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I am submitting herewith a dissertation written by David D. Parker, Jr., entitled "Peptide Uptake in Yeast: Glycolytic Energy Requirement and Noninvolvement of External Peptidase." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

J. M. Becker, Major Professor

We have read this dissertation
and recommend its acceptance:

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Graduate Studies and Research

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PEPTIDE UPTAKE IN YEAST: GLYCOLYTIC ENERGY REQUIREMENT AND NONINVOLVEMENT OF EXTERNAL PEPTIDASE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

David D. Parker, Jr.

March 1980

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DEDICATION

This manuscript is dedicated respectfully to several of the author's close personal friends, Dr. Jonathan D. Martin, Dr. Steven D. Brown, and Dr. Mark L. E. Camblin, all of whom have been of great personal value in sharing the "good times" as well as the "hard times."

This manuscript is also dedicated sincerely to the author's ex-wife, Nancy H. Parker, for her tremendous understanding, patience, and dedication to the author throughout most of his graduate education. Without her support, this manuscript would have never been possible. May she have all the happiness she truly deserves because the graduate education was a rough experience for them both. The author will never forget the support she gave, both emotionally and financially, and will love her always. For the good memories, thank you.

For the most part, this manuscript is dedicated to the author's daughter, Jennifer Ann Parker. Although she only endured part of the graduate experience, she has been the "sunshine" in the author's life. It is for her that this manuscript was finished and there will never be a stronger love than the one the author has for her. To "my cute little redhead," your "Daddy" wishes you all the happiness the world has to offer. Thank you for the love for I will cherish it for the rest of my life.

Finally, to all of the author's friends not mentioned, thank you for just being there. I love you all.

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ABSTRACT

A fermentable energy source was required for the uptake of trimethionine by log phase cells of the yeast, Saccharomyces cerevisiae 139. The deletion of fermentable energy during uptake resulted in an eight-fold reduction in transport rate. Starvation of the cells under aerobic conditions resulted in an 80% loss of transport activity. This loss in activity was not reversed by treatment of cells with reducing agents. However, the loss of activity was prevented by starvation under a nitrogen atmosphere. Cells with a repressed mitochondrial system (e.g. glucose or anaerobically grown cells, petite mutants) had the greatest peptide transport capacity. In contrast, cells grown on an oxidizable energy source (e.g. glycerol, acetate) would not transport trimethionine, and cells grown on D-mannose, which is both fermented and oxidized, transported trimethionine but at a reduced rate. In addition, inhibitors of glycolysis such as arsenate or iodoacetic acid prevented transport. These data indicate that energy for peptide transport in S. cerevisiae is provided initially by glycolysis.

Recent studies showed that an external peptidase activity, aminopeptidase II, which hydrolyzes amino acid-p-nitroanilides and several di- and tripeptides is present in Saccharomyces cerevisiae. We have investigated the relationship between this peptidase activity and peptide transport in yeast. We found that intact cells of S. cerevisiae 139 hydrolyze amino acid-p-nitroanilides (PNA) by an activity similar to that of aminopeptidase II. In contrast, intact

cells of S. cerevisiae 139 did not hydrolyze trimethionine and the peptidase activity toward this substrate was localized in the soluble fraction of the yeast. These results and competition studies demonstrate that the external peptidase in S. cerevisiae 139 can hydrolyze amino acid-p-nitroanilides but not $(Met)_3$. Kinetic analysis showed that the Vmax for the intracellular hydrolysis of $(Met)_3$ was 120 nmoles Met released/min/mg dry wt whereas the Vmax for $(Met)_3$ transport was 8.3 nmoles $(Met)_3$ transported/min/mg dry wt. We conclude that this tripeptide is taken up by S. cerevisiae intact and rapidly hydrolyzed in the cytoplasm.

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PART I

PEPTIDE UPTAKE IN YEAST: GLYCOLYTIC ENERGY REQUIREMENT FOR THE UPTAKE OF RADIOACTIVE TRIMETHIONINE

CHAPTER I

INTRODUCTION

The energetics of amino acid transport in yeast has been studies extensively. Previously published results demonstrate that amino acid uptake can be observed in freshly-harvested yeast in medium without a fermentable carbon source (1-8), although glucose did stimulate uptake six to eight fold (1,8). Depletion of ATP reserves either by starvation or by treatment with antimycin and 2-deoxyglucose caused a decrease in transport rate but the cells retained sufficient uptake capacity so that amino acid transport could be studied (2-9). A hydrogen ion gradient appeared to be responsible for the amino acid transport observed in Saccharomyces carlsbergensis (1-5).

