH_3); 1.40·(9H,s, 3 x CH_3C); 1.40-2.08(6H,c, 2 x βCH_2,γCH); 3.92-4.24(1H,m, α-CH); 4.50-4.82(1H,m, α-CH); 4.84-5.12(1H,d,NH); 5.14(2H,s,benzyl CH_2); 6.40-6.68(1H,d,NH); 7.32(5H,s,phenyl H). One gram (2.3 mmole) of Boc-$(Leu)_2$-OBzl was dissolved in approximately 25 ml of ether and stirred at room temperature. Dry HCl gas was bubbled through the reaction mixture for one hour. The product that precipitated from the reaction mixture was filtered on a fritted glass funnel of coarse porosity, washed with ether, and dried in a vacuum dessicator. The yield was 91% ($[\alpha]_D^{25}$ = -6.66 [C 1.0, $CHCl_3$]). The product was judged to be pure on silica thin layers ($CHCl_3$-MeOH 2:1; Rf = 0.75) and homogeneous on a high voltage paper electrophoresis chromatogram of a 2 mg/ml solution of the peptide.

3) Preparation of $(Leu)_2$-Eth

Boc-$(Leu)_2$-OBzl(13 g, 0.23 mmole) was dissolved in 100 ml of t-butanol/dioxane (4:1 by volume) with stirring. Catalyst(0.6 g, 10% Pd/C) was added and the solution was hydrogenated under approximately 50 lbs. pressure (Parr apparatus) for 7½ hours. The crude product was freeze dried, taken up in ethyl acetate and extracted with 5% $NaHCO_3$. The aqueous layer was acidified to pH 4 with 10% citric acid, extracted again into ethyl acetate, dried with magnesium sulfate, filtered, and the solvent removed under vacuum. The product, produced in 45% yield, yielded a single spot on silica thin layers, developed in iodine vapors. Rf = 0.69, (MeOH-$CHCl_3$, 2:1), and

$[\alpha]_D^{25}$ = -27.77 (C 1.0 in $CHCl_3$). The structure was confirmed using NMR (10% in $CDCl_3$) δ 0.80-1.16(12H,m, 4 x δ-CH_3); 1.26(9H,s, 3 x CH_3C); 1.55-2.24(6H,c, 2 x β-CH_2, γ-CH); 4.00-4.44(1H,m, α-CH); 4.48-4.80 (1H,m,α-CH); 5.24-5.56(1H,d,NH); 6.80-7.12(1H,d,NH); 8.34(1H,s,broad, OH). Boc-$(Leu)_2$-OH (4.29 g, 12.5 mmole) and 2.57 g of dicyclohexyl-carbodiimide (12.5 mm) were dissolved in a minimum of DMF. N-Hydroxy-succinimide (1.434 g,12.5 mmole) was added, and the resulting solution stirred and cooled in an ice/salt bath. After ½ hr. the solution was removed from the bath and allowed to stir at 4°C for 36 hours. The solution was filtered, solvent removed under reduced pressure and the residue was taken up in ethyl acetate. Two drops of glacial acetic acid were added, and the solution was allowed to stand at 4°C for 1½ hr. The dicyclohexyl urea was removed by filtration and the solution was extracted with water and then dried with Na_2SO_4. The solvent was removed under vacuum leaving a viscous oil. The oil was taken up in ethyl acetate/hexane and the resulting oil that appeared after removal of solvent solidified immediately under high vacuum. $[\alpha]_{25}^{D}$ = -52.44 (C 1.03 in $CHCl_3$). The structure was confirmed using NMR (10% in $CDCl_3$). δ 0.80-1.12(12H,m, 4 x δ-CH_3); 1.44(9H,s, 3 x CH_3C); 1.52-2.14 (6H,c, 2 x β-CH_2,γ-CH); 2.8(4H,s,CH_2-CH_2); 3.96-4.24(1H,m,α-CH); 4.76-5.24 (2H,c,α-CH,NH); 6.64-6.88(1H,d,NH). Ethionine (0.1848 g,1.134 mmole) and 0.1910 g of $NaHCO_3$ (2.268 mmole) were dissolved in about 8 ml water. 0.500 g of Boc-$(Leu)_2$-OSu (1.134 mmole) was dissolved in a mixture of 12.5 ml dioxane and 2.5 ml water. The solution containing Boc-$(Leu)_2$-OSu was added to the ethionine solution and the reaction mixture was stirred for 24 hours at room temperature. The solvent was removed under vacuum, and the product taken up in a minimum of water. The solution

was acidified with 10% citric acid to pH 2, and the product filtered, washed with a small portion of water, and dried overnight in a vacuum dessicator. The yield was 0.350 g (63%), and the lack of any starting material confirmed by the absence of a UV sensitive spot on a thin-layer chromatography plate (silica thin layers, solvent = ethyl acetate). The structure was confirmed using NMR (10% $CDCl_3$). δ 0.78-1.10 (12H,m, 4 x δ-CH_3); 1.14-1.42(3H,t,S-CH_2CH_3); 1.62(9H,s, 3 x CH_3C); 1.52-1.86 (8H,c, 2 x β-CH_2, 2 x γ-CH, Leu; 2 x β-CH_2, Eth); 1.88-2.32(2H,m, γ-CH_2); 2.42-2.72(2H,q,ε-CH_2); 3.94-4.32(1H,m,α-CH); 4.42-4.82(2H,m, 2 x α-CH); 5.12-5.62(1H,s,broad,NH); 7.14-7.58(1H,s,broad,NH) 7.70-8.06 (1H,s,broad,NH). Glacial acetic acid (11.5 ml) saturated with HCl was added to 300 mg of Boc-$(Leu)_2$-Eth and stirred for 45 minutes. The product was precipitated with ether, filtered, and dried overnight in a vacuum dessicator. Unpurified HCl$(Leu)_2$-Eth (100 mg) was dissolved in 7.5 ml H_2O. Insoluble material was removed by filtration with a very fine fritted glass funnel. The clear aqueous solution was neutralized to pH 6 with LiOH. Absolute ethanol was added to precipitate the peptide. The product was filtered, washed with ethanol, ether, and then dried for 3 days in a vacuum dessicator. The yield was 37.5 mg (41%). The homogeneity was confirmed by a single spot on a high voltage paper electrophoresis chromatogram of 100 μg of applied peptide, and by thin layer chromatography (silica thin layers, 2-propanol:water, 7:3), Rf = 0.73.

D. Electrophoresis

Electrophoresis was carried out in a model LT-36 electrophoresis tank with EC-123 coolant and a HV-5000 power supply (Savant Instruments, Inc., Hicksville, N.Y.). Pyridine-acetate buffer (pH 3.5) was prepared

from glacial acetic acid-pyridine-water (10:1:189 vol/vol). For some assays, a pH 1.9 buffer of formic acid-glacial acetic acid-water (6:24:270 vol/vol) was used. Peptides, amino acids, and/or samples from the peptidase assays were applied to Whatman 3 MM paper and run at a gradient of approximately 37 volt/cm for 1.5 to 3 hours. After electrophoresis, the paper was dried in a solution of ninhydrin (0.5% wt/vol) in 95% aqueous acetone, and developed in a heated and ventilated chromatography oven. The ninhydrin stains were fixed by dipping the paper in a solution of $Cu(NO_3)_2$ (1%) and HNO_3 (0.2%) in acetone-ethanol (2:1).

E. Preparation of Z1-2D Cell Extract

1. Four one liter batches of cells were grown up in minimal medium [yeast nitrogen base + leucine (60 µg/ml) + lysine (20 µg/ml)]. The cells were grown at room temperature for 28 hours with shaking (New Brunswick Model VS) to a mid/late log phase of 110-140 Klett units. An aliquot of each liter of cells was plated out as described earlier to shown maintenance of the requirements for leucine and lysine. The cells were harvested by centrifuging at 5000 rpm for 5 min. The supernatant was decanted and the resulting wet paste filtered to a hard solid mass on a millipore 0.45 µ filter, yielding 6.28 g of cells. The cells were resuspended, washed twice with cold distilled water, and centrifuged. A 0.05 M buffer of Tris·HCl, pH 7.3 was prepared by dissolving 6.85 g of Trizma·HCl and 0.80 g of Trizma Base in water and diluting to 1 liter. The washed cells were then resuspended in 50 ml of the Tris buffer, and poured into a 500 ml Omni-mixer cup (Ivan Sorvall, Inc., Norwalk, Conn.) with 200 gm of glass beads (Sigma, 50-70 µm in diameter). The cells were homogenized at 4°C

at speed setting no. 8 for 10 minutes. The glass beads were removed by filtration with a coarse fritted glass filter. The cell debris was then centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant solution (the cell extract) was dialyzed with stirring against 2000 ml of the Tris buffer. After 2 hours, a fresh change of buffer was made, and dialysis continued for 3 hours, and then for 20 hours with a final change of buffer. Protein concentration was determined to be 3.8 mg/ml by the Lowry method[47] with bovine serum albumin as the standard. Aliquots of the cell extract were stored frozen at -30°C.

2. After isolation of the peptide transport-deficient mutant, strain Z1-2D Eta^r, new cell extracts of both parent and mutant strain were prepared. Ten ml starter cultures of Z1-2D and Z1-2D Eta^r were grown to 200 Klett units. The cells were washed twice with sterile distilled H_2O and resuspended in 5 ml of sterile distilled water. Four ml of each suspension was used to inoculate 1 liter quantities of minimal medium + leucine + lysine. The remaining portion of cells from each starter culture was plated out to check for maintenance of desired growth characteristics. In particular the mutant's growth on SM + Eth-Ala and lack of growth of SM + Eth and SM + $(Leu)_2$ was noted. The 1 liter flasks of cells grew to mid log phase (107 and 112 Klett units) in 22 hours. An aliquot of the resulting cells were tested in an identical plating procedure as described above. The rest of each group of cells was harvested as described in section 1 above. The yield was 1.4 gm of Z1-2D cells and 1.3 gm of Z1-2D Eta^r cells. However the cells were suspended in a 40 mM potassium phosphate buffer pH 7, rather than the Tris buffer, before breaking. Similarly,

the two cell extracts were dialyzed against 4 changes of the same phosphate buffer over a 4 day period. The 65 mls of mutant cell extract and 70 mls of parent extract were stored in 1 ml aliquots at -30°C. Protein concentrations were determined to be 0.39 mg/ml for Z1-2D CE, and 0.30 mg/ml for Z1-2D Eta^r CE.

F. Peptidase Assays

1. Non-Growth Substrates

A 200 µl portion of cell extract (Z1-2D, 3.8 mg/ml) was incubated at 30°C with an equal volume of peptide solution (usually 2 mg/ml in distilled, sterilized H_2O). At various times 50 µl, 100 µl, or 200 µl samples of the assay mixture were removed, immediately frozen (dry ice-acetone), and stored at -30°C until the assay was completed. Similar assays were performed with the more dilute extracts of the mutant and parent strains to compare peptidase activities.

2. Competition Assays

Peptide mixtures of $(Met)_2$:$(Leu)_2$ and $(Ala)_2$:$(Leu)_2$ at molar ratios of 10:1 in each case were prepared. Z1-2D cell extract (100µl) was added to approximately four mls of each peptide mixture, and 200 µl aliquots withdrawn at various times, frozen, and electrophoresed.

$(Met)_3$ and $(Leu)_3$ were mixed (1:1.2 molar ratio), and 200 µl of Z1-2D cell extract added to make a total volume of 1.0 ml. Aliquots(100µl) were sampled over a 2 hour period, frozen, and electrophoresed.

3. Fluorescence Peptidase Assay of Dileucine Hydrolysis

This assay, a modification of the procedure of M. Roth,[67] was used to quantitate the hydrolysis of various substrates. The assay

mixture consists of a 25 µl aliquot of cell extract added to a solution of 0.9 ml of 40 mM phosphate buffer pH 7 plus 0.1 ml of a 0.01 M dileucine solution (in the phosphate buffer). The fluorescent reagent consists of 90 ml of a 0.05 M sodium tetraborate solution, pH 9.5 plus 1.5 ml of a 10 mg/ml o-phthaldehyde solution in ethanol, plus 1.5 ml of a 5 µl/ml (v/v) solution of β-mercaptoethanol in ethanol. At various times, 0.1 ml of the assay mixture is added to 3 ml of the fluorescent reagent, and the fluorescence read after 5 minutes. The fluorescence of solutions with known amounts of leucine and dileucine were measured to calibrate the hydrolysis of the peptide by mutant and parent cell extracts. Fluorescence measurements were made on an Aminco Bowman Spectrophotofluorometer, excitation λ = 340 nm, emission λ = 450 nm, with a 3-2-3 slit arrangement. The assays were done at 30°C as with the qualitative peptidase assays. Fluorescence measurements were made relative to a 1 µg/ml solution of quinine sulfate in 0.1 N H_2SO_4.

G. Crystal Tests

1. The toxicity of various amino acid analogues and peptides containing these amino acid analogues was tested with a crystal test procedure. Approximately 10^6 cells were evenly distributed on an SM + Leu + Lys plate. After the plate was dry a small crystal of the substrate to be tested was applied to the plate using a sterile inoculating loop. The point of applying the crystal was marked, and the plate allowed to incubate for two days at 30°C. A zone of inhibition around the crystal was evidence that the analogue was toxic to the yeast.

2. Sometimes the growth response to leucine and lysine peptides was measured by using crystal tests. For leucine peptides, cells were distributed on SM + Lys plates and crystals applied. For lysine peptides, crystals were applied to cells that were uniformly streaked on a SM + Leu plate. For the peptide Lys-Leu, crystals were applied to cells on an SM plate. A positive growth response would be judged by a region of growth around the spot where the peptide was applied.

H. Isolation of a Peptide Transport-Deficient Mutant of Z1-2D

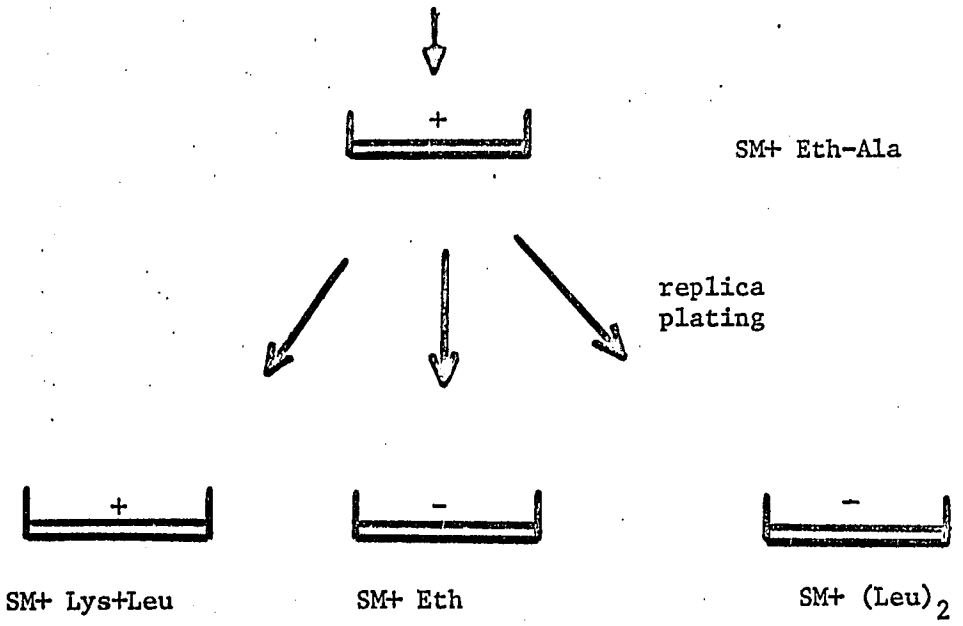
Approximately 10^6 cells of a stationary phase culture of strain Z1-2D grown on 0.23 mM leucine, 0.11 mM lysine, 0.23 mM Eth-Ala, and 1.61 mM $(Leu)_2$ were plated out on a minimal media agar plate containing leucine, lysine and Eth-Ala (see Figure 2, and Figure 14, results section). Thirteen colonies grew on this plate and were individually transferred to plates containing lysine and leucine; lysine and $(Leu)_2$; and lysine, leucine and ethionine. Eleven colonies were resistant to ethionine. The two remaining colonies, as schematically illustrated in Figure 2, were sensitive to ethionine and did not grow on the plate containing $(Leu)_2$. One of these colonies, designated Z1-2D Eta^r, was transferred to a YEPD slant and stored at 4°C.

I. Revertant Analysis of Z1-2D Eta^r

A 5 ml starter culture of Z1-2D Eta^r was washed, and resuspended in an equal volume of sterile distilled water. A viable count was made by an appropriate series of dilutions followed by pipetting 0.1 ml onto an SM + Lys + Leu plate. 0.1 ml aliquots of the resuspended starter culture cells were applied to an SM + $(Leu)_2$, and an SM + $(Leu)_3$ plate and incubated at 30°C for 4 days. The frequency of revertants

Figure 2

Isolation of Z1-2D Eta^r (a)



(a) In each case, the cells applied to the minimal media plate were allowed to incubate at 30°C for two days. A (+) symbol on the SM + Eth-Ala plate indicates the positive growth response of 13 colonies. Two of these thirteen colonies exhibited the displayed growth response [(+)=growth, (-)= no growth] when replica plated to the other minimal media plates.

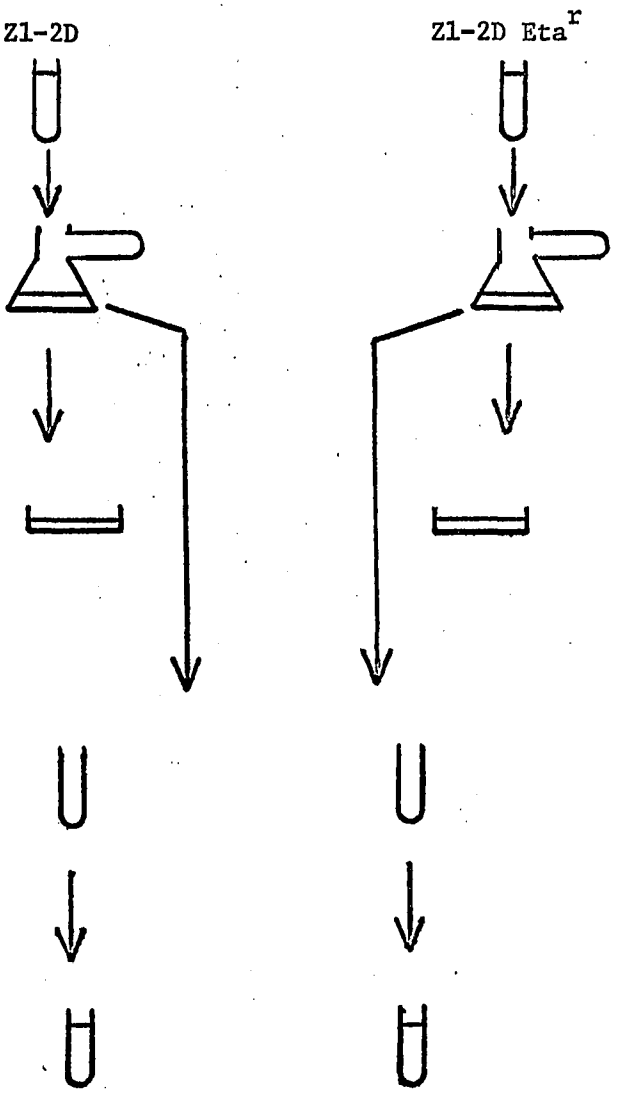
for growth on the SM + $(Leu)_2$, and the SM + $(Leu)_3$ plates was calculated as an average of the number of colonies that grew on each plate. Each colony that grew on the SM + $(Leu)_2$ plate was transferred to a grid on another SM + $(Leu)_2$ plate. Revertant colonies from the SM + $(Leu)_3$ plate were similarly transferred to a grid on an SM + $(Leu)_3$ plate. Both plates were incubated for another 4 days. Then each colony from the SM + $(Leu)_2$ grid was replica plated onto plates of SM + Lys + Leu, SM + Lys, SM + Leu, SM + Eth, SM + Eth-Ala, and SM + $(Leu)_3$. Colonies from the SM + $(Leu)_3$ grid were transferred to plates of SM + Lys + Leu, SM + Lys, SM + Leu, SM + Eth, SM + Eth-Ala and SM + $(Leu)_2$. The plates were scored after 2 days.