Previous reports from this laboratory have described the uptake of peptides by yeasts (10-15) and more directly, the optimal physical parameters for the uptake of $(Met)_3$ by S. cerevisiae 139. Initial experiments, using inhibitors of energy metabolism, indicated that cellular energy was required for peptide uptake (10). However, no systematic study has been reported on the energetics of peptide uptake in fungi, bacteria, or mammalian tissue. In the absence of this data, we may only speculate on the possible modes of energy coupling to peptide uptake. In this paper, we attempt to elucidate the source of energy for peptide uptake in S. cerevisiae.

CHAPTER II

MATERIALS AND METHODS

Yeast strains and maintenance. Saccharomyces cerevisiae Fleishman strain 139 (ATCC 9896) was employed. This strain and the petite mutants mentioned below were maintained on agar slants (YEPD) of 1% yeast extract, 2% bacto-peptone (Difco), and 2% glucose. Every month the organisms were transferred to fresh medium, incubated 24 h at 30°C, and stored at 4°C. Isolation of petite mutants (designated PM1 and PM2) was carried out by previously established procedures using ethidium bromide (16). Lack of growth on glycerol was used to test for maintenance of the petite characteristic.

Growth media. The mineral growth medium used for most experiments was Vogel's-N-Medium (17) modified by the addition of inositol (36 ug/ml), pantothenic acid (2 ug/ml), pyridoxine-HCl (2 ug/ml), thiamine (4 ug/ml), biotin (o.5 ng/ml) and proline (4 mg/ml) and by the elimination of NH_4NO_3. For most experiments, a sterile solution of glucose was added to a final concentration of 2% (w/v) (VGlu). In some experiments, sodium acetate (2% w/v) (VAc) or glycerol (4% w/v) (VGly) was used as the carbon source.

For anaerobic growth, VGlu was supplemented with Tween 20 (0.3 mg/ml), Tween 80 (1.25 mg/ml), ergosterol (40 ug/ml), sodium thioglycollate (0.5 mg/ml) and resazurin (1 ug/ml) (VAn). The medium was flushed with N_2 (approximately 2 h) before inoculation. For growth of the petite mutants, the medium was VGlu supplemented with 1% yeast extract (VGluYE).

Growth conditions. For growth, the yeast was taken from a YEPD slant, inoculated into VGlu medium grown for 24 h at 30°C, and used directly or stored at 4°C for up to two weeks. A portion (1 ml) of the 24 h culture was inoculated into 50 ml of the medium (VGlu, VAc, or VGly) and grown for 16 h at 30°C with stirring. A portion (20 ml) was then inoculated into 200 ml of the appropriate medium, which was contained in a 500 ml Erlenmeyer flask fitted with a sidearm, and grown at 30°C with stirring. Growth was determined by the increase in turbidity at 400-420 nm (blue filter) using a Klett-Summerson photoelectric colorimeter.

For anaerobic growth, 1 ml of the 24 h culture was inoculated into 50 ml of VGlu medium and grown at 30°C for 24 h. A portion (20 ml) was then inoculated into 200 ml VAn medium and grown at 30°C for 24 h under a N_2 atmosphere. A control flask (VAn medium) was also inoculated with 20 ml of the 24 h culture and incubated 24 h at 30°C with aeration.

Transport studies. Cells at 100-150 Klett units (approximately 4 h in VGlu) were harvested by centrifugation at 6300 x g for 10 min at 5°C and washed twice in distilled water. The cells were resuspended in distilled water so that a 1:10 dilution of the resuspended cells (1 mg dry wt/ml) gave a Klett reading of 30-40. For uptake studies, 1.5 ml cells were added to 1.5 ml 0.1M phosphate buffer, pH 5.5, containing 0.5% (w/v) glucose (unless indicated otherwise) and incubated for 5 min at 37°C. Uptake was initiated by the addition of 30 ul ^{14}C-$(Met)_3$ (1 mg/ml) or 20 ul ^{14}C-Met (1 mg/ml) to the cell suspension. The mixture was vortexed and at approximately

15 sec, 30 sec, 1 min, 1.5 min, and 2 min, 0.5 ml samples were removed and applied to 0.45 um Amicon filters (25 mm diameter, Amicon Corp., Lexington, MA) supported by a Yeda manifold (Yeda Scientific Instr., Rehovot, Israel). After filtration, the cells were washed twice with 2 ml distilled water. The filters were removed from the manifold, placed in 5 ml Bray's solution (Research Products Intern. Corp., Elk Grove Village, IL) and counted in a Beckman Liquid Scintillation counter, Model LS7000,or a Searle Scintillation counter, Isocap/300. Initial velocities in cpm were converted to nmole/min/mg dry wt of cells by taking into account specific activity (1 mCi/mmole for $(Met)_3$), counting efficiency (80%), and the weight of cells sampled (0.25 mg dry wt). Transport rates varied (2.6-3.2 nmoles/min/mg dry wt cells) from one batch of cells to another. Thus, controls were included in each set of experiments.

Chemicals. The peptide L-Met-L-Met-L(^{14}C)Met (1 mCi/mmole) was prepared by a previously described procedure (10). L-Met-L-Met-L-Met was purchased from Bachem (Marina Del Ray, CA). Radioactive methionine (L-(methyl-^{14}C)-methionine, 50 mCi/mmole) was purchased from Amersham (Arlington Heights, IL). Chloramphenicol and sodium thioglycollate were purchased from Sigma (St. Louis, MO). Iodoacetic acid was purchased from Schwarz/Mann (Orangeburg, NY). All other chemicals were the purest commercially available.

CHAPTER III

RESULTS

Growth stage. Cells were grown on VGlu to various stages of growth, harvested and analyzed for their ability to transport radioactive trimethionine and radioactive methionine. The results indicated that log (both early and mid-log) phase cells transport both $(Met)_3$ and methionine approximately 40X and 10X, respectively, faster than stationary phase cells when glucose was present in the uptake buffer (Table 1). When glucose was absent from the uptake buffer the transport rates were greatly reduced, yet early log phase cells still transported $(Met)_3$ approximately 25X and methionine, 10X, faster than stationary cells. Therefore, log phase cells were used in all subsequent experiments. The data in Table 1 also indicate that the deletion of glucose from the uptake medium has a greater effect on peptide transport than on amino acid transport.

Requirements for fermentable substrate. Cells were grown in VGlu medium and harvested as described previously. Radioactive trimethionine uptake was carried out with various concentrations of glucose in the uptake buffer. As shown in Fig. 1, increasing concentrations of glucose resulted in an increased transport rate up to approximately 0.04% glucose. For many of the following experiments, a final concentration of 0.25% glucose was employed so that an excess of glucose was available to the cells.

TABLE 1. Effect of growth stage on the transport of Trimethionine and Methionine.

Source of cells	Transport Rate			
	$(Met)_3$		Met	
	+ Glucose	- Glucose	+ Glucose	- Glucose
Early log phase	2.41	0.24	10.76	3.87
Mid-log phase	2.65	0.29	6.76	2.47
Stationary phase	0.06	0.01	0.81	0.29

A portion of the 24 h culture (20 ml) was inoculated into one liter VGlu. The early log phase cells were harvested 4-10 h after inoculation, the mid-log cells were harvested 12-20 h after inoculation and the stationary cells were harvested from 30-4- h cultures. Cells were washed as described in Materials and Methods and uptake was carried out in 50 mM phosphate buffer, pH 5.5, with or without 0.25% glucose. The initial concentrations of $(Met)_3$ and Met were 2.4×10^{-5}M and 4.5×10^{-5}M, respectively. Results were expressed as nmoles substrate transported/min/mg dry wt cells.