J. Radioactive Uptake Assays (see Figure 3).

For the growth of cells to be used in uptake assays with radioactively labeled substrates, proline at an equivalent nitrogen contration replaced ammonium sulfate in the minimal medium. Therefore Difco yeast nitrogen base without amino acids and without ammonium sulfate was used, but supplemented with 87.1 mg/ml proline (10 times strength concentration). The single strength medium (SM/Pro) contains the required amounts of leucine + lysine and dextrose. Cultures of Z1-2D + Z1-2D Eta^r were inoculated in this medium, and grown to mid-log phase (60-80 Klett units) for all uptake assays. Either a 5 or 7.5 ml starter culture was prepared. The cells were transferred to Klett flasks and diluted with the SM/Pro medium to approximately 20 Klett units. The flasks were shaken at 30°C for 3-5 hours to allow the cells to grow to 30 Klett. At this time a portion of each batch of cells, Z1-2D or Z1-2D Eta^r was plated out to check for the maintenance of the desired growth characteristics. Then 5 ml portions of mutant and

Figure 3

Method to Assay Uptake of Radioactively Labeled Substrates



Grow starter cultures in SM/Pro media, 5-7.5 ml

At mid-log phase(60-80 Klett units), transfer to Klett flasks.

Dilute with SM/Pro media to 20 Klett units. Grow 3-5 hours to 30 Klett units.

Plate out aliquots of Z1-2D, Z1-2D Eta^r to check growth characteristics.

Transfer 5 ml portions into assay tubes, centrifuge, wash twice with sterile, distilled water and decant.

Resuspend cells in either:
a. SM/Pro(0.5% dextrose)
or b. 0.1M phosphate buffer, (0%, or 1% dextrose).

Add competing substrate, (if used). Add labeled amino acid or peptide, mix rapidly and begin assay.

parent cells were transferred to assay tubes, spun down, washed twice with sterile distilled water and resuspended in either the SM/Pro medium (with 0.5% dextrose), or 0.1 M phosphate buffer, pH 5.5,[12] containing 1% dextrose. Some assays with [U-^{14}C]leucine were performed without dextrose in the phosphate buffer. The assay tube was maintained at 30°C. Any competing substrate was added, followed by the addition of the labeled substrate with rapid mixing to begin the assay. In almost all cases, the amount of labeled and unlabeled substrates added was less than 5% of the total assay volume.

The apparatus used for all uptake assays was the Yeda Multiple Membrane Filter, Tel Aviv Israel, in conjunction with a Millipore vacuum pump, Bedford, Mass. Routinely, 0.5 ml aliquots of cells were withdrawn from the assay mixture and filtered on 0.45 μ Millipore filters on the Yeda apparatus. Immediately the filter was washed with two 1 ml portions of water, and after the assay transferred to a scintillation vial. Fifteen ml of Aqueous Counting Scintillent (Amersham, Arlington Heights, Ill.), was added and after 6-24 hours, counted for 2 minutes in a Nuclear Chicago Mark II Liquid Scintillation Counter. The particular assay conditions are summarized in table 2. Competition experiments were performed with [U-^{14}C] leucine and [1-^{14}C] Gly-Leu. In each case where a competing amino acid, or peptide is added a control assay with the labeled peptide or amino acid is run simultaneously at the same substrate concentration. Methionine, phenylalanine and leucine were tested for their ability to compete with labeled leucine at concentration ratios (relative to [U-^{14}C]leucine) of 20:1 and 40:1. $(Leu)_2$, $(Leu)_2$-OBzl, and $(Leu)_2$-OMe were examined at 10:1 and 20:1 molar ratios to [1-^{14}C]Gly-Leu in other

Table 2

Assay Conditions for the Uptake of

Radioactively Labeled Substrates

Substrate	Concentration	Assay Medium	Dextrose Concentration
[1-^{14}C] methionine 10 mCi./mmole	10^{-6}M	SM/Pro	0.5%
[U-^{14}C] leucine 51 mCi./mmole	10^{-7}M, 10^{-6}M, and 10^{-5}M	phosphate buffer, 0.1M, pH 5.5	1% or 0%
[1-^{14}C] Gly-Leu 20 mCi./mmole	10^{-6}M	SM/Pro	0.5%
Met-Met-[1-^{14}C]Met 1 mCi./mmole	10^{-6}M, 10^{-5}M	SM/Pro	0.5%

competition assays performed in the SM/Pro medium. The uptake assays on [1-^{14}C]Gly-Leu and [1-^{14}C]methionine were run concurrently on separate portions from each batch of mutant and parent cells.

The efficiency of the liquid scintillation counter was determined by counting known amounts of [1-^{14}C]methionine, [1-^{14}C]Gly-Leu, [U-^{14}C]leucine and [^{14}C]toluene. Standard solutions of the first three labeled substrates were prepared as follows: a) 10 µl of [1-^{14}C] methionine (10 µCi/µmole) is added to 5 ml H_2O, mixed, and a 0.25 ml aliquot transferred to a scintillation vial. b) 20 µl of [1-^{14}C]Gly-Leu (20 µCi/µmole) was added to 5 ml of H_2O, mixed, and a 0.1 ml aliquot transferred to a scintillation vial. c) 50 µl of [U-^{14}C] leucine (51 µCi/µmole) was added to 5 ml of H_2O, mixed, and a 50 µl aliquot transferred to a scintillation vial. The water in the scintillation vials in a), b) and c) was allowed to evaporate under a heat lamp. Then the scintillant (15 ml) was added. The labeled toluene standard (10λ, 4.24×10^5 dpm/ml) was added directly to the scintillation vial after the scintillant was introduced. A clean filter, dried on the uptake apparatus was added to each vial. The samples were counted for 10 minutes and the efficiency for each substrate determined by dividing the number of counts by the theoretical amount predicted by the specific activity and concentration. The resultant efficiency is an average of these efficiencies calculated for each of the 4 substrates and was determined to be 86%.

The cell density corresponding to 30 Klett units was determined. Starter cultures of Z1-2D were grown to mid log phase, transferred to Klett flasks and diluted with SM/Pro to ~ 20 Klett units. The flasks were incubated at 30°C with shaking for 3 hours. At 30 Klett, 50 ml

portions of the starter cultures were filtered on preweighed 0.45 μ filters that were brought to constant dry weight in a dessicator. The filters plus cells were transferred to the dessicator and weighed daily until the dry weight was constant. In this manner, the cell density corresponding to 30 Klett units was found to be 0.1 mg cells/ml.

III. RESULTS

A. Determination of the Minimal Concentrations of Leucine and Lysine Required for Maximum Growth of Z1-2D.

Two series of growth flasks were prepared. One series (Figure 4) contained minimal medium, lysine (20 µg/ml) and concentrations of leucine ranging from 0-100 µg/ml. The other series (Figure 5) contained minimal medium, leucine (60 µg/ml) and concentrations of lysine ranging from 0-50 µg/ml. The maximum Klett values reached after a 40 hour growth period (when all growth flasks had reached a stationary phase) were plotted as a function of the leucine and lysine concentrations. It was determined that the minimal concentration of leucine needed for maximum growth was 60 µg/ml. However, to make the assay more sensitive, it was decided to measure growth of the yeast relative to a leucine concentration of 30 µg/ml (0.23 mM), as indicated by the arrow in Figure 4. The minimal concentration of lysine required for maximum growth was 15 µg/ml (0.11 mM).

B. Growth Response of Z1-2D to 0.23 mM Leucine and 0.11 mM Lysine.

Growth curves A in Figures 6 and 7 illustrate the growth response of Z1-2D to the minimal concentrations of leucine and lysine. The final Klett values of 140-150 are equivalent to 2.5 - 3 x 10^7 cells/ml as determined by viable counting procedures. The average generation time for growth on leucine and lysine was 2.2 hours (6 independent experiments).

C. Growth Response of Z1-2D to Leucine and Lysine Peptides.

1. Leucine Peptides

Many leucine peptides are growth substrates for strain Z1-2D. These peptides satisfy the nutritional requirement of the organism for

Figure 4

Determination of the minimal concentration of leucine required for the maximum growth of S.cerevisiae. Each growth flask contained lysine (20 µg/ml) and various concentrations of leucine(0,2,5,10,20,30,40,60,or 100 µg/ml). Turbidity, in Klett units, was measured for each growth flask at 40 hours. The minimal concentration required for maximum growth was 60 µg /ml. The arrow indicates the leucine concentration (30 µg/ml) that was used to make the assay more sensitive.

Figure 4

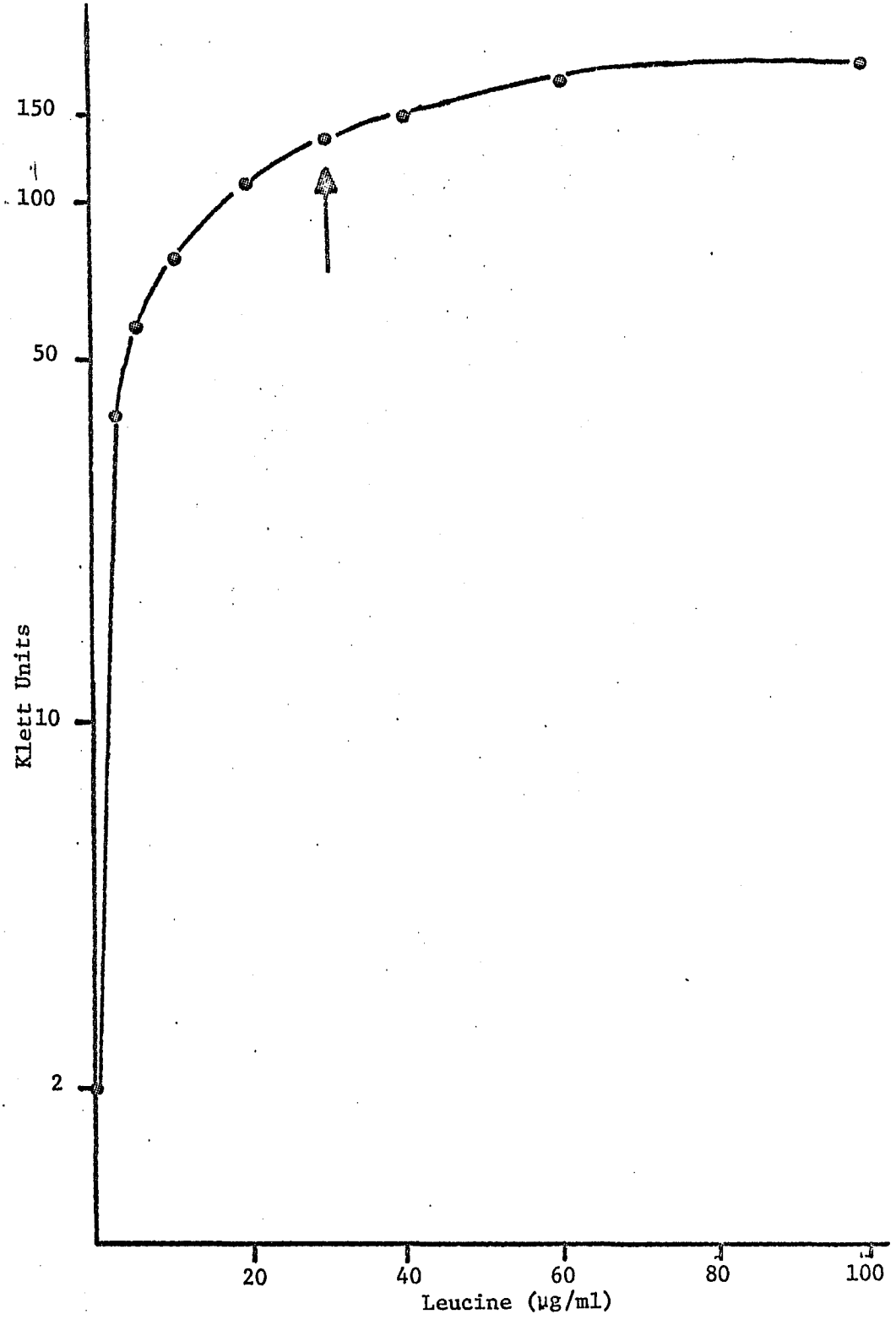
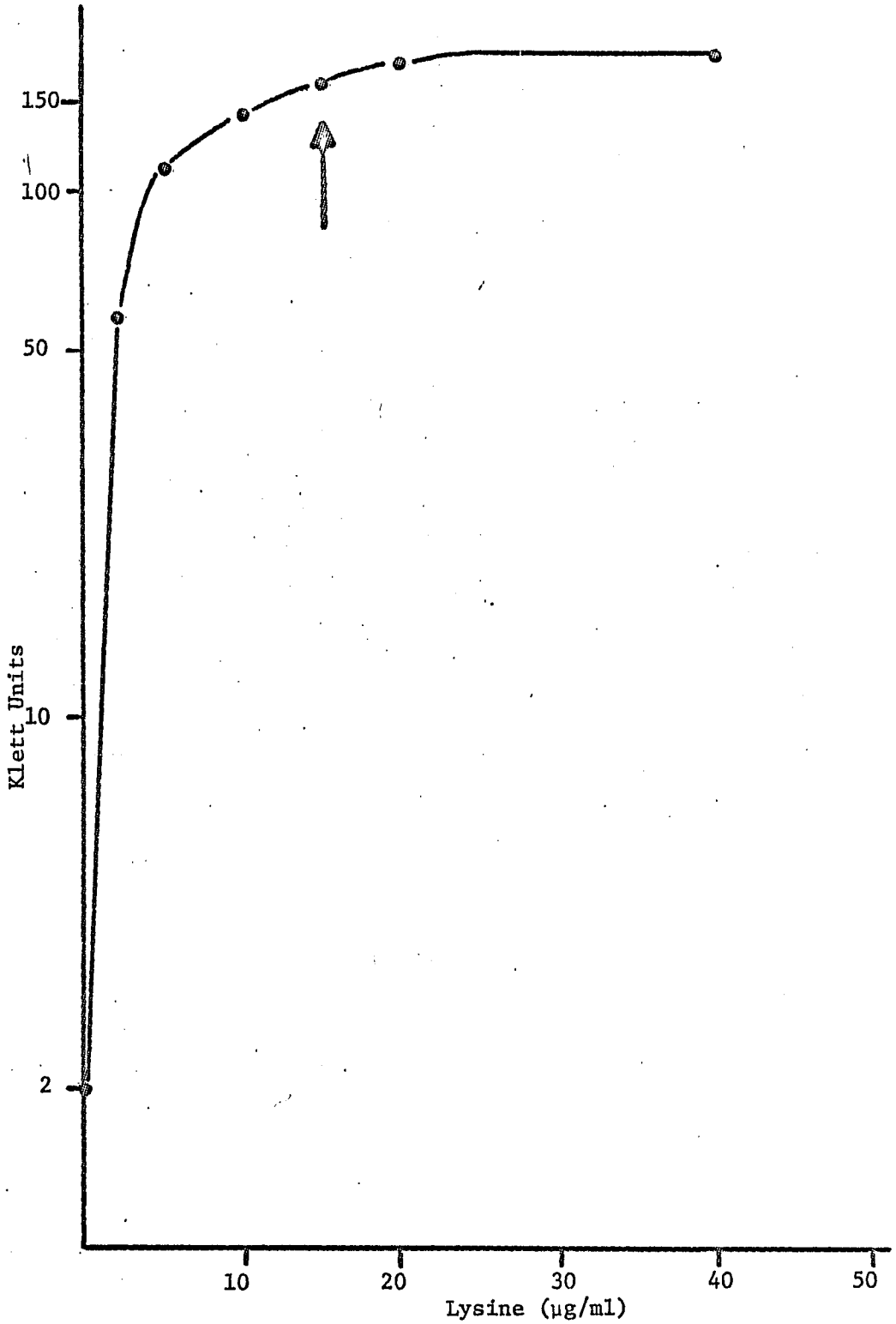


Figure 5

Determination of the minimal concentration of lysine required for the maximum growth of S.cerevisiae Z1-2D. Each growth flask contained leucine(60 µg/ml) and various concentrations of lysine(0,2,5,10,15,20,or 50 µg/ml). Turbidity, in Klett units, was measured for each growth flask at 40 hours. The arrow represents the minimal concentration of lysine required for maximum growth.

Figure 5



leucine when supplied in its place at the equivalent leucine concentration of 0.23 mM. There were 3 categories of growth response to leucine peptides. A number of peptides elicited a growth response equivalent, or nearly so, to that of leucine. Growth on two of these peptides is illustrated by curves B and C (Figure 6). These good growth peptides, $(Leu)_2$ and $(Leu)_3$ always exhibit a slight lag (5-7 hours) when compared to growth on leucine and lysine alone. The generation time for these peptides is about 0.5 hour longer (2.7 hr.) than growth on the required free amino acids. Curve D illustrates the growth response of an intermediate growth substrate, Gly-Leu. The generation time on these substrates was nearly equivalent to that of the good growth peptides, but they exhibit a lag period of 25 hours or more. Other leucine peptides, such as Gly-Leu-Gly, and Gly-Leu-Gly-Leu were not growth substrates over the 40 hour test period.

2. Lysine Peptides

Several lysine peptides were also tested as growth substrates in place of free lysine (Figure 7). Lys-Gly, Lys-Leu (curves B,C), and Lys-Gly-Gly (not shown) were good growth substrates. $(Lys)_n$ peptides, where n = 2,3,4,5 were not growth substrates for strain Z1-2D (D). The growth response to all leucine and lysine peptides is summarized in Table 3.

D. Tests to Determine the Toxicity of Non-growth Substrates.

It is possible that the peptides listed in Table 3 as non-growth substrates were transported and toxic to Z1-2D. To test for this possibility, each non-growth-supporting peptide was supplied in a growth flask along with the normal amount of the required amino acids. As seen in Table 4, all non-growth substrates examined did not prevent

Figure 6

Growth of S.cerevisiae Z1-2D on leucine peptides. Each growth flask contained lysine(0.11mM) and leucine(0.23mM) or the peptide under investigation. Turbidity, in Klett units, was measured for growth on leucine(+), $(Leu)_2$(□), $(Leu)_3$(○), Gly-Leu(▲), and Gly-Leu-Gly or Gly-Leu-Gly-Leu(△). Each growth flask was supplemented with peptide so that the leucine content was equal to 0.23mM of the free amino acid.

Figure 6

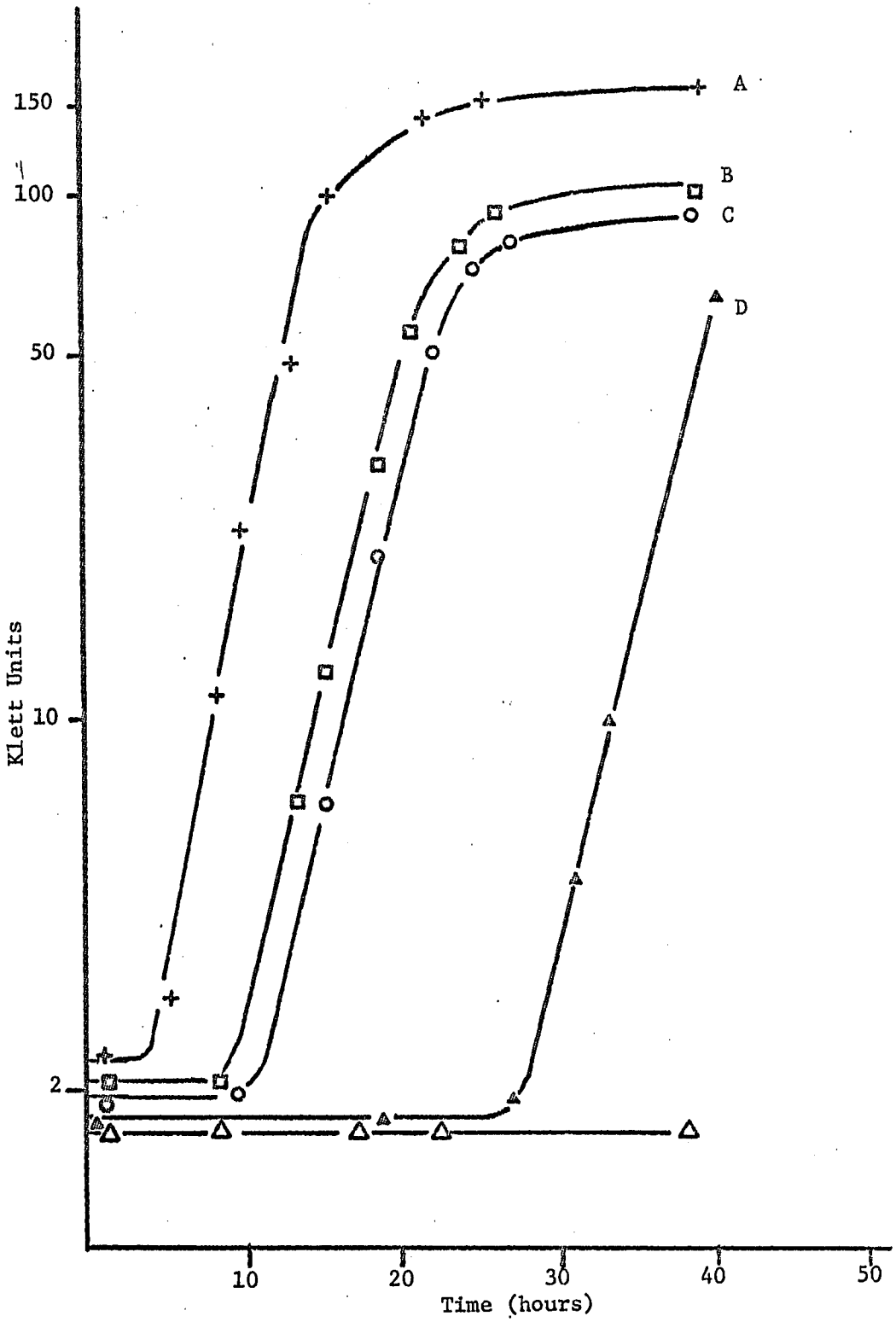


Figure 7

Growth of S.cerevisiae Z1-2D on lysine peptides. Each growth flask contained leucine(0.23mM) and lysine(0.11mM) or the peptide under investigation. Turbidity, in Klett units, was measured for growth on lysine(+), Lys-Leu(□), Lys-Gly(○), and $(Lys)_n$,n=2-5(△). Each growth flask was supplemented with peptide so that the lysine content was equivalent to 0.11mM of the free amino acid.

Figure 7

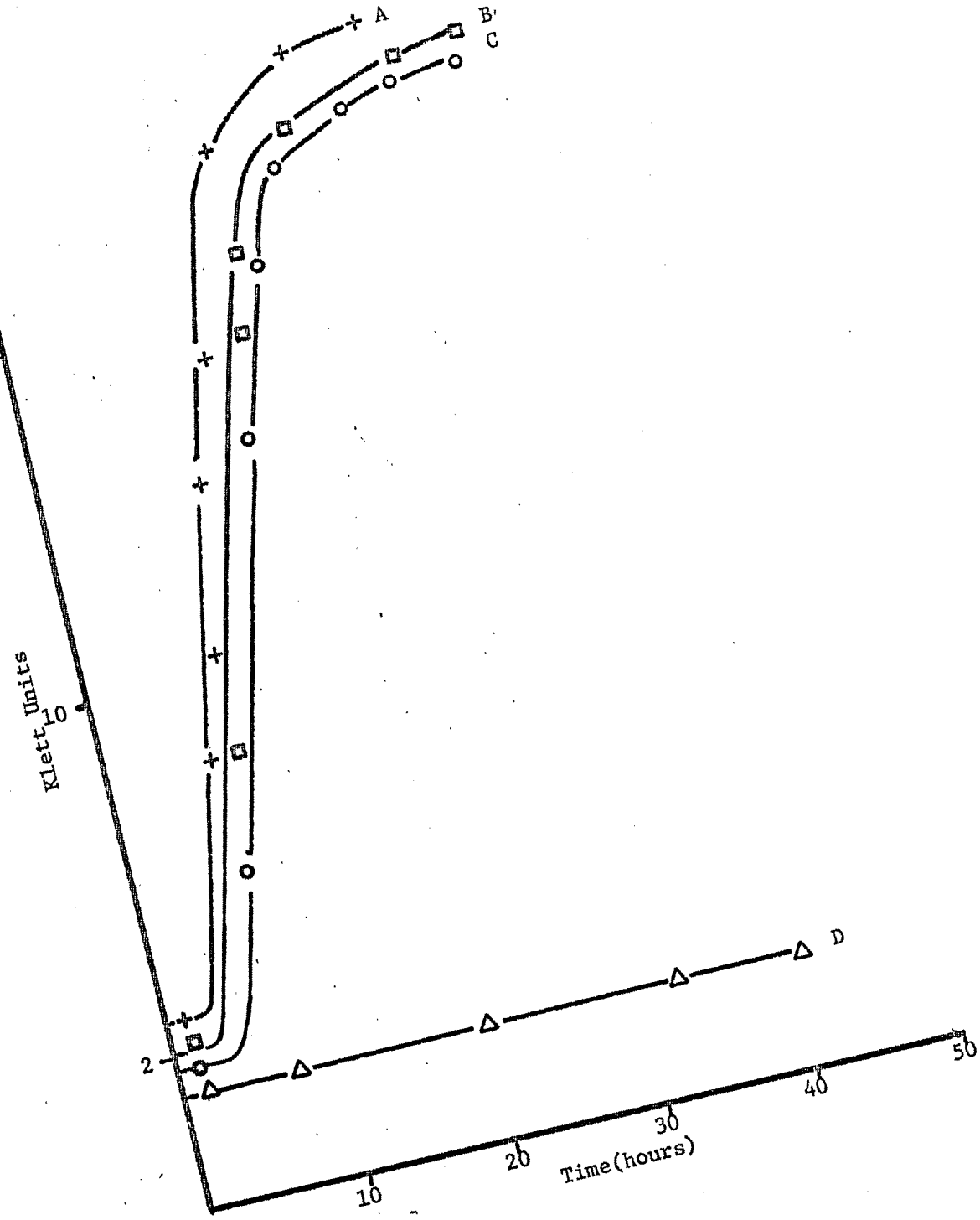


Table 3

Growth Response of Z1-2D to Leucine and Lysine Peptides

Good Growth Substrates[a]	Intermediate Substrates[b]	Non-Growth[c] Substrates
Leu-Leu	Gly-Leu	L-Leu-D-Leu
Leu-Leu-OBzl	Met-Leu	D-Leu-L-Leu
Leu-Ala	Pro-Leu	Ac-Gly-Leu
Ala-Leu		Ac-Leu-Leu-Leu
Leu-Gly		
Val-Leu		Gly-Gly-Leu
Leu-Val		Gly-Leu-Gly
Lys-Leu		Leu-Gly-Gly
Leu-Pro		Pro-Pro-Leu
Leu-Leu-Leu		Pro-Leu-Gly-NH_2
Leu-Gly-Leu		Met-Met-Leu
Leu-Leu-Gly		Gly-Leu-Gly-Gly
Lys-Gly		Gly-Leu-Leu-Gly
Lys-Leu		Gly-Leu-Gly-Leu
Lys-Gly-Gly		Leu-Leu-ε-aminocaproic acid
		Leu-Leu-OMe
		Leu-Leu-Leu-OMe
		Tyr-Gly-Gly-Phe-Leu
		Gly-Lys
		Lys-Lys
		Lys-Lys-Lys
		Lys-Lys-Lys-Lys
		Lys-Lys-Lys-Lys-Lys

[a] Peptide elicits a growth response nearly equivalent to that of leucine and lysine.

[b] Peptide elicits a positive growth response after a lag period of 25 to 40 hours.

[c] No positive growth response over 40 hour testing period.

Table 4

Toxicity Tests on Non-Growth-Supporting Peptides[a]

Non-Growth-Substrate	40 Hour Klett Units for Substrates Plus Leucine and Lysine
None	146
Met-Met-Leu	146
Gly-Leu-Gly	145
Ac-Leu-Leu-Leu	149
Pro-Pro-Leu	169
Gly-Leu-Gly-Gly	142
Gly-Leu-Gly-Leu	143
L-Leu-D-Leu	151
D-Leu-L-Leu	153
Lys-Lys	143
Lys-Lys-Lys	133
Lys-Lys-Lys-Lys	136
Leu-Leu-OMe	167
Leu-Leu-Leu-OMe	160

[a] Klett values are normalized to an initial reading of 2. Leucine and leucine-containing peptides are supplied in the liquid media at an equivalent leucine concentration of 0.23mM. Lysine and lysine-containing peptides are supplied at an equivalent lysine concentration of 0.11mM.

normal growth in the presence of leucine and lysine.

E. Growth Response of Z1-2D to High Concentrations of Non-Growth-Supporting Peptides.

Several peptides that were not growth substrates at the equivalent leucine concentration of 0.23 mM were supplied in the growth media at higher concentrations. The growth response to peptides under these conditions is listed in Table 5. It is apparent that the tripeptide Gly-Leu-Gly is a growth substrate at the higher concentrations. Growth studies reported in the next section will examine the factors that can account for a positive growth response at these concentrations. The tetrapeptide Gly-Leu-Gly-Gly (at 20 x) is not a leucine source for this auxotroph. The high concentration of the tetrapeptide does not impart any toxic effect to Z1-2D as shown by the positive growth response of Z1-2D to the normal amount of leucine with 4.6 mM Gly-Leu-Gly-Gly included in the growth medium. Dileucyl-ε-aminocaproic acid is an intermediate growth substrate at 2.3 mM.

F. Effect of Competing Amino Acids on the Growth of Z1-2D on Leucine Peptides.

1. Phenylalanine

Phenylalanine inhibited the growth of Z1-2D on leucine. As shown in Figure 8, a 1:1 molar ratio of phenylalanine to leucine produced a slight lag in the growth. At a 10:1 ratio considerable inhibition resulted, and no growth was evident over a 40 hour period when a 46:1 ratio was used. However a 92:1 ratio of phenylalanine to $(Leu)_2$ didn't inhibit growth compared to $(Leu)_2$ alone (Figure 9). Similarly, a 30:1 molar ratio of phenylalanine to $(Leu)_3$, and a 92:1 molar ratio of phenylalanine to $(Leu)_2$-OBzl yielded no inhibition of

Table 5

Growth Response of Z1-2D to High Concentrations of Non-Growth-Supporting Peptides

Non-Growth Substrate[a]	Concentration	Klett Units, 40 Hours
Gly-Leu-Gly	2.3mM	139
Gly-Leu-Gly	6.9mM	154
Leu-Leu-ε-aminocaproic acid	2.3mM	73
Gly-Leu-Gly-Gly	2.3mM	2
Gly-Leu-Gly-Gly	4.6mM	2
Gly-Leu-Gly-Gly + Leucine	4.6mM .23mM	150

[a] Each growth flask contained, in addition, the normal amount of lysine(0.11mM).

Figure 8

Growth of S.cerevisiae Z1-2D in the presence of various concentrations of phenylalanine. Each growth flask contained leucine(0.23mM) and lysine(0.11mM). Turbidity, in Klett units, was measured for growth in the presence of no phenylalanine(+), phenylalanine at equimolar concentration to leucine(□), phenylalanine at a 10-fold molar excess to leucine (O), and phenylalanine at a 46-fold molar excess to leucine (Δ).

Figure 8

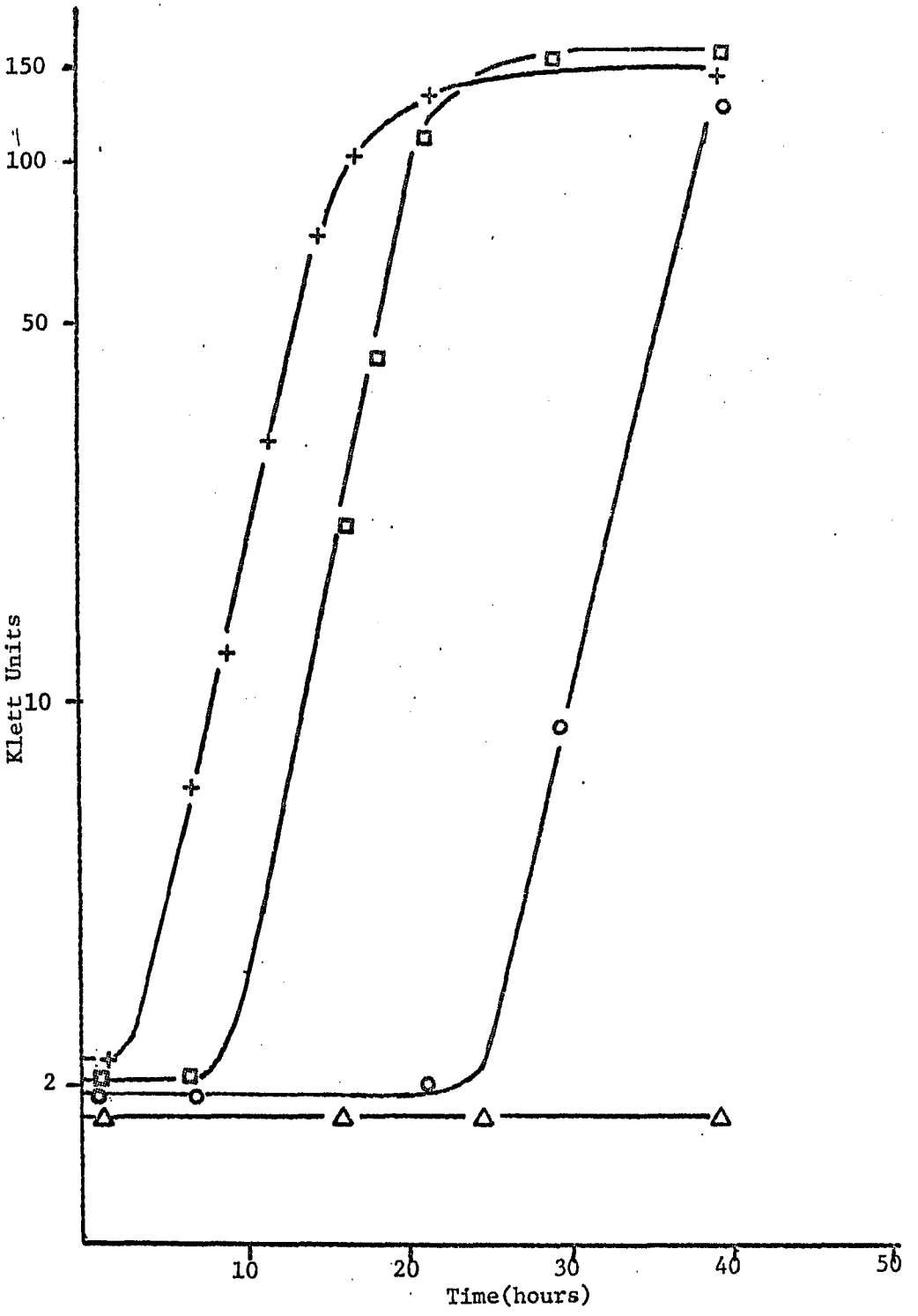
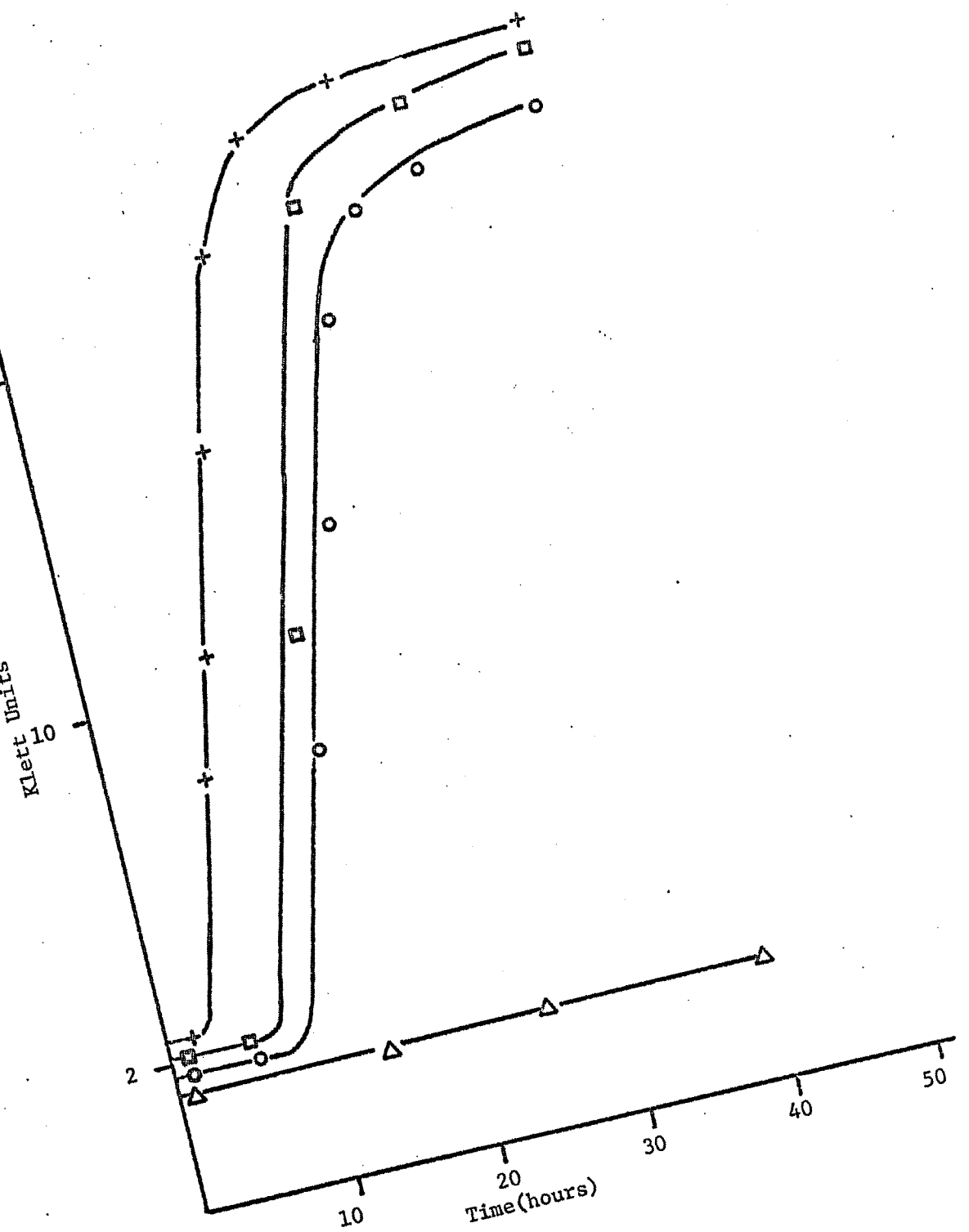


Figure 9

Growth of S.cerevisiae Z1-2D on leucine and $(Leu)_2$ in the presence and absence of phenylalanine. Each growth flask contained lysine(o.11mM). Leucine and $(Leu)_2$ were supplied in the appropriate growth flasks so that each flask contained a leucine content equivalent to 0.23mM of free amino acid. Turbidity, in Klett units, was measured for growth on leucine (+), leucine + phenylalanine at a 46-fold molar excess to leucine(Δ), $(Leu)_2$(○), and $(Leu)_2$+ phenylalanine at a 92-fold molar excess to $(Leu)_2$(□).