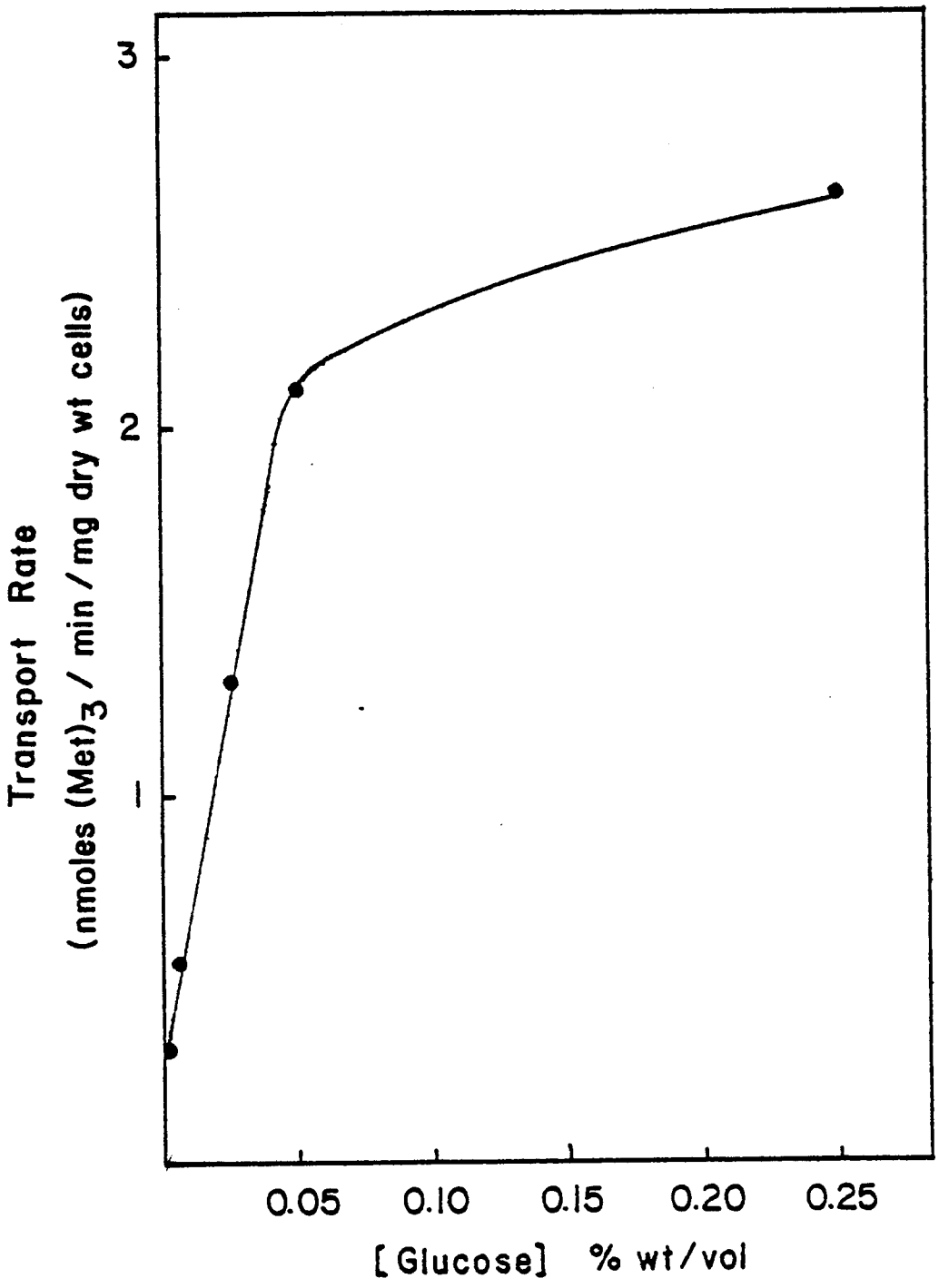


Figure 1. Transport of $(Met)_3$ in S. cerevisiae in response to increasing concentrations of glucose.

Cells were grown on VGlu and uptake was in 50 mM phosphate buffer, pH 5.5, containing the concentrations of glucose indicated.

The K_m and V_{max} of peptide transport in buffer with and without glucose was determined (Fig. 2). The K_m (3.12×10^{-5}M) with and without glucose was identical indicating that the affinity of $(Met)_3$ in the rate limiting step, presumably the carrier protein affinity, was the same under both sets of conditions. The V_{max} in the reaction mixture without glucose was much lower (0.83 nmoles/min/mg dry wt cells) than the reaction mixture containing glucose (6.2 nmoles/min/mg dry wt cells).

Several other compounds were tested for their ability to serve as energy sources for peptide transport (Table 2). ∝-methyl glucoside (a nonmetabolizable glucose analogue) and glycerol (an oxidative energy source for growth) did not provide the energy required for uptake. D-mannose was an effective energy source while xylose, sucrose, sorbose, and arabinose were not. It should be noted that D-mannose could serve as a carbon source for growth, sucrose was assimilated but only after an appreciable lag period, and D-xylose, L-sorbose, and D-arabinose could not be assimilated or fermented by this yeast strain.