Figure 9



the normal growth response to these compounds.

In the last section, Gly-Leu-Gly at 30 times the leucine concentration normally used in growth experiments (0.23 mM) was found to be a growth substrate. In competition experiments with a 16-fold excess of phenylalanine present in the growth medium (Figure 10), a slight lag in the growth curve results compared to growth on high concentrations of the peptide alone. This lag is negligible when compared to the total inhibition of growth on a 30-fold concentration of leucine in the presence of the same excess of phenylalanine. The reason for the increased lag in the growth on Gly-Leu-Gly in the presence of phenylalanine is not well understood. One explanation is that phenylalanine inhibits a minor leucine impurity in Gly-Leu-Gly from entering Z1-2D, and the observed lag is due to the remaining equivalent amount of leucine available in the peptide. Another possibility is that the 110.4 mM phenylalanine causes a small inhibition in growth unrelated to the transport of leucine, or Gly-Leu-Gly. However I believe that phenylalanine does not directly inhibit the uptake of Gly-Leu-Gly.

2. Methionine

Methionine also inhibited growth of Z1-2D on leucine, but to a much lesser extent than phenylalanine. In Figure 11 we see how a 10:1 molar ratio of methionine to leucine had virtually no effect, while molar ratios of 46:1 and 92:1 produced only a 10-15 hour lag period on the growth on leucine. However a much greater molar ratio of methionine to $(Leu)_2$, 184:1, (Figure 12) has no inhibitory effect on the growth response of Z1-2D compared to $(Leu)_2$ alone. A molar ratio of 276:1, methionine to $(Leu)_3$, also produced no inhibition in

Figure 10

Growth of S.cerevisiae Z1-2D on high concentrations of leucine and Gly-Leu-Gly in the presence and absence of phenylalanine. Each growth flask contained lysine(0.11mM). Leucine and Gly-Leu-Gly were supplied in the appropriate growth flasks so that each flask contained a leucine content equivalent to 6.9mM of the free amino acid. Turbidity, in Klett units, was measured for growth on leucine(+), leucine + phenylalanine at a 16-fold molar excess to leucine(Δ), Gly-Leu-Gly(□), and Gly-Leu-Gly + phenylalanine at a 16-fold molar excess to Gly-Leu-Gly(o).

Figure 10

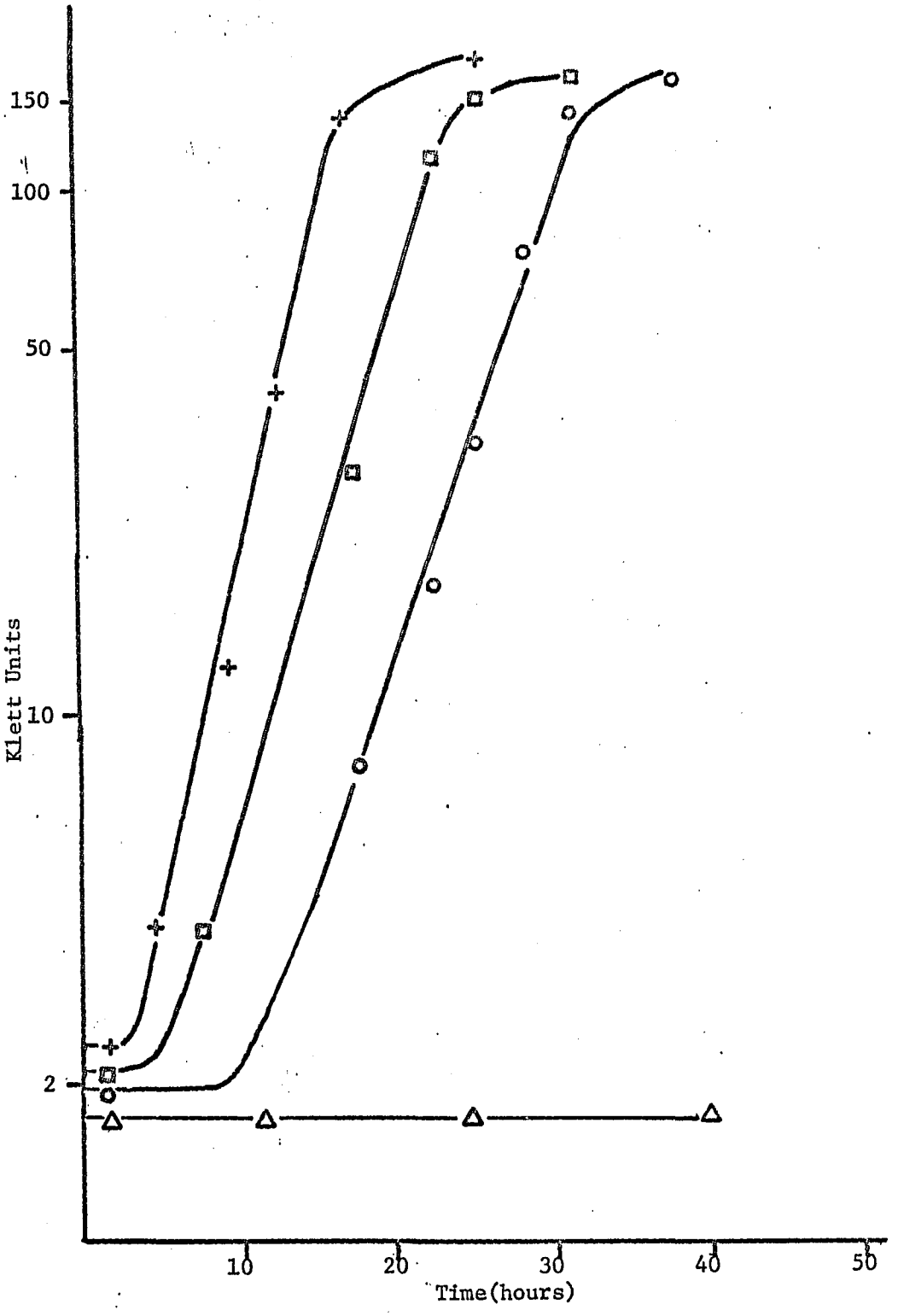


Figure 11

Growth of S.cerevisiae Z1-2D in the presence of various concentrations of methionine. Each growth flask contained lysine (0.11mM) and leucine(0.23mM). Turbidity, in Klett units, was measured for growth in the presence of no methionine(+), methionine at a 10-fold molar excess to leucine(□), methionine at a 46-fold molar excess to leucine(O), and methionine at a 92-fold molar excess to leucine(Δ).

Figure 11

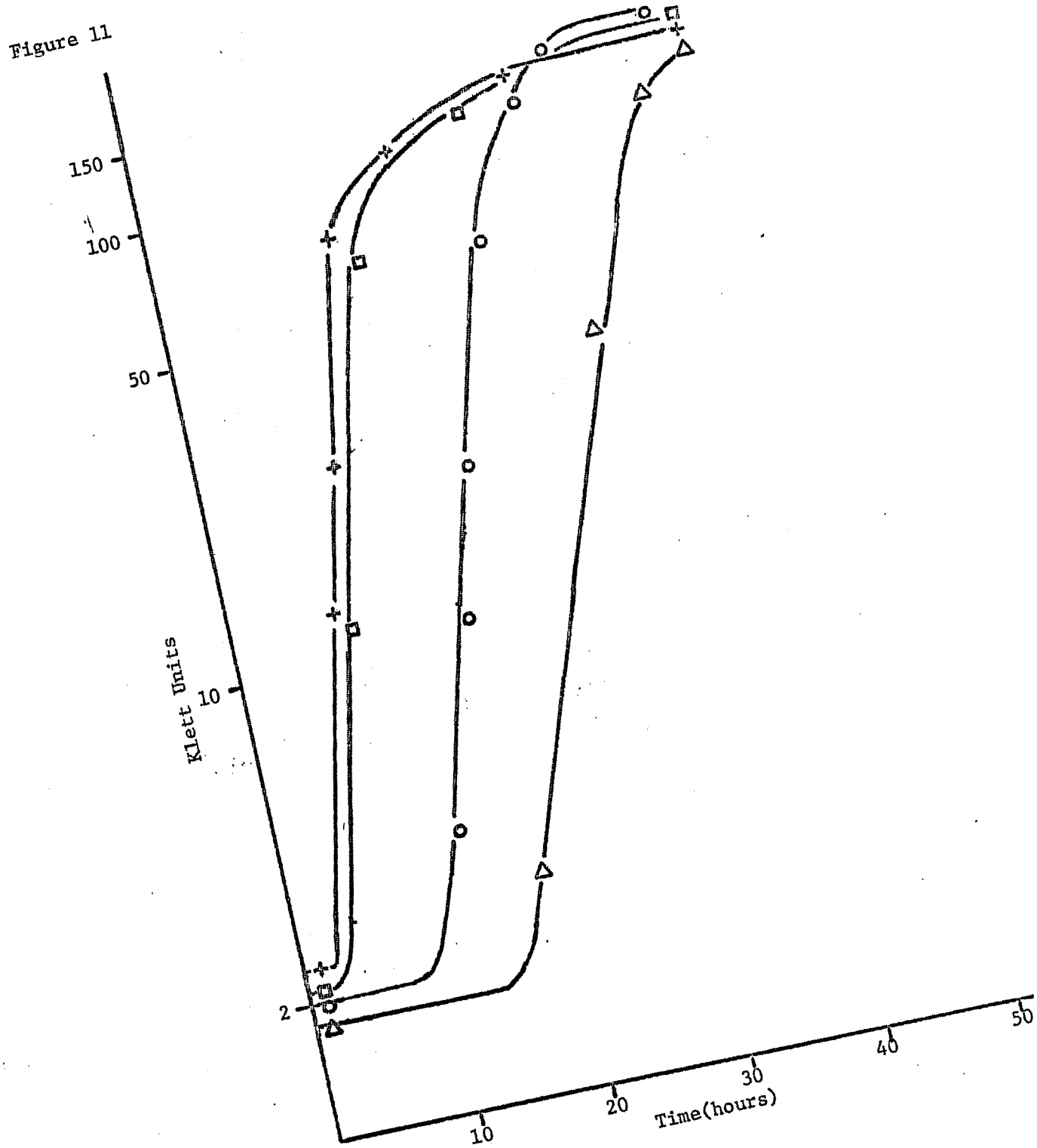
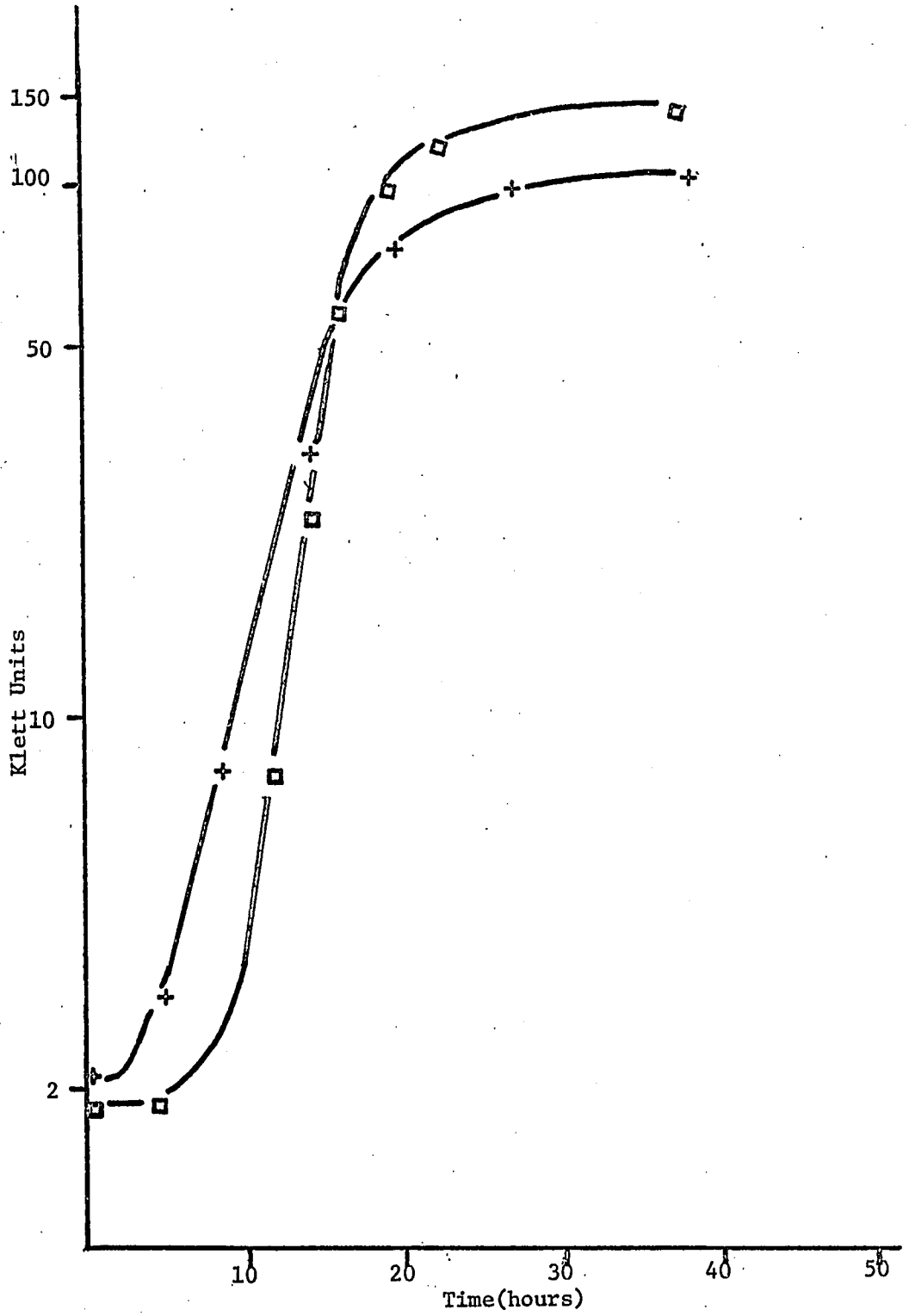


Figure 12

Growth of S.cerevisiae Z1-2D on $(Leu)_2$ in the presence and absence of methionine. Each growth flask contained lysine (0.11mM). $(Leu)_2$ was supplied so that each growth flask contained a leucine content equivalent to 0.23mM of free amino acid. Turbidity, in Klett units, was measured for growth in the presence of no methionine(+), and methionine at a 184-fold molar excess to $(Leu)_2$(□).

Figure 12



growth. Therefore both methionine and phenylalanine appeared to exert their inhibitory effect only on leucine and not on the peptides $(Leu)_2$, $(Leu)_2$-OBzl, and $(Leu)_3$.

G. Competition for the Uptake of [U-^{14}C]leucine by Leucine, Methionine and Phenylalanine.

The uptake of [U-^{14}C]leucine over the 20-25 minute assay period was linear with time. However the rate of uptake at one particular concentration varied from one batch of cells to another. For example, the initial rate of uptake of [U-^{14}C]leucine varied from 0.083 nmole/mg-min to 0.138 nmole/mg-min, at 10^{-6}M leucine, depending on the particular batch of cells used in the growth experiment. Therefore calculations of percent inhibition of [U-^{14}C]leucine uptake by the competing amino acid are always related to the particular rate for the control run simultaneously with the same concentration of labeled leucine. The rate of uptake of [U-^{14}C]leucine depends on its concentration in the assay medium, as shown in Table 6. The effect of different amino acids on [U-^{14}C]leucine uptake at various concentrations is summarized in Table 7. The results indicate that phenylalanine and methionine inhibit the uptake of [U-^{14}C]leucine to the same extent as unlabeled leucine, and that the % inhibition is greatest when the [U-^{14}C]leucine concentration is 10^{-5}M. Also note that the presence or absence of dextrose has only a minor effect on the inhibitory effect of 40:1 concentrations of phenylalanine on 10^{-6}M [U-^{14}C]leucine. The results suggest that the ability of phenylalanine and methionine to inhibit growth of Z1-2D on leucine is due to their competition with leucine for entry into the cell.

Table 6

Rate of [U-^{14}C]leucine Uptake in S.cerevisiae Z1-2D

[U-^{14}C]leucine Concentration	Rate[a]	n[b]
10^{-7}M	0.00694[c]	1
10^{-6}M	0.114[d]	4
10^{-5}M	0.463[c]	3

[a] nmole/mg cells-minute

[b] number of determinations

[c] 1% dextrose

[d] the rate of [U-^{14}C]leucine uptake with and without dextrose present in the assay medium.

Table 7

Inhibition of [U-^{14}C]leucine Uptake in S.cerevisiae Z1-2D by Amino Acids

[U-^{14}C]leucine Concentration	Amino Acid	Ratio[a]	Percent Inhibition[b]	n[c]
10^{-7}M	phenylalanine	40:1	52.7[d]	1
10^{-6}M	phenylalanine	20:1	47.1[e]	2
	leucine	20:1	55.4[e]	2
	methionine	20:1	45.7[e]	1
	phenylalanine	40:1	68.1[f]	2
	leucine	40:1	67.4[e]	1
	methionine	40:1	65.2[e]	1
10^{-5}M	phenylalanine	40:1	87.8[d]	2
	methionine	40:1	69.2[d]	1

[a] The molar ratio of unlabeled amino acid to [U-^{14}C]leucine.

[b] $$\% \text{ inhibition} = \frac{\text{Rate [U-}^{14}\text{C]leucine} - \text{Rate [U-}^{14}\text{C]leucine plus amino acid}}{\text{Rate[U-}^{14}\text{C]leucine}} \times 100$$

[c] number of determinations

[d] 1% dextrose

[e] 0% dextrose

[f] % inhibition determined with and without dextrose present in the assay medium.

H. Competition Studies

1. Non-Growth-Supporting Leucine-Containing Peptides

The effect of several non-growth substrates on the growth response of Z1-2D to leucine peptides was examined. As Table 8 indicates, of all the competing peptides examined, only Met-Met-Leu had a noticeable affect on $(Leu)_2$, $(Leu)_3$, or $(Leu)_2$-OBzl.

2. Other Peptides

Peptides not containing leucine or lysine were examined for their ability to inhibit growth on leucine of lysine peptides. Of these peptides, $(Met)_3$ and $(Met)_2$ had a considerable inhibitory effect on the growth response to $(Leu)_3$, $(Leu)_2$, $(Leu)_2$-OBzl, Lys-Gly and Lys-Leu (Table 9). Compared with $(Met)_3$, Ac-$(Met)_3$ and $(\underline{L}\text{-Ala})_3$ were only weakly inhibitory, and $(Gly)_3$ was a non-competitor. A similar trend was seen in comparing the competition of $(Met)_2$, $(Ala)_2$, and $(Gly)_2$ with $(Leu)_3$, $(Leu)_2$, or $(Leu)_2$-OBzl. Although $(\underline{L}\text{-Ala})_3$ at a 10 fold molar excess inhibited the growth of S. cerevisiae on $(Leu)_3$, neither $(\underline{D}\text{-Ala})_3$ or $\underline{L}$-Ala-$\underline{L}$-Ala-$\underline{D}$-Ala had any inhibitory effect. It is significant that methionine at ratios of $\geq$ 10:1 did not inhibit growth on $(Leu)_2$, $(Leu)_3$, $(Leu)_2$-OBzl, Lys-Leu, or Lys-Gly. Examples of the different growth curves obtained during competition experiments are illustrated in Figure 13.

I. Competition Studies with Radioactively Labeled [1-^{14}C]Gly-Leu

These studies were performed on cells suspended in the SM/Pro buffer (dextrose = 0.5%) in the presence of leucine and lysine. Table 10 shows the percent inhibition of [1-^{14}C]Gly-Leu uptake exhibited by three leucine peptides. The absolute rate of [1-^{14}C]Gly-Leu uptake varied from one run to another, as observed for the uptake of

Table 8

Competition Studies With Leucine-Containing Non-Growth-Supporting Peptides

Growth Substrate[a]	Competing Peptide[b]	Ratio[c]	Effect[d]
$(Leu)_3$	Gly-Leu-Gly	3:1	None
	Met-Met-Leu	3:1	+++
	Pro-Pro-Leu	3:1	None
	Gly-Leu-Gly-Gly	3:1	None
	Gly-Leu-Gly-Leu	3:2	None
	Ac-$(Leu)_3$	1:1	None
$(Leu)_2$	Gly-Leu-Gly	2:1	None
	Met-Met-Leu	2:1	++
	D-Leu-L-Leu	2:1	None
	L-Leu-D-Leu	2:1	None
	Gly-Leu-Gly-Gly	2:1	None
	Gly-Leu-Gly-Leu	1:1	None
	Leu-Leu-OMe	1:1	None
$(Leu)_2$-OBzl	Met-Met-Leu	2:1	+++

[a] This peptide was supplied in the growth media at a concentration of 0.23mM in leucyl residue equivalents.

[b] The competing peptide did not support the growth of S.cerevisiae Z1-2D when supplied as the sole source of leucine.

[c] The ratio represents the molar concentration of competing peptide relative to the molar concentration of growth substrate in the culture medium.

[d] None- indicates no affect on growth
(+) - indicates that the competing peptide when present at the indicated ratio inhibited the onset of growth from 10 to 25 hours.
(++)- indicates that the competing peptide inhibited the onset of growth for greater than 25 hours but less than 40 hours.
(+++)- indicates no growth during the 40 your test period.

Table 9

Competition Studies in S.cerevisiae Z1-2D

With Peptides Not Containing Leucine[a]

Growth Peptide	Competing Peptide	Ratio	Effect
$(Leu)_2$	$(Met)_2$	1:1	+
	$(Met)_2$	10:1	+++
	$(Met)_3$	1:1	++
	$(Met)_3$	6.3:1	+++
	Ac-$(Met)_3$	10:1	None
	$(Ala)_2$	10:1,(1:1)	None
	$(Ala)_3$	10:1	+
	$(\underline{\underline{D}}\text{-Ala})_3$	10:1	None
	$\underline{\underline{L}}$-Ala-$\underline{\underline{L}}$-Ala-$\underline{\underline{D}}$-Ala	10:1	None
	$(Gly)_2$	10:1	None
	$(Gly)_3$	10:1	None
	$(Glu)_2$	10:1	None
	$(Glu)_3$	10:1	None
	$(Lys)_2$	10:1	None
	$(Lys)_3$	10:1	None
$(Leu)_2$-OBzl	$(Met)_2$	1:1	+
	$(Met)_2$	10:1	+++
	$(Met)_3$	1:1	+++
	$(Met)_3$	10:1	+++
	Ac-$(Met)_3$	10:1	+
	$(Ala)_2$	10:1,(1:1)	None
	$(Ala)_3$	10:1,(1:1)	None
	$(\underline{\underline{D}}\text{-Ala})_3$	10:1	None
	$\underline{\underline{L}}$-Ala-$\underline{\underline{L}}$-Ala-$\underline{\underline{D}}$-Ala	10:1	None
	$(Gly)_2$	10:1	None
	$(Gly)_3$	10:1	None

Table 9 (continued)

Growth Peptide	Competing Peptide	Ratio	Effect
$(Leu)_2$-OBzl	$(Glu)_2$	10:1	None
	$(Glu)_3$	10:1	None
	$(Lys)_2$	10:1	None
	$(Lys)_3$	10:1	None
$(Leu)_3$	$(Met)_2$	1.5:1	++
	$(Met)_2$	15:1	+++
	$(Met)_3$	1:1	+++
	$(Met)_3$	10:1	+++
	Ac-$(Met)_3$	10:1	+
	$(Ala)_2$	10:1	++
	$(Ala)_3$	10:1	++
	$(\underline{D}\text{-Ala})_3$	10:1	None
	$\underline{L}$-Ala-$\underline{L}$-Ala-$\underline{D}$-Ala	10:1	None
	$(Gly)_2$	10:1	None
	$(Gly)_3$	10:1	None
	$(Glu)_2$	10:1	None
	$(Glu)_3$	10:1	None
	$(Lys)_3$	10:1	None
Lys-Gly[e]	$(Met)_2$	1.2:1	+
	$(Met)_2$	11.5:1	+++
	$(Met)_3$	7.6:1	+++
Lys-Leu	$(Met)_2$	2.5:1	+++
	$(Met)_3$	3.3:1	+++

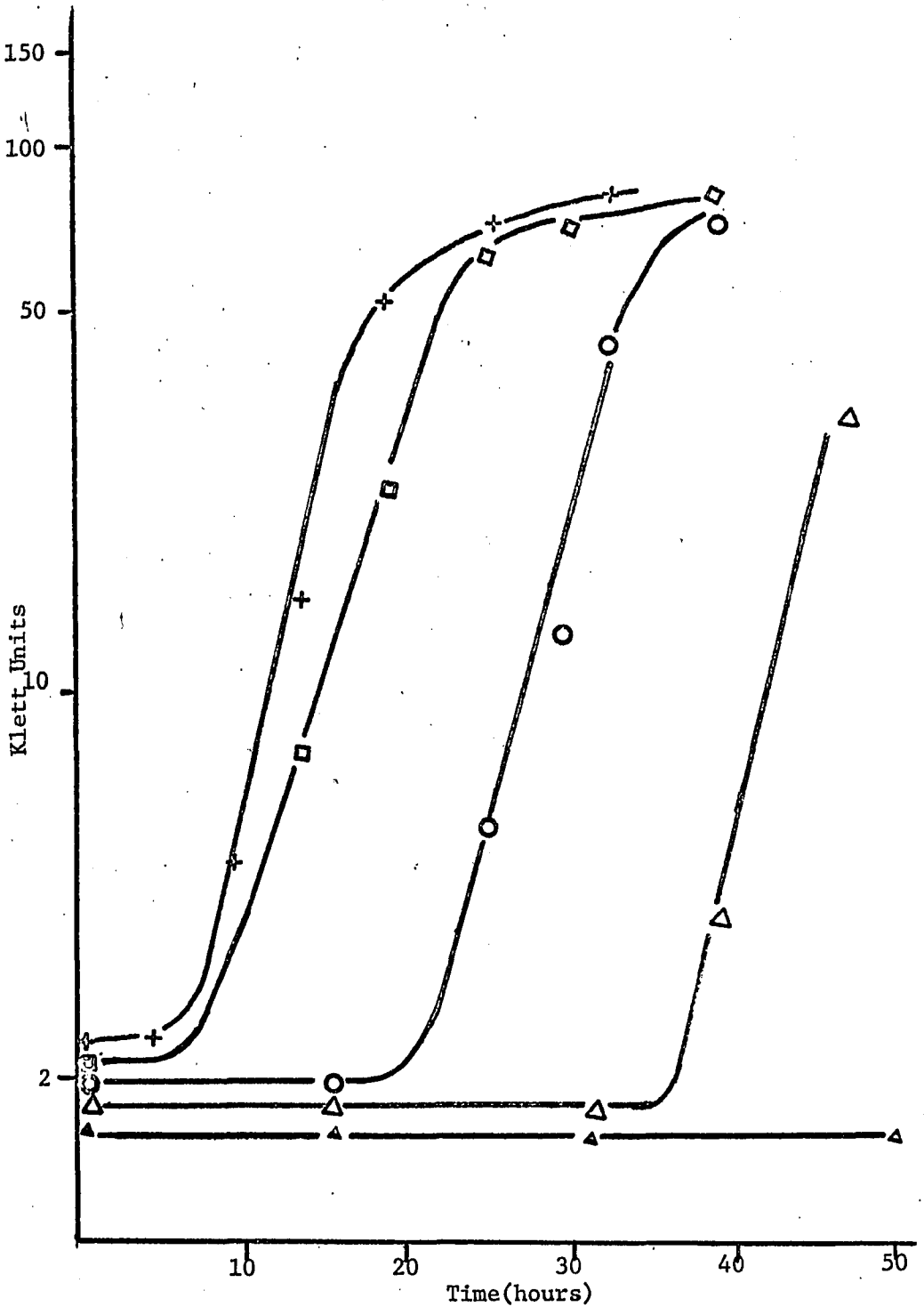
[a] See footnotes to table 8.

[e] This peptide was supplied in the growth medium at a concentration of 0.11mM in lysyl residue equivalents.

Figure 13

Competition studies with S.cerevisiae Z1-2D. Each growth flask contained lysine(0.11mM) and $(Leu)_3$, so as to give a leucine concentration of 0.23mM. Turbidity, in Klett units, was measured for growth in the presence of no competing peptide(✣), and 10-fold molar excesses of $(Gly)_3$(□), Ac-$(Met)_3$(○), $(Ala)_2$(△), and $(Met)_3$(▲).

Figure 13



[U-^{14}C]leucine, and the appropriate control was always included in order to calculate percent inhibition. It is interesting to note that the only peptide that was not inhibitory to the uptake of [1-^{14}C]Gly-Leu, $(Leu)_2$-OMe, was also unable to support growth of Z1-2D.

J. Studies with Toxic Amino Acid Analogues and Peptides Containing These Analogues.

1. As part of the scheme to isolate a peptide transport deficient mutant, it was necessary to obtain a substrate that would be toxic to Z1-2D. First, crystal tests with substrates containing potentially toxic agents were performed. The results of these tests are shown in Table 11. Only ethionine and Eth-Ala are shown to be toxic from these tests.

2. The effects of the above substrates, and other potentially toxic agents were examined also in liquid medium. Table 12 presents the effects of these amino acid analogues and peptides on the growth response of Z1-2D. The normal amounts of leucine and lysine were included in the growth medium with each of the substrates being tested. From Tables 11 and 12 the toxicity of ethionine and ethionine-containing peptides to Z1-2D is evident. The failure of Met-Met-Eth to be toxic is apparently due to the ability of the methionine to specifically inhibit the intracellular effects of its structural analogue. This hypothesis is supported by the fact that free methionine, at a 2:1 molar ratio to ethionine prevents the toxicity of the analogue from being exhibited (Table 12).

3. Competition studies with the toxic agents ethionine, Eth-Ala, and Leu-Leu-Eth were performed. For example, in Figure 14, a 7:1 molar ratio of $(Leu)_2$ to Eth-Ala is able to prevent the analogue from

Table 10

Inhibition of [1-^{14}C]Gly-Leu Uptake in S.cerevisiae Z1-2D by Leucine Peptides

[1-^{14}C]Gly-Leu Concentration	Peptide	Ratio[a]	Percent Inhibition[b]	n[c]
10^{-6}M	$(Leu)_2$-OMe	10:1	0%	1
	$(Leu)_2$-OBzl	10:1	44.3%	1
	$(Leu)_2$	10:1	42.0%	1
10^{-6}M	$(Leu)_2$-OMe	20:1	0%	1
	$(Leu)_2$-OBzl	20:1	54.2%	1
	$(Leu)_2$	20:1	40.8%	1

[a] Molar ratio of unlabeled peptide to [1-^{14}C]Gly-Leu in the assay medium

[b] $$\% \text{ inhibition} = \frac{\text{Rate [1-}^{14}\text{C]Gly-Leu Uptake} - \text{Rate [1-}^{14}\text{C]Gly-Leu Uptake With Leucine Peptide}}{\text{Rate [1-}^{14}\text{C]Gly-Leu Uptake}} \times 100$$

[c] Number of determinations.

Table 11

Crystal Tests on S.cerevisiae Z1-2D

With Amino Acid and Peptide Analogues [a]

Amino Acid Analogue	Growth Response
norvaline	+
ethionine	-

Peptide Analogue	Growth Response
Gly-Gly-Nle[b]	+
$(Orn)_3$	+
Nle-Gly-Gly	+
Nle-Ala	+
Gly-Nva[b]	+
Gly-Nle	+
Nva-Gly-Gly	+
Eth-Ala	-

[a] Approximately 10^6 cells were applied to SM+Lys+Leu plates. A small crystal of each analogue was placed on the surface of the plate and allowed to incubate at 30°C for two days. Inhibition of growth, as indicated by a void area(no growth) about the point where the crystal was applied, is designated in this table by a (-) symbol. A (+) symbol indicates normal growth in the area where the crystal was applied.

[b] Nle= norleucine, Nva= norvaline

Table 12

Growth Response of S.cerevisiae Z1-2D

To Amino Acid and Peptide Analogues[a]

Amino Acid Analogue	Concentration	Growth Response
norleucine	1.16mM	+
ethionine	0.23mM	-
ethionine + methionine	0.23mM 0.46mM	+
β-alanine	0.23mM, 2.3mM	+
ε-aminocaproic acid	0.23mM, 2.3mM	+
D-leucine	0.23mM	+
D,L-p-fluorophenyl-alanine	0.23mM, 1.16mM	-

Peptide Analogue	Concentration	Growth Response
Met-Met-Eth	0.23mM, 1.16mM	+
Pro-Pro-D,L-p-F-Phe	0.23mM, 1.16mM	+
Met-Met-D,L-p-F-Phe	0.23mM, 1.16mM	+
Eth-Ala	0.23mM	-
Leu-Leu-Eth	0.23mM	-

[a] Growth as determined by an increase in turbidity(Klett units) over a 40 hour test period. Leucine(0.23mM) and lysine(0.11mM) are present in each growth flask. A (+) symbol indicates growth equivalent to that obtained with leucine, lysine and no analogue. A (-) symbol indicates no growth over the 40 hour test period.

exhibiting its toxic effect. However a 14:1 molar ratio of $(Leu)_2$ to ethionine has no such effect. (Note that, as indicated in Figure 14 and in the experimental section, the mutant strain Z1-2D Eta^r was subsequently isolated from the cells of the competition experiment between Eth-Ala and $(Leu)_2$.) In other studies, a 7:1 molar ratio of $(Leu)_3$ to Leu-Leu-Eth, or Eth-Ala was shown to prevent either analogue from being toxic to Z1-2D. Therefore these peptide analogues most likely enter the yeast through the same system as the peptides $(Leu)_2$ and $(Leu)_3$. The results are consistent with those obtained from the competition studies with methionine peptides detailed in Table 9. They suggest that $(Leu)_2$ and $(Leu)_3$ compete with Eth-Ala and Leu-Leu-Eth for entry into Z1-2D in the same manner as $(Met)_2$ and $(Met)_3$ inhibit growth on these leucine peptides - i.e. by a specific inhibition of the transport of the toxic analogue or growth substrate.

K. Peptidase Assays with Z1-2D Cell Extract.

1. The ability of the cell extract of Z1-2D to hydrolyze non-growth substrates was examined. Several peptides that are good growth substrates, such as $(Leu)_3$ and Leu-Gly, establish a base line for the release of leucine in these assays. All of the non-growth-supporting peptides showed release of free leucine in the assay mixture. In Figure 15 the hydrolysis of Leu-Gly, Gly-Leu, $(Leu)_3$ and Gly-Leu-Gly by Z1-2D cell extract (3.8 mg protein/ml) is compared. This schematic representation of the results of the high voltage paper electrophoresis chromatogram demonstrates that leucine is released from the intermediate (Gly-Leu) and non-growth substrates (Gly-Leu-Gly) as readily as from the good growth substrates [$(Leu)_3$ and Leu-Gly]. After a two hour incubation, all four substrates were completely

Figure 14

Growth of S.cerevisiae Z1-2D in the presence of ethionine, Eth-Ala, and $(Leu)_2$. Each growth flask contained lysine (0.11mM). Ethionine or Eth-Ala was supplied so that the ethionine content was 0.23mM of the free amino acid. Turbidity in Klett units was determined for $(Leu)_2$(⊡), leucine + Eth-Ala + $(Leu)_2$ at a 7-fold molar excess to Eth-Ala(✥), and leucine + ethionine, or leucine + Eth-Ala, or ethionine + $(Leu)_2$ at a 14:1 molar excess of $(Leu)_2$ to ethionine(Δ). The arrow represents the point in the growth experiment with Eth-Ala and $(Leu)_2$ where an aliquot of cells at 45 hours was removed and used to isolate Z1-2D Eta^r, as described in Figure 2.