Energy starvation. In S. cerevisiae 139 starved with aeration for three hours, there was no loss in viability yet the cells lost about 80% of their ability to transport $(Met)_3$ and methionine (Fig. 3). Starvation was previously shown to decrease glycine transport 70% in another yeast (1). If glucose was not added to the uptake buffer after cells had been starved for 3 h, there was no detectable there was no detectable transport of $(Met)_3$ (data not shown).

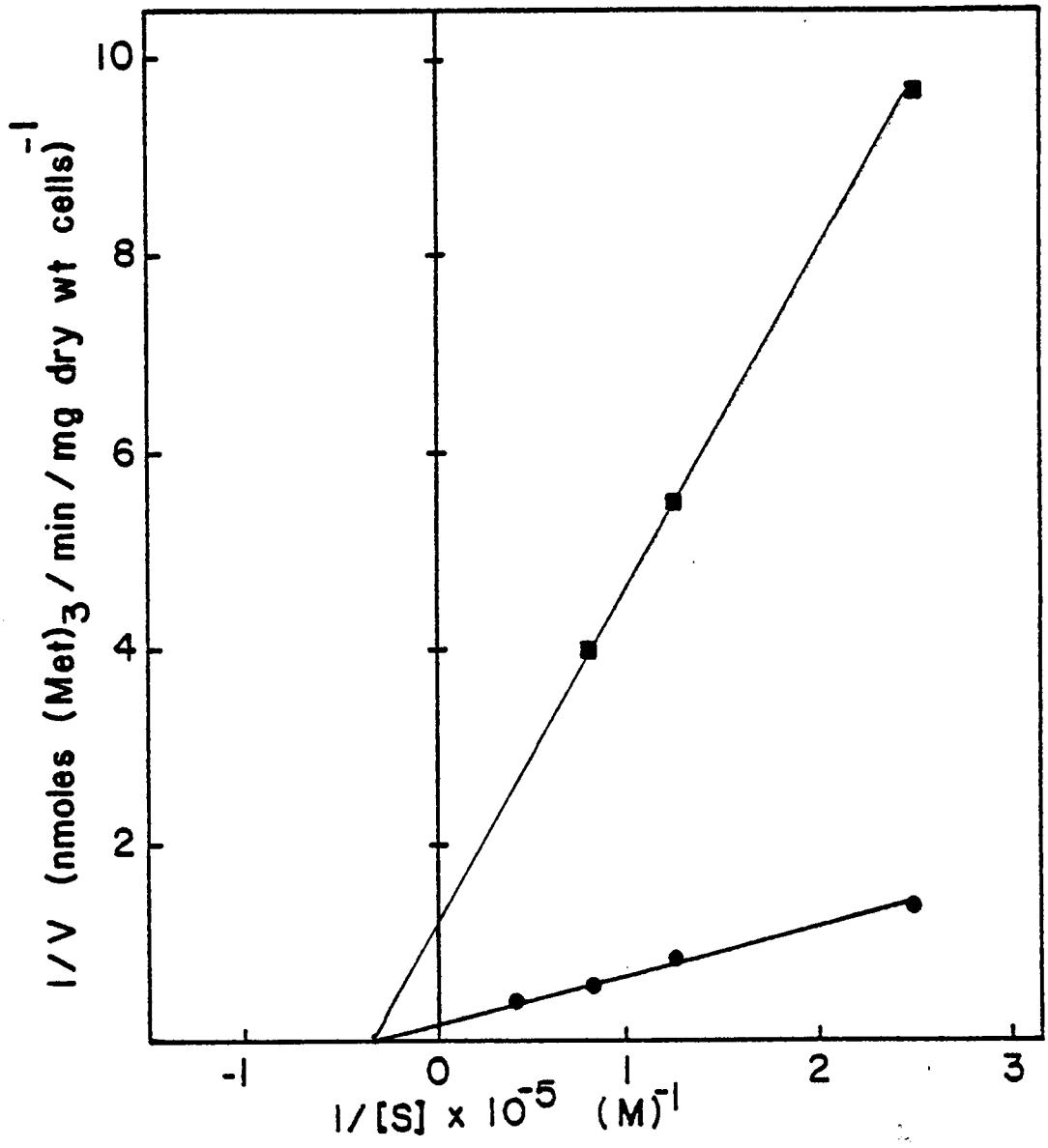


Figure 2. Km of $(Met)_3$ transport in S. cerevisiae.