Figure 14

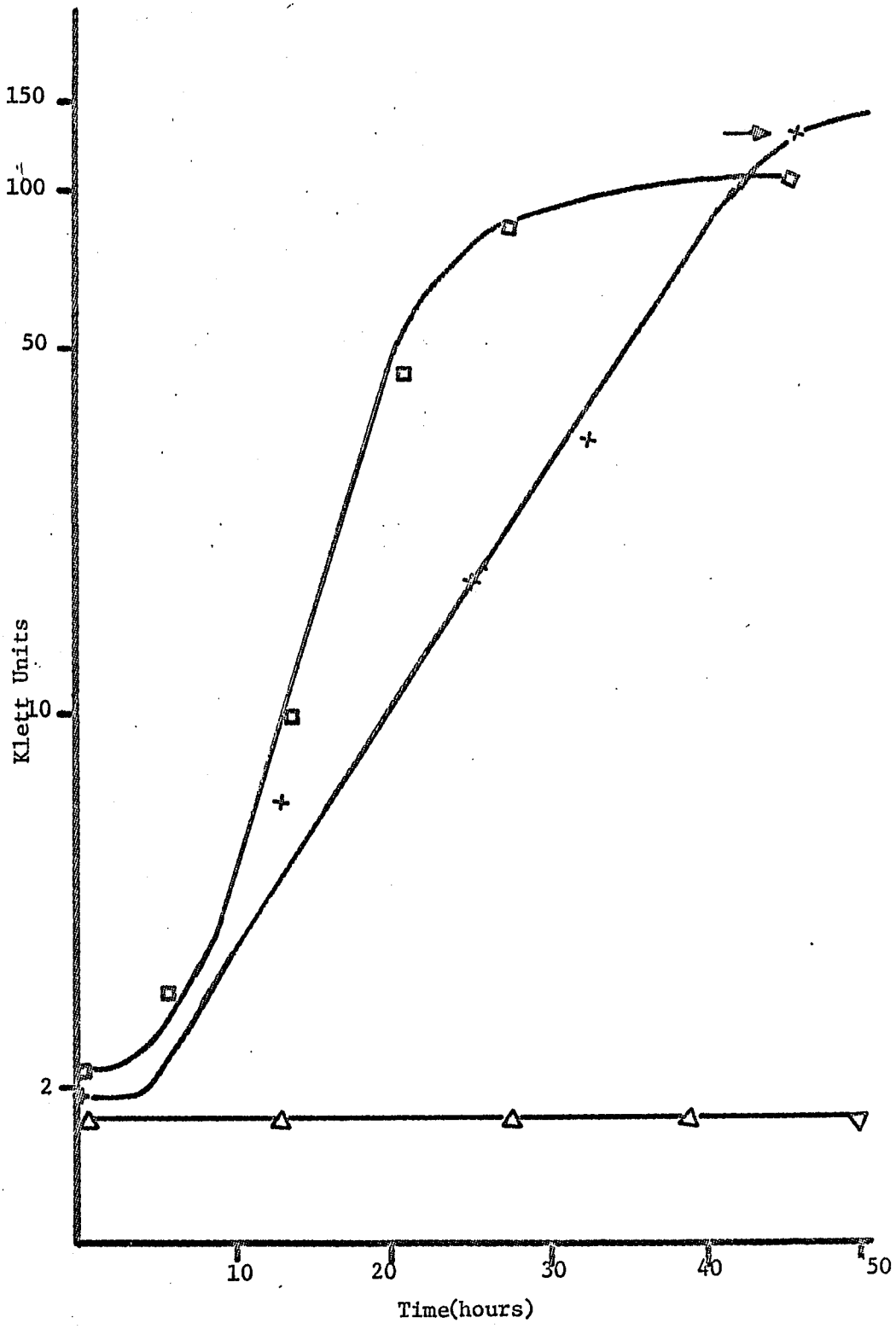
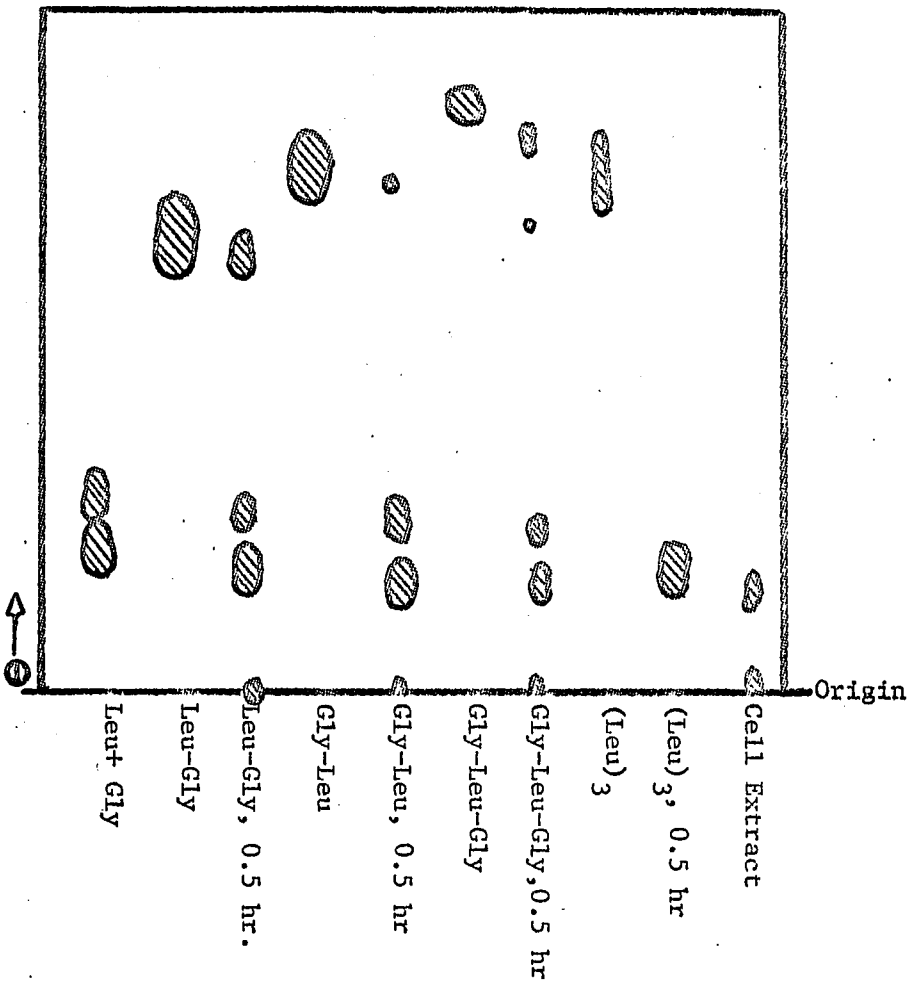


Figure 15

Hydrolysis of Peptides by Z1-2D Cell Extract[a]



[a] Schematic representation of a high voltage paper electrophoresis chromatogram of 0.5 hour incubation mixtures of a cell extract from Z1-2D and $(Leu)_3$, Gly-Leu-Gly, Gly-Leu, and Leu-Gly. Markers are represented by Leu + Gly, Leu-Gly, Gly-Leu, Gly-Leu-Gly and $(Leu)_3$. The assays were performed at 30°C and the chromatogram run at pH 3.5 for 3.5 hours at 3000 volts.

hydrolyzed. Other assays with lysine peptides showed that all such substrates that were non-growth supporting were cleaved by the cell extract to release lysine. Several non-growth substrates (Pro-Pro-Leu, L-Leu-D-Leu, D-Leu-L-Leu and Pro-Leu-Gly-NH_2 were hydrolyzed slowly as judged by the presence of undergraded peptide in the incubation mixture after 24 hours. The toxic analogue Eth-Ala is rapidly degraded by the cell extract as noted by the absence of a peptide spot after a 0.5 hour incubation with Z1-2D cell extract.

2. Assays were performed to measure the hydrolysis of growth essential peptides in the presence of a non-essential peptide. In Table 13, we see that several peptides used in growth competition experiments led to an increase in the time required for the complete hydrolysis of $(Leu)_2$. However both $(Met)_2$, a growth competitor, and $(Ala)_2$, a non-competitor, caused the same increase in the hydrolysis time of $(Leu)_2$. Thus, the effect of the competing peptide on the hydrolysis of $(Leu)_2$ is independent of the ability of the peptide to compete in the growth experiment. In another assay a nearly 1:1 ratio of $(Met)_3$ to $(Leu)_3$, conditions which produced a complete inhibition of growth on $(Leu)_3$ in liquid culture, did not prevent the hydrolysis of this growth substrate. Both $(Met)_3$ and $(Leu)_3$ were cleaved completely within two hours when the concentration of cell extract in the assay medium was 0.76 mg protein/ml. Peptidase assays on Z1-2D Eta^r cell extract will be presented separately.

L. Characterization of the Growth Response of Z1-2D Eta^r to Leucine and Lysine Peptides.

As indicated in the experimental section, the mutant Z1-2D Eta^r was isolated on the basis of its simultaneous resistance to the toxic

Table 13

Hydrolysis by S.cerevisiae Z1-2D Cell Extract of $(Leu)_2$ in the Presence of $(Met)_2$ or $(Ala)_2$[a]

Peptide Substrate	Protein Concentration[b]	Competing Substrate	Ratio[c]	Hydrolysis Time[d]
$(Leu)_2$	0.085	None	-	3.5
$(Leu)_2$	0.085	$(Met)_2$	10:1	6.0
$(Leu)_2$	0.139	$(Ala)_2$	10:1	6.0

[a] The cell extract and peptide (2 mg/ml), with or without competing substrate, were incubated at 30°C. At 0, 0.5, 1.5,3.5,6 and 72 hours, portions were removed, spotted on paper and electrophoresed.

[b] mg/ml of assay mixture.

[c] The ratio represents the molar concentration of competing peptide relative to the molar concentration of peptide substrate present in the incubation mixture.

[d] The hydrolysis time is the time, in hours, for the complete hydrolysis of the peptide substrate as judged by the absence of a ninhydrin positive spot at the R_f of the substrate.

effects of Eth-Ala and to growth on $(Leu)_2$. To further characterize the properties of the mutant, its growth response to leucine and lysine peptides was measured. In Table 14, the growth characteristics of Z1-2D and Z1-2D Eta^r are compared. The mutant grows well on leucine and lysine with the same generation time, lag phase and maximum cell density at 40 hours as the parent strain. It maintains its growth requirement for both amino acids. However it does not grow on $(Leu)_2$, $(Leu)_3$, $(Leu)_2$-OBzl, or Lys-Gly-Gly. The mutant also resists the toxic effects of Leu-Leu-Eth. Crystal tests summarized in Table 15 show that nine peptides that are growth substrates for Z1-2D do not support growth of Z1-2D Eta^r. Therefore the mutant is resistant to growth on all peptides examined. The mutant Z1-2D Eta^r is not a petite strain, as judged from its positive growth response to plates containing glycerol (2.4% v/v), glucose (.025%), yeast extract (1%), peptone (1%), and agar (2%).

M. Uptake of Radioactively Labeled Amino Acids and Peptides by Z1-2D and Z1-2D Eta^r

These studies were performed on growing cells in SM/Pro medium in the presence of leucine and lysine (and 0.5% dextrose). The uptake of [1-^{14}C]-Gly-Leu and Met-Met[1-^{14}C]-Met by Z1-2D was linear over the 40 minute assay period. [1-^{14}C] Methionine uptake was measured simultaneously with the uptake of [1-^{14}C]-Gly-Leu to serve as a control for the mutant cells' viability under the conditions of assay. Since the uptake of [1-^{14}C]methionine by Z1-2D and Z1-2D Eta^r was not linear over the 40 minute assay period, rates are calculated from the initial data points during the first 10-15 minutes of the assay by linear regression analysis. In Table 16 the rates

Table 14

Growth Response of S.cerevisiae Z1-2D and Z1-2D Eta^r to Peptides and Amino Acids[a]

Peptide	Amino Acids	Z1-2D	Z1-2D Eta^r
-	leucine + lysine	144	143
-	leucine	2	2
-	lysine	2	2
$(Leu)_2$	lysine	110	2
$(Leu)_3$	lysine	103	2
$(Leu)_2$-OBzl	lysine	131	10
Lys-Gly-Gly	leucine	131	2
-	leucine + lysine + ethionine	2	2
Eth-Ala	leucine + lysine	4	154
Leu-Leu-Eth	leucine + lysine	2	152

[a] Growth as determined by an increase in turbidity(Klett units) over a 40 hour test period. Klett values are normalized to an initial reading of 2. Leucine, ethionine, and related peptides are supplied in the liquid media at an equivalent leucine concentration of 0.23mM, lysine and Lys-Gly-Gly at an equivalent lysine concentration of 0.11mM.

Table 15

Crystal Tests Measuring the Growth Response of S.cerevisiae Z1-2D and Z1-2D Eta^r to Leucine and Lysine Peptides.[a]

Plate	Peptide	Z1-2D Growth Response	Z1-2D Eta^r Growth Response
SM + Lys	Leu-Ala	+	-
	Ala-Leu	+	-
	Leu-Gly	+	-
	Leu-Leu-Gly	+	-
	Val-Leu	+	-
	Leu-Val	+	-
	Leu-Pro	+	-
SM + Leu	Lys-Gly	+	-
SM	Lys-Leu	+	-

[a] Approximately 10^6 cells of a stationary phase culture of either Z1-2D or Z1-2D Eta^r are uniformly applied to each of the above plates. A small crystal of each peptide is placed on the appropriate plate, and the point of application noted. After two days incubating at 30°C, the plates are scored. A (+) symbol indicates a region of growth around the area where the crystal was placed. A (-) symbol indicates that no growth was noted.

Table 16

Uptake of [1-^{14}C]methionine, [1-^{14}C]Gly-Leu And Met-Met-[1-^{14}C]Met by Z1-2D and Z1-2D Eta^{r}

Strain	Substrate	Concentration	Rate[a]	n[b]
Z1-2D	[1-^{14}C]methionine	10^{-6}M	0.120	2
Z1-2D Eta^{r}	[1-^{14}C]methionine	10^{-6}M	0.076	2
Z1-2D	[1-^{14}C]Gly-Leu	10^{-6}M	0.0105	2
Z1-2D Eta^{r}	[1-^{14}C]Gly-Leu	10^{-6}M	0.00[c]	2
Z1-2D	Met-Met-[1-^{14}C]Met	10^{-6}M	0.054	2
Z1-2D Eta^{r}	Met-Met [1-^{14}C]Met	10^{-6}M	0.00[c]	2
Z1-2D	Met-Met-[1-^{14}C]Met	10^{-5}M	0.233	2
Z1-2D Eta^{r}	Met-Met-[1-^{14}C]Met	10^{-5}M	0.00[c]	2

[a] nmole/mg cells-minute

[b] number of determinations

[c] no increase in counts during the assay period.

for the uptake of all three labeled substrates are presented. Although the initial rates indicate that Z1-2D Eta^r accumulates [1-^{14}C]methionine at a slightly lower rate than Z1-2D, the total amount of labeled substrate taken up by both strains at the end of the 40 minute assay (Figure 16) is almost identical. However under identical conditions of assay, Z1-2D takes up [1-^{14}C]Gly-Leu, and Met-Met-[1-^{14}C]Met (at 10^{-6}M, and 10^{-5}M), but Z1-2D Eta^r does not take up either of these labeled peptides. The uptake of both labeled substrates by Z1-2D is illustrated in Figure 17. It is interesting to note that Met-Met-[1-^{14}C]Met is taken up at a substantially higher rate than the same concentration of [1-^{14}C]Gly-Leu. $(Met)_3$ was a very strong competitor of leucine peptides in growth experiments, (Table 9) and Gly-Leu was only an intermediate growth substrate (Table 3). The uptake rate for [1-^{14}C]methionine at 10^{-6}M (0.120 nmole/mg-min) is nearly the same as the rates reported in Table 6 for the uptake of the same concentration of [U-^{14}C]leucine (0.114 nmole/mg-min).

N. Peptidase Activity of Z1-2D Eta^r Cell Extract and Comparison with Z1-2D Cell Extract.

A fresh supply of parent cell extract and mutant cell extract were prepared. Phosphate buffer was used in the dialysis step because Tris-HCl would interfere in the quantitative fluorescence peptidase assay. The cell extracts of mutant and parent strains were much more dilute than the original preparation of parent extract (Z1-2D CE = 0.39 mg/ml, Z1-2D Eta^r = 0.30 mg/ml).

The ability of both mutant and parent extracts to hydrolyze dileucine and trileucine was examined. In Figure 18 the equivalent hydrolysis of dileucine by mutant and parent extracts is demonstrated.

Figure 16

Uptake of [1-^{14}C]methionine in Z1-2D and Z1-2D Eta^{r}. The concentration of substrate in the SM/Pro assay medium is 10^{-6}M. Each curve represents data for two experiments measuring uptake in Z1-2D(✣) and Z1-2D Eta^{r}(□).

Figure 16

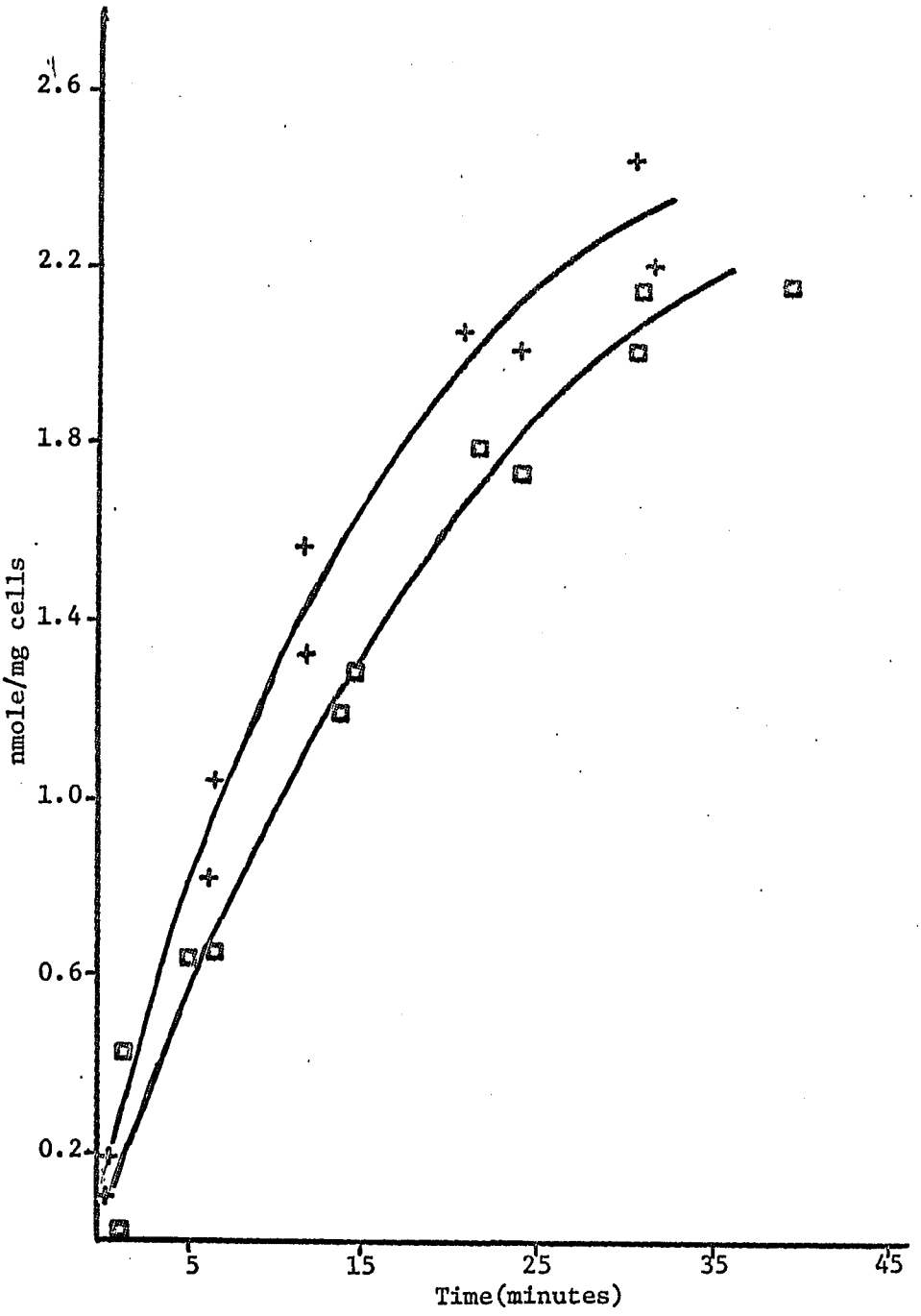
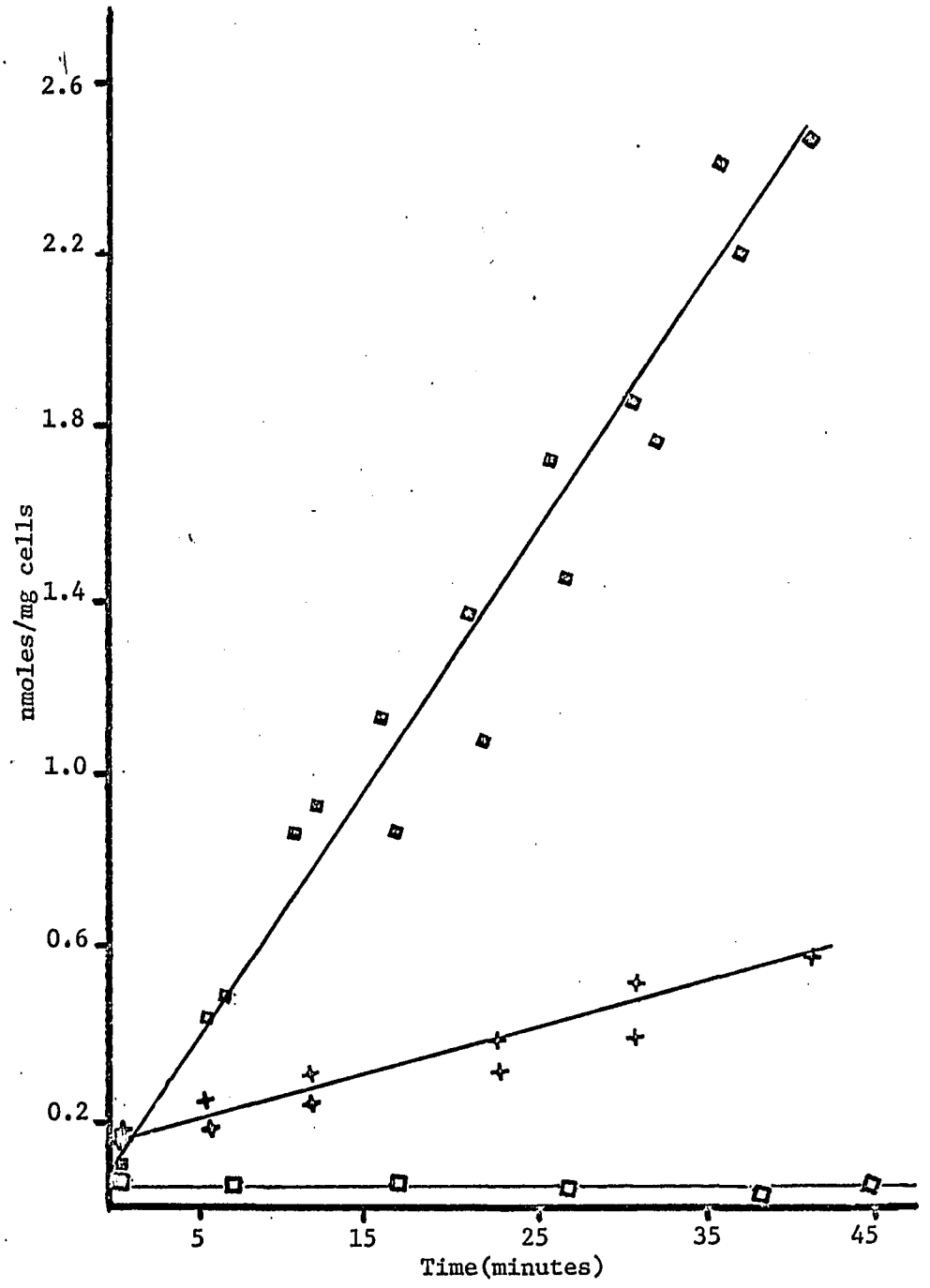