Double reciprocal plot of $(Met)_3$ transport in 50 mM phosphate buffer, pH 5.5, ■; and buffer containing 0.25% glucose, ●.

TABLE 2. Effect of various energy sources on the uptake of rimethionine.

Additions to Uptake buffer (50 mM phosphate, pH 5.5)	% Initial Rate
-	10
0.25% Glucose	100[a]
0.25% ∝-methyl glucoside	7
0.5% Glycerol	11
0.25% D-mannose	58
0.25% D-xylose	8
0.25% Sucrose	8
0.25% L-sorbose	8
0.25% D-arabinose	8

Cells were grown in VGlu. The sugars were suspended in the uptake buffer at the final concentrations indicated. The cells were incubated 5 min at 37°C before the addition of trimethionine.

[a]Initial rate approximately 3.2 nmoles/min/mg dry wt cells.

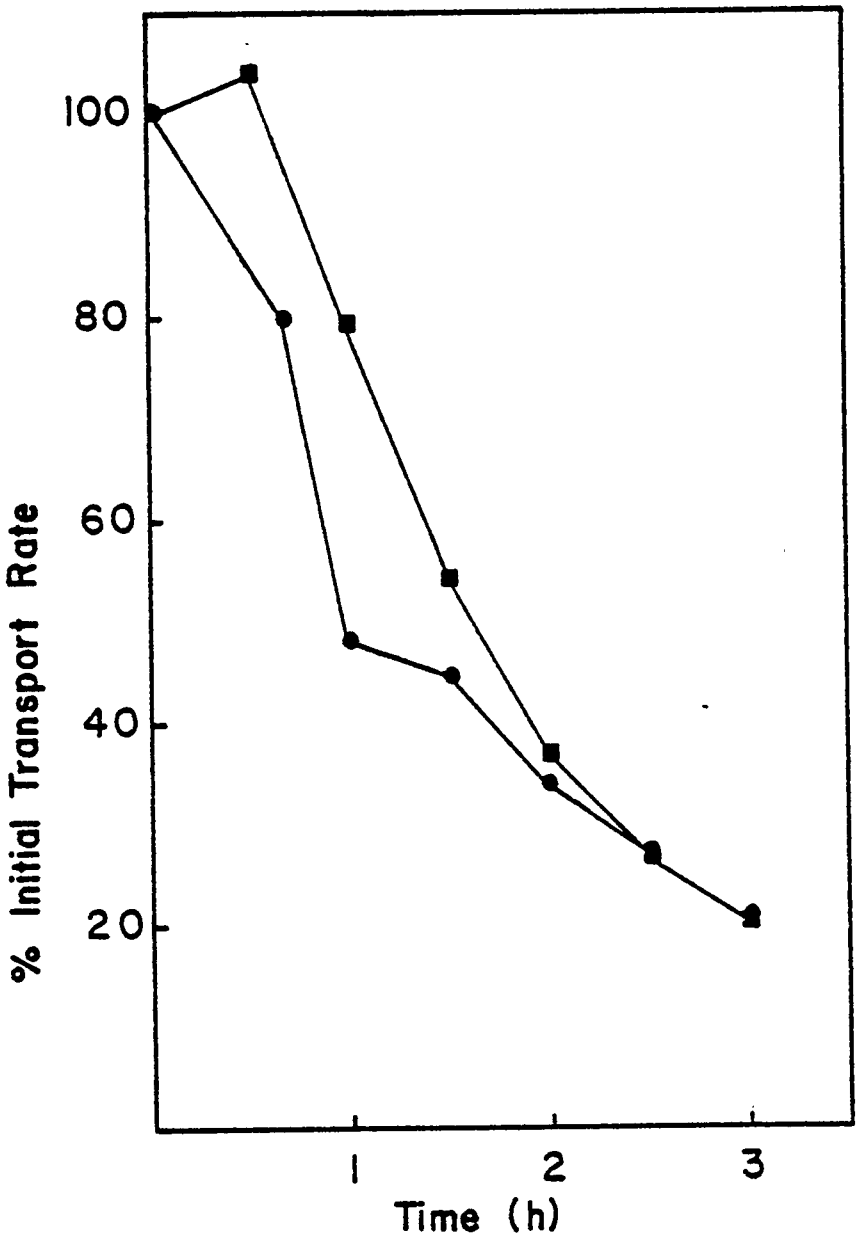


Figure 3. Decline of $(Met)_3$ and Met transport activity during starvation of cells in distilled water with aeration.