Figure 17

Uptake of Met-Met-[1-^{14}C]Met and [1-^{14}C]Gly-Leu in Z1-2D and Z1-2D Eta^{r}. All substrates are supplied in the SM/Pro assay medium at 10^{-6}M. Each curve represents data from two experiments measuring the uptake of Met-Met-[1-^{14}C]Met in Z1-2D(●), [1-^{14}C]Gly-Leu in Z1-2D(▼), and Met-Met-[1-^{14}C]Met or [1-^{14}C]Gly-Leu in Z1-2D Eta^{r}(■).

Figure 17



A similar chromatogram revealed that the hydrolysis of $(Leu)_3$ by both extracts was equivalent.

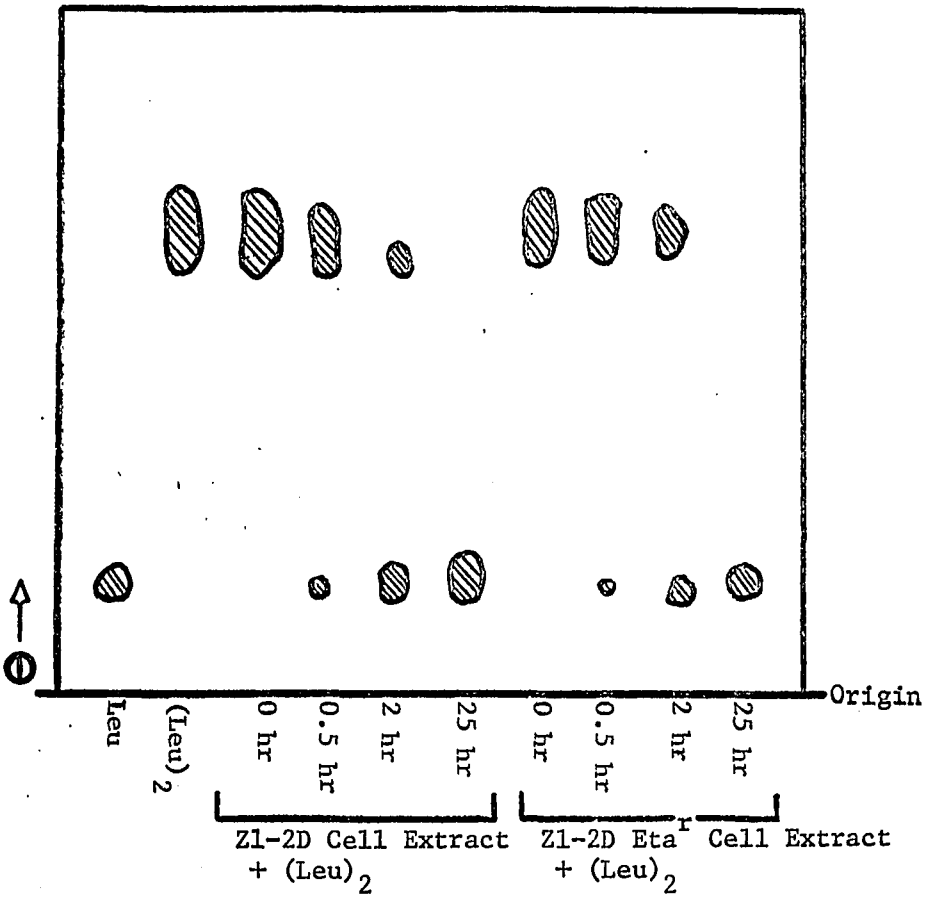
A quantitative measure of the peptidase activity of both extracts against $(Leu)_2$ was provided by the fluorescence assay method, described earlier in the experimental section. The mutant's specific activity for hydrolyzing dileucine was equal to the release of 0.10 µmole L-leucine per minute per mg protein. This was only about ½ the rate at which the parent extract cleaves this peptide (0.26 µmole L-leucine released/min per mg protein). This reduced rate, however, is much higher than the rate at which L-lysyl-L-leucine, a good growth substrate for the parent, is hydrolyzed by the parent cell extract. Therefore Z1-2D Eta^r has the enzymatic activity to cleave $(Leu)_2$, and $(Leu)_3$ although these peptides are unable to serve as growth substrates.

O. Revertant Frequency Analysis of Z1-2D Eta^r

Viable counting procedures determined that 4.68×10^6 cells of Z1-2D Eta^r were pipetted into SM + $(Leu)_2$ and SM + $(Leu)_3$ plates. After 4 days at 30°C, 21 and 20 revertant colonies had appeared on the dileucine and trileucine plates, respectively. Each revertant colony demonstrated the identical growth characteristics of Z1-2D: requirements for lysine and leucine, sensitivity to ethionine and Eth-Ala, and growth on $(Leu)_2$ [for an SM + $(Leu)_3$ revertant, or growth on $(Leu)_3$ for the SM + $(Leu)_2$ revertant]. The frequency of revertants was calculated to be 4.3×10^{-6}.

Figure 18

Hydrolysis of $(Leu)_2$ by Cell Extracts of Z1-2D and Z1-2D Eta^r (a)



(a) Schematic representation of a high voltage paper electrophoresis chromatogram of the hydrolysis products of $(Leu)_2$ by Z1-2D and Z1-2D Eta^r cell extracts. Markers are represented by Leu(0.100mg) and $(Leu)_2$(0.156mg). Each time point represents 50 µl of a 50:50 assay mixture of $(Leu)_2$(6.5 mg/ml) and cell extract. Protein concentrations of the cell extracts were 0.39 mg/ml for Z1-2D and 0.30 mg/ml for Z1-2D Eta^r. Samples were incubated at 30°C, spotted on the chromatogram, and electrophoresed at pH 3.5 for 3 hours at 3000 volts.

IV. DISCUSSION

The ability of a lysine/leucine auxotroph of S. cerevisiae to transport peptides has been demonstrated using several techniques. Indirect evidence was provided by growth studies. A variety of leucine peptides and several lysine peptides supplied strain Z1-2D with amino acids necessary for growth. Other peptides not containing leucine or lysine inhibited the growth of the yeast on the peptide growth substrates. Peptides containing toxic amino acid analogues exhibited the same lethal effect as their unconjugated counterparts. Before each of the above criteria is discussed in detail, it is pertinent to review the arguments that validate the use of this indirect evidence as proof of a peptide transport process in Z1-2D.

There are no extracellular peptidases in S. cerevisiae capable of acting on the substrates examined. One would expect based on studies of the broad specificities of microbial peptidases[26] that if extracellular peptidases were involved in the hydrolysis of Leu-Gly, Leu-Gly-Leu and Leu-Leu-Gly (all good growth substrates), they would also cleave Gly-Leu-Gly, Gly-Leu-Gly-Gly and Gly-Leu-Gly-Leu (all non-growth substrates). Other evidence that argues against extracellular hydrolysis in Z1-2D is the results of the competition studies with phenylalanine and methionine. Each of these amino acids showed the ability to inhibit growth of Z1-2D on leucine, but not on $(Leu)_2$ or $(Leu)_3$. If free leucine were produced in the growth media, the competing amino acid would be able to inhibit growth due to competition for the amino acid transport system.[80] Investigators[11,53] examining other S. cerevisiae strains with the use of osmotic shock methods were unable to show the presence of any peripheral peptidase

activity in the organisms tested. Claims[30,49] that extracellular peptidase activity exists in S. cerevisiae have several limitations. In these studies no activity has been measured against free peptides, only aminoacyl-β-naphthalamides and aminoacyl-p-nitroanilides. The exact localization of purified enzyme is unknown. Furthermore, the use of snail gut enzymes in spheroplast preparation allows for the distinct possibility that any measured hydrolysis results from the activity of contaminating peptidases from the snail gut preparation. Therefore it is likely that peptides are not hydrolyzed externally in Z1-2D.

The second aspect of the growth studies to be clarified is the mutual independence of peptide transport and peptidase activity. Growth experiments can be correlated with transport only when the assumption that 'sufficient' peptidase activity exists for the release of amino acids required for growth from all substrates is valid. Figure 15 demonstrates in a qualitative manner that an intermediate growth substrate (Gly-Leu) and non-growth substrate (Gly-Leu-Gly) are hydrolyzed as rapidly as $(Leu)_3$ and Leu-Gly, which are good growth substrates. The quantitative hydrolysis of peptides by Z1-2D cell extract was measured using the o-phthaldehyde procedure. These studies showed that Leu-Gly, a good growth substrate is hydrolyzed 4 times slower than Gly-Leu (an intermediate growth substrate). Also, Lys-Leu, another good growth substrate, was cleaved 20 times more slowly than Gly-Leu. (B. Rose, unpublished data.) Thorough qualitative in vitro assays performed on non-growth-supporting peptides showed the release of leucine or lysine in every such substrate examined. The peptidase activities measured on the mutant extract by a qualitative assay

(Figure 18) and by quantitative assays present the most compelling argument for the independence of hydrolysis and transport. It is clear that the mutant strain does not accumulate peptides, but retains the ability to hydrolyze substrates at nearly the same rate as the parent organism. Therefore peptide transport and hydrolysis are distinctly independent processes in strain Z1-2D.

It can be concluded that a positive growth response is evidence of peptide transport into the cell. In addition, a negative growth response indicates the failure of the substrate to be transported into the cell, or at least a low affinity for the system that brings the peptide to the site of peptidase activity inside the cell. An exception to this latter conclusion would exist if a peptide was transported, but then toxic to the yeast. However Tables 4 and 5 present data that eliminates this possibility. Specifically, all non-growth substrates allow growth in the presence of the required amino acids.

The third assumption behind the interpretation of the results of growth experiments is that in competition studies the competition occurs at the point of uptake, and not at the level of hydrolysis. Table 13 presents qualitative evidence from <u>in vitro</u> peptidase assays that suggests that hydrolysis rates are not the limiting factor in growth inhibition experiments with leucine peptides. Both $(Met)_2$, a competitor, and $(Ala)_2$, a non-competitor, equally increased the hydrolysis time for $(Leu)_2$. Therefore, growth inhibition in the competition experiment results from competition for the peptide transport system in this organism. As Figure 13 indicates, all cases where inhibition was observed, it was reflected as an increase in the lag

time of the yeast auxotroph. Similar findings of increased lag times have been observed in peptide competition studies with bacteria. This phenomenon has been extensively discussed in the literature.[60] The increased lag is due to competition at the level of transport. The competing peptide is transported and hydrolyzed by intracellular peptidases. After the concentration of competing peptides is depleted by this process, the peptide containing the required amino acid can enter, and growth commences. Therefore the validity of the growth studies and competition experiments to measure peptide transport is evident.

An important result of these studies is that the system that transports peptides into the cell operates independently of the system used for the uptake of amino acids. Several observations support this conclusion. The transport of amino acids by Z1-2D has been characterized by growth studies and experiments with labeled substrates. In the growth experiments, phenylalanine and methionine inhibit the growth of Z1-2D on leucine. The ability of phenylalanine and methionine to utilize the same uptake system in another strain of S. cerevisiae has been demonstrated by isolating mutants in a single step that were resistant to ethionine, and simultaneously, to the phenylalanine analogue p-F-phenylalanine.[73] The growth studies with Z1-2D demonstrate that phenylalanine and methionine do not inhibit growth on any leucine peptide examined (Figures 9,11). Other studies with Z1-2D showed that $(Met)_2$ and $(Met)_3$ did not inhibit growth on leucine. Uptake studies with [U-^{14}C]leucine demonstrate that phenylalanine and methionine inhibit growth on leucine by competing with the amino acid for transport into the yeast. In Table 7, the data

reveals that both amino acids inhibit [U-^{14}C]leucine uptake 65-88%, depending on the substrate concentration and inhibitor. The rates of uptake for [U-^{14}C]leucine (Table 6) and [1-^{14}C]methionine (Table 16) by Z1-2D at 10^{-6}M are similar despite the different assay conditions. Studies with the toxic analogue ethionine also indicate that uptake of peptides and amino acids occurs by separate processes. In Figure 14 the toxicity of Eth-Ala is prevented by a 7:1 molar ratio of $(Leu)_2$ to peptide analogue in the growth media, but a 14:1 molar ratio of $(Leu)_2$ to ethionine can not inhibit the toxicity of the amino acid analogue. Therefore the ability of $(Leu)_2$ to compete with Eth-Ala for entry into Z1-2D, but not with ethionine is a strong indication of the different transport mechanisms operating for amino acids and peptides. The most convincing evidence regarding the individuality of amino acid and peptide uptake is the result of studies with Z1-2D Eta^r. This strain, grows normally on leucine and lysine. It is as sensitive to ethionine as the parent strain. It accumulates [1-^{14}C]methionine at nearly the same rate as Z1-2D (Figure 16). However Z1-2D Eta^r does not utilize any leucine or lysine peptides that were growth substrates for the parent strain (Table 14, 15), and resists the lethal effects of Leu-Leu-Eth and Eth-Ala. The mutant does not take up [1-^{14}C]Gly-Leu or Met-Met-[1-^{14}C]Met, in contrast to Z1-2D. Therefore the mutant, deficient in the transport of every peptide substrate examined, retains its ability to grow on amino acids, and to transport similarly labeled substrates. Therefore the existence of individual uptake processes for peptides and amino acids in Z1-2D is clearly demonstrated. There is a considerable amount of data that suggests that the peptide transport system in Z1-2D

recognizes both dipeptides and tripeptides. From competition studies in growth experiments (Table 9), both $(Met)_2$ and $(Met)_3$ inhibit growth on $(Leu)_2$, $(Leu)_3$, and $(Leu)_2$-OBzl. $(Met)_3$ inhibited growth on Lys-Gly and Lys-Leu. $(Ala)_3$ inhibited growth on $(Leu)_2$ and $(Leu)_3$. These results indicate a common system of entry for both dipeptides and tripeptides. Studies with the toxic peptides also show the propensity of dipeptides and tripeptides for one uptake system. In addition to Figure 14 illustrating the competition between $(Leu)_2$ and Eth-Ala for uptake into Z1-2D, other evidence not shown reveals that $(Leu)_3$ had equal ability to prevent the toxicity of Eth-Ala and Leu-Leu-Eth from being exhibited. The studies with Z1-2D Eta^r show that it simultaneously lost the ability to grow on dipeptides and tripeptides, and to take up the label from [1-^{14}C]Gly-Leu, or Met-Met-[1-^{14}C]Met. Since this mutant was isolated by a one step selection procedure, these results are evidence that all these peptides shared a common uptake system. (The argument for a single step mutation will be presented later on.)

The affinity of dipeptides and tripeptides for a single transport system in S. cerevisiae is in distinct contrast to the previously cited studies in E. coli,[9] and other evidence in P. putida,[16] S. cremoris[43] and S. typhimurium[87] implicating separate systems in the uptake of these two classes of substrates. However other tissues and organisms of greater complexity show that dipeptides and tripeptides share a 'general' oligopeptide transport system. For example, in the germinating barley scutellum[72] Gly-Sar and Gly-Sar-Sar both compete with each other, and a variety of other oligopeptides for transport. A recent study with the barley embryo system[36] revealed that alanine

peptides of four and five residues competed for uptake by this general peptide transport system. In hamster[1] and mammalian[2] jejunum, studies with the labeled sarcosine peptides alluded to above, and with glycine peptides demonstrated that both dipeptides and tripeptides exhibit an affinity for a common mode of transport. Therefore this phenomena, characterizing peptide transport in Z1-2D, represents an important criteria distinguishing procaryotic and eucaryotic cells.

It is clear that the growth studies and uptake assays give valuable information about the structural specificity of the general peptide transport system in Z1-2D. These results are important in implicating a carrier-mediated uptake process for these substrates.

Most lysine peptides are not substrates for the peptide transport system in Z1-2D. Only 3 peptides, Lys-Gly, Lys-Leu and Lys-Gly-Gly could supply the cell with lysine needed for growth. The growth-supporting lysine dipeptides share the same uptake system as do leucine peptides, as evidenced by the equivalent ability of $(Met)_2$ and $(Met)_3$ to inhibit growth on all of these compounds (Table 9). The other lysine peptides examined [$(Lys)_n$, n = 2-5] are not growth substrates. Neither $(Lys)_3$ nor $(Lys)_2$ is able to inhibit growth on $(Leu)_2$, $(Leu)_3$ or $(Leu)_2$-OBzl. These results in Z1-2D are consistent with evidence from other yeasts. Three other S. cerevisiae strains[11] were not able to use lysine-containing peptides. In another strain of S. cerevisiae[12] $(Lys)_3$ did not inhibit the uptake of labeled $(Met)_3$ under conditions where the same concentration of $(Leu)_3$ inhibited uptake of the labeled peptide 80%. Also, in the fungus C. albicans[46] most lysine peptides tested were unable to support growth of this lysine auxotroph. The low affinity of these substrates for the peptide transport systems in

S. cerevisiae and C. albicans is in sharp contrast to the specificity for transport in the procaryote E. coli. In the bacteria, the Opp system responsible for the uptake of a wide variety of oligopeptides in E. coli shows a high affinity for lysine peptides.[58] Hydrophobic peptides appear to have the highest affinity for the peptide transport system in Z1-2D. Competition studies with non-growth essential peptides provide the most thorough evidence for this concept. Table 9 shows that methionine peptides are powerful inhibitors of growth on the leucine or lysine peptides. Methionine peptides are more effective competitors than are alanine peptides, which are still more effective than the glycine peptides. Lysine peptides and glutamic acid peptides are without any inhibitory effect on leucine containing peptides. Therefore the ability of these non-essential peptides to inhibit growth on leucine or lysine peptides, and thus compete for entry into the cell by the peptide transport system, appears to be greatest for these peptides with large hydrophobic side chains. This observation appears to be consistent with the aforementioned evidence that Met-Met-[1-^{14}C]Met is taken up at a greater rate into Z1-2D than [1-^{14}C]Gly-Leu. The general preference for hydrophobic substrates is indicated in Table 3. If one eliminates from consideration non-growth-supporting peptides with blocked amine or carboxyl termini, peptides with D-residues, or peptides with greater than three residues (all cases which will be examined in detail later on), the remaining non-growth peptides have a) glycine residues at two or more positions of the peptide, or b) lysine residues at the carboxyl terminus of the peptide. Lys-Gly-Gly is the only growth substrate or competitor having two glycyl residues present. In fact growth on this

peptide is interesting in that its more hydrophobic counterpart, Leu-Gly-Gly is a non-growth substrate. Pro-Pro-Leu and Met-Met-Leu are two hydrophobic peptides whose inability to support growth is puzzling. Therefore each of these peptides will be discussed separately. Nevertheless the case for a general peptide transport system with a high affinity for hydrophobic peptides is valid. The affinity of the transport system of Z1-2D for hydrophobic peptides is consistent with studies on other strains of S. cerevisiae.[11,12] In this regard, its specificity resembles the Opr I system of E. coli,[9] the restrictive system for oligopeptide transport mediating in the uptake of substrates such as $(Met)_3$ and $(Leu)_3$.

Evidence suggests that the sequence of amino acids in a peptide may determine the affinity for transport. For example, according to the data in Table 3, Leu-Gly is a good growth substrate, Gly-Leu an intermediate substrate. The same observation is true for Leu-Pro and Pro-Leu. Similarly Lys-Gly is a good growth substrate, but Gly-Lys, a non-growth-supporting peptide. Other studies with S. cerevisiae[12,53] strains showed that the growth response to methionine peptides depended on the peptide sequence and the ability of peptides to compete with $(Met)_3$ into the yeast depended on the amino acid sequence and composition. As indicated above, two dipeptides containing proline are growth substrates, suggesting that peptides composed of imino acids are transported. However Pro-Pro-Leu and Pro-Leu-Gly-NH_2 are not utilized by Z1-2D. Pro-Pro-Leu did not compete with $(Leu)_3$ (Table 8) at a 3:1 molar ratio in the growth medium. Also, the peptide Pro-Pro-p-F-Phe is not toxic to Z1-2D at five times the equivalent leucine concentration. Therefore only dipeptides with imino

acid residues appear to be substrates for the peptide transport system in Z1-2D.

The results in Table 8 are mostly consistent with the suggestion that peptides that are non-growth-supporting should not compete with the growth-supporting peptides for access to the peptide transport system in Z1-2D. However Met-Met-Leu is anomalous in this respect. This peptide inhibits growth on $(Leu)_2$, $(Leu)_2$-OBzl and $(Leu)_3$. It is not surprising that Met-Met-Leu would have access to the transport system, since it is a hydrophobic substrate not very different from $(Met)_3$ or $(Leu)_3$. But its failure to support growth of Z1-2D and still compete with the growth-supporting peptides is unusual. Since competition represents an affinity for transport, and Met-Met-Leu is subject to hydrolysis by intracellular peptidases, the reason that Met-Met-Leu fails to support growth is not obvious. It is possible that some secondary intracellular barrier (i.e.,vacuole) prevents the Met-Met-Leu from reaching its site of peptidase activity. Although a particular aminopeptidase of broad specificity is partially localized in the vacuole,[30,50] the reason for the vacuolar membrane to exclude this particular peptide is not clear.

A free carboxyl terminus is not required for peptide transport in Z1-2D. Primarily this is evidenced by the positive growth response to $(Leu)_2$-OBzl. However not all peptide esters have the ability to enter this strain. $(Leu)_2$-OMe and $(Leu)_3$-OMe are not growth substrates. Furthermore, uptake studies (Table 10) demonstrate the failure of $(Leu)_2$-OMe to inhibit the uptake of [1-^{14}C]Gly-Leu at concentrations of ten and twenty times that of the labeled peptide. However at these same concentrations both $(Leu)_2$ and $(Leu)_2$-OBzl effectively inhibit the

uptake of [1-^{14}C]Gly-Leu. $(Leu)_2$-OMe does not inhibit growth of Z1-2D on $(Leu)_2$ (Table 8). Therefore the uptake of peptide esters in Z1-2D may be quite specific. The poor growth response to peptide methyl esters in Z1-2D is consistent with another strain of S. cerevisiae.[53] In that organism, although $(Met)_3$-OMe, and even $(Met)_4$-OMe and $(Met)_5$-OMe supported growth of the methionine auxotroph, longer lag times were exhibited in the growth curves. In fact, if the criteria from the footnotes to Table 3 were applied to growth curves exhibited by these peptide esters, all would be classified as intermediate growth substrates. The ability of $(Leu)_2$-OBzl to support growth of Z1-2D does not mean it diffuses into the cell because of its high hydrophobic content. Considering the specific inhibition of growth on $(Leu)_2$-OBzl by free peptides 8, 9), the ability of $(Leu)_2$-OBzl to inhibit [1-^{14}C]Gly-Leu uptake to the same degree as $(Leu)_2$ (Table 10) and its mutual exclusion along with $(Leu)_2$ and the other peptides from Z1-2D Eta^r, the transport of this peptide is regulated by a specific uptake process.

The requirement of a protonatable amine terminal residue on a peptide for transport is evident. Both Ac-$(Leu)_3$ and Ac-Gly-Leu do not support growth of Z1-2D. Ac-$(Leu)_3$ does not compete with $(Leu)_3$ (Table 8). In addition, Ac-$(Met)_3$ is a weak competitor (Table 9) compared to $(Met)_3$. This contrasts with the observation[53] that many acylated peptides, such as Ac-$(Met)_3$ and Ac-Gly-Met were growth substrates for a methionine auxotroph of S. cerevisiae G1333. However in another strain of S. cerevisiae[12] although uptake of labeled Ac-$(Met)_3$ was demonstrated, a twenty-fold ratio of Ac-$(Met)_3$ to Met-Met-[1-^{14}C]Met produced no inhibition in the uptake of the

unblocked, labeled peptide. Therefore one may argue that the peptide transport system in this yeast strain has a different specificity than in G1333, and that the ability to transport the acylated peptides represents the presence of an additional system that is absent in Z1-2D.

The existence of a stereochemical requirement for peptide transport in Z1-2D is indicated by growth studies and competition experiments. L-Leu-D-Leu and D-Leu-L-Leu are not growth substrates for this organism. They do not inhibit growth on $(Leu)_2$ when present in the growth medium in 2-fold excess (Table 8). In addition, neither $(D\text{-Ala})_3$ nor L-Ala-L-Ala-D-Ala effectively prevents $(Leu)_3$ from serving as a growth substrate, whereas $(L\text{-Ala})_3$ under similar conditions, inhibits growth on $(Leu)_3$ for 25 hours (Table 9). Previous studies on peptide utilization in *E. coli*,[10,61] and *C. albicans*,[6] gave evidence for stereospecific uptake processes in these organisms. In *E. coli* these studies showed that the L-L-D form of a tripeptide had the greatest affinity for transport of the peptides with D-residue substituents at different positions in the peptide. In *C. albicans* the higher affinity of D-Met-L-Met-L-Met for uptake was noted. Evidence in another *S. cerevisiae* strain[12] revealed that L-Met-L-Met-D-Met and L-Met-D-Met-L-Met inhibited the uptake of labeled $(L\text{-Met})_3$, although the percent inhibition was much lower than that obtained using cold $(L\text{-Met})_3$. An auxotroph of *S. cerevisiae* (F. Naider, unpublished results) can utilize L-Met-L-Met-D-Met as a source of methionine. These results indicate that in *S. cerevisiae* and other organisms D-residues in a peptide generally reduce the affinity of a peptide for transport. The ability of *E. coli* and *S. cerevisiae* to transport peptides with D-residues in the carboxyl

terminal position is consistent with the observations that the terminal carboxyl group is not required for transport (some peptide esters are growth substrates). C. albicans can accommodate an amine terminal acylated group, and therefore can transport D-residues at this position in the peptide. This organism did not utilize peptide esters, and therefore the failure to utilize L-Met-L-Met-D-Met is consistent with its requirement for a free carboxyl group.

The existence of a size limit to peptide transport in Z1-2D is still not clear. Previous studies with microorganisms concluded that size limits to peptide uptake could result from either a sieving action of the cell-envelope[9,59], or from the failure of the peptide transport system to bind peptides above a given size.[83] The size restriction to peptide transport in E. coli is thought to be affected at the level of the lipopolysaccharide or peptidoglycan.[9] In N. crassa, mutants containing porous cell walls were used to show that the peptide transport system itself is incapable of handling peptides that contain on the average more than five amino acid residues.[83]

In the present study, all tetrapeptides containing leucine or lysine failed to serve the nutritional requirements of strain Z1-2D. While two non-growth supporting tripeptides, Gly-Leu-Gly, and Leu-Leu-ε-aminocaproic acid were growth supporting when supplied at high concentrations in the growth medium, Gly-Leu-Gly-Gly at 20 times the minimal concentration of leucine still is not a growth substrate (Table 5). The complete inability of tetrapeptides or larger substrates to enter Z1-2D, as well as their failure to compete with the uptake of $(Leu)_3$ (Table 8), suggests a size limit for transport at the tetrapeptide level. However all the leucine tetrapeptides that

were examined have two or more glycyl residues in the peptide. The low affinity of glycine-containing peptides for transport is apparent from the evidence that Leu-Gly-Gly, Gly-Gly-Leu and Gly-Leu-Gly are not growth substrates, and that $(Gly)_2$ and $(Gly)_3$ do not inhibit growth on any leucine peptides. Thus its possible that the failure of the tetrapeptides to support growth represents the sequence and composition requirement of the peptide transport system rather than a size limit to transport. In addition, the molecular weight of $(Leu)_3$ and $(Leu)_2$-ε-aminocaproic acid is greater than some of the non-growth-supporting tetrapeptides. It is also probable that these leucine peptides have a hydrodynamic volume equal to or greater than that of Gly-Leu-Gly-Gly. These results imply that if a size limit to transport exists in Z1-2D it does not depend on the hydrodynamic volume of the peptide. In another strain of S. cerevisiae,[53] several tetrapeptides and $(Met)_5$ supported growth of this methionine auxotroph. Other studies showed the yeast cell to be permeable to a polyethylene glycol as large as molecular weight 700. Therefore conclusions about the factors responsible for a size limit to transport in S. cerevisiae are tentative. The use of substrates containing residues with a higher affinity for transport [e.g. Lys-$(Leu)_n$, n = 2,3,4,5] will provide a better estimate of the size limit in Z1-2D, and confirm whether or not it differs from the above-mentioned studies in other yeast strains.

The existence of a size limit to peptide transport in S. cerevisiae may be important in explaining the mechanism by which peptide hormones interact with cells. Recent evidence[32,42] shows that fragments of several peptide hormones are accumulated inside cells or tissues.

S.cerevisiae cells of α-mating type produce a tridecapeptide hormone[15] hormone which has the ability to arrest cells of a-mating type in the G1 phase of the cell cycle. Studies on the mechanism of action of this peptide using ^{125}I-labeled and fluorscein isothiocyanate(FITC)-labeled[77] α-factor suggested that a-mating type cells could take up the label from this hormone, although the accumulation of intact α-factor has not been demonstrated. If a size limit to transport in a-mating type cells is determined, it may explain whether α-factor can be taken up through the peptide transport system intact, or if partial cell surface is necessary for the transport[76] of α-factor fragments. An alternative possibility is that the hormone is accumulated through a separate transport system.

The isolation and characterization of the mutant, Z1-2D Eta^r, represented an important step in characterizing the peptide transport system in S.cerevisiae Z1-2D. The method used to isolate the mutant deserves some comment. As are other strains of S.cerevisiae, Z1-2D is quite sensitive to ethionine. It is now known[69] that ethionine causes repression of endogenous methionine biosynthesis and consequently arrests cells of S.cerevisiae in the G1 phase of the cell cycle. However, mutation to ethionine resistance has been widely reported in the literature.[29,33,73] These studies reveal that one permease mutation is expressed by an inability to transport ethionine (along with methionine, p-F-phenylalanine, and canavanine). Two other classes of mutation confer resistance specifically to ethionine by a mechanism unrelated to the transport of the toxic analogue. The studies reported here demonstrate that the ethionine peptides are toxic to Z1-2D because of ethionine released inside the cell. Also, based on competition

studies, Eth-Ala and Leu-Leu-Eth have an affinity for the peptide transport system in this organism. Therefore the isolation of a mutant resistant to the toxic peptides, but sensitive to ethionine would be presumptive evidence (pending confirmation of sufficient peptidase activity) of a peptide transport-deficient mutant. The methods chosen to isolate the mutant were either from liquid culture, or solid media by natural selection. However in solution experiments where Eth-Ala was supplied in the growth media with normal amounts of leucine and lysine, no resistant colonies were evident during the 40 hour growth period. When stationary phase cultures of Z1-2D grown on leucine and lysine were distributed on SM + Eth-Ala plates, the only colonies to appear had attained a resistance to ethionine and not the toxic peptide. It was therefore fortuitous that the mutant was isolated in the manner described in the experimental section. Although it appears that two steps were used to select Z1-2D Eta^r, the first step of growing the parental cells with Eth-Ala and an excess of $(Leu)_2$ was most probably coincidental with the success in obtaining the mutant. The actual selection more than likely occurred in the second step of this procedure when the cells were applied to the plate containing Eth-Ala. This is the analagous procedure to the one used in E. coli to obtain mutants resistant to triornithine from a minimal media agar plate containing 100 µg/ml of the toxic peptide.[7] These authors reported a relatively high frequency (10^{-4}) of isolating a TOR mutant from unselected E. coli populations; approximately 100-fold higher than normal. This is much higher than the low success rate obtained with Z1-2D by the procedures outlined above. However, in E. coli, every colony growing on the

triornithine plate was determined to be an oligopeptide transport-deficient mutant. Contrast this with the finding in this S. cerevisiae strain that most Eth-Ala resistant colonies were actually resistant to ethionine, and not the peptide analogue. In fact, in the experiment successfully reporting the isolation of the transport mutant (Figure 2), eleven of the thirteen colonies that grew on the SM + Eth-Ala plate were resistant to ethionine and only two displayed the characteristics of Z1-2D Eta^r. The mutant's revertant frequency is 4.3×10^{-6}. All revertant colonies have regained the ability to utilize both dipeptides and tripeptides. The revertant frequency is much larger than the probability that would be expected for obtaining spontaneous revertants in two independent systems (10^{-12}).[9] The mutant grows at the same rate as Z1-2D, takes up a labeled amino acid at nearly the same rate as the parent strain, and also had the same levels of peptidase activity as Z1-2D.

Therefore Z1-2D Eta^r represents a peptide transport-deficient mutant. Its characteristics confirms that dipeptides and tripeptides share the general peptide transport system in Z1-2D, and therefore are simultaneously excluded from the mutant by a single step selection procedure. Z1-2D Eta^r is the first organism isolated to be resistant to dipeptide and tripeptide uptake by this single step process. In E. coli, it was suggested[63] that the TOR mutation represents a deficiency in an energy-coupling component and the Opr I system represented facilitated diffusion of the leucine and methionine peptides where the peptide carrier continues to function in the absence of energy coupling. Even though the genetic mutation for Opp has been mapped, only the isolation of the specific gene product would be

conclusive evidence for an energy deficient mutant. Therefore the precise nature of the peptide transport mutant in S. cerevisiae as characterized by a deficiency in a binding protein or membrane carrier protein, or energy-coupling component will remain unanswered until the results of osmotic shock studies, genetic analyses, and binding studies with labeled substrates are completed.

In summary, S. cerevisiae Z1-2D represents one of many organisms and tissues possessing a functional peptide transport system. As in most cases of peptide utilization, the uptake is distinct from that of amino acids. Most peptides are not accumulated inside the cell but are hydrolyzed by ample levels of cytoplasmic peptidases. Extracellular peptidases have no detectable activity on free peptides in this organism. However it should be pointed out that in general, the existence of peptide transport in an organism does not preclude the activity of selective extracellular hydrolysis against certain substrates. The 'evolutionary' argument is that the most efficient way for an organism to concentrate substrates necessary for survival is to employ both methods (peptide transport, or peptide hydrolysis followed by amino acid uptake) to adapt to conditions of a rapidly changing environment. For example, in E. coli, although acetyl-blocked peptides are not substrates for the peptide transport system,[9] a periplasmic membrane bound dipeptidyl carboxypeptidase hydrolyzes Ac-$(Ala)_3$ to provide the cell with a nitrogen source.[28] This enzyme has no hydrolytic activity against free peptides. In N. crassa[81] there is a functional oligopeptide transport system although many dipeptides in the same organism are hydrolyzed extracellularly. Finally, in the human jejunum[2] $(Gly)_2$ and $(Gly)_3$ are transported by an oligopeptide

transport system, but $(Gly)_4$ is mostly hydrolyzed extracellularly.

The characteristics of peptide transport in Z1-2D are listed in Table 17. Based on the detailed structural specificity exhibited by Z1-2D, and the studies isolating and characterizing Z1-2D Eta^r, it is probable that a carrier-mediated process is responsible for the transport of peptides in this organism. These studies in S. cerevisiae have, in many aspects, bridged the gap in the knowledge between peptide transport in E. coli and the lack of information about these processes in more complex organisms. Preliminary evidence from uptake studies in mammalian systems[1,9] reveal several important similarities in the transport of peptides by yeast and these more complex eucaryotic organisms, including man. For example, mammalian systems appear to have a common mode of uptake for dipeptides and tripeptides, as well as a preference for hydrophobic residues. Therefore these studies in S. cerevisiae have great promise for future work. It is likely that the isolation and characterization of the cellular components of the peptide transport system in yeast will be a major step in understanding how transport is regulated in more complex systems. The S. cerevisiae strains Z1-2D and Z1-2D Eta^r will provide a well defined system in which to conduct these studies.

Table 17

Characteristics of Peptide Transport

In S.cerevisiae Z1-2D

1. Peptide uptake is distinct from amino acid uptake.
2. Peptide hydrolysis and peptide transport are not related.
3. Dipeptides and tripeptides share a single uptake system.
4. Acidic and basic residues have a lower affinity for transport than hydrophobic substrates.
5. The sequence of amino acids in a peptide may determine its affinity for transport.
6. Imino acid dipeptides are transported.
7. A protonatable amine terminal is required for transport.
8. D-residues lower the affinity of a peptide for transport.
9. A free carboxyl terminal is not required for transport.
10. The size limit to transport is not related to the hydrodynamic volume of the peptide.
11. The transport system can be eliminated by a single step mutation.

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