Uptake was in 50 mM phosphate buffer, pH 5.5, containing 0.25% glucose. $(Met)_3$ transport, ●; 100% value was 3.3 nmoles/min/mg dry wt cells: Met transport, ■; 100% value was 17.1 nmoles/min/mg dry wt cells.

When cells were washed and starved under other conditions (e.g. 0.85% NaCl, pH 5.5; 100 mM phosphate, 0.6 M mannitol, pH 5.5; complete Vogel's without glucose) there was still an 80-99% decline in transport activity after a 3 h starvation. This showed that a diffusable ion or an osmotic effect was not responsible for the observed decline in transport rate. After starvation in water for 3 h, cells placed in a buffered glucose solution up to 1 h or Vogel's (without inositol or biotin) up to 30 min had no observable restoration of transport activity. It should also be noted that cells could be stored up to 4 h without aeration with no observable decline in transport activity.

We did observe that during starvation, the presence of a N_2 atmosphere prevented the decay of transport activity (Table 3). If the starved cells (aerobic conditions) were incubated with dithiothreitol (DTT) or 2-mercaptoethanol, no restoration of activity was observed. Furthermore, cells starved (2 h) in the presence of DTT or 2-mercaptoethanol exhibited a 40-70% decline in $(Met)_3$ uptake. This indicated that oxidation of surface proteins was probably not responsible for the observed decay of transport activity during starvation.

Fermentative involvement. As shown previously, cells grown in the presence of glucose (VGlu) (a fermentable carbon source that represses mitochondrial function), transport both $(Met)_3$ and methionine (Table 4). In addition, chloramphenicol, which represses the synthesis of several cytochromes, causes only a small decrease

TABLE 3. Effect of nitrogen and reducing agents on the transport activity in starved cells.

Starvation conditions	% Initial Transport Rate		
	0 min	75 min	180 min
Aerobic atmosphere	100[a]	79	32
Dithiothreitol[b]	100	-	31
2-mercaptoethanol[b]	100	-	31
Nitrogen atmosphere	100[a]	119	102

Cells were grown in VGlu and starved in distilled water at 30°C.

[a]Inititial rate was 2.6 nmoles/min/mg dry wt cells.

[b]Cells were harvested at the time indicated and incubated with reducing agent for 30 min. The cells were removed from the reducing agent and the uptake performed with glucose (0.25%) present. Initial rate was 3.2 nmoles/min/mg dry wt cells.

TABLE 4. Effect of various growth conditions on the uptake of trimethionine and methionine.

Growth conditions	Strain	% Initial Rate	
		$(Met)_3$	Met
VGlu	139	100[b]	100[c]
VGlu-chloramphenicol[a]	139	73	47
VGly	139	0	7
VAc	139	0	4
VAn-aerobic	139	100[d]	-
VAn-anaerobic	139	100	-
VGluYE	139	100[e]	100[f]
VGluYE	PM1	90	39
VGluYE	PM2	96	58

[a]Cells were grown in VGlu containing 2 mg/ml chloramphenicol.

[b]$(Met)_3$ initial rate was 2.8 nmoles/min/mg dry wt cells.

[c]Met initial rate was 18.2 nmoles/min/mg dry wt cells.

[d]Initial rate was 0.21 nmoles/min/mg dry wt cells.

[e]Initial rate was 1.8 nmoles/min/mg dry wt cells.

[f]Initial rate was 4.2 nmoles/min/mg dry wt cells.

in $(Met)_3$ uptake although methionine transport is inhibited by 50%. However, when cells were grown in the presence of glycerol (VGly) or acetate (VAc) (growth rate approximately one-half of cells grown on glucose), both of which are utilized oxidatively with full derepression of mitochondrial function (18,19), there was no uptake of $(Met)_3$. In order to assess the effect of growth rate, cells were grown on VGlu at 35°C (approximately one-half the growth rate at 30°C). Trimethionine transport was found to be unaffected due to the decreased generation time. Cells grown aerobically in VAn transported $(Met)_3$ as well as cells grown anaerobically in VAn (no functional cytochrome system). The lower initial transport rate in these cells was probably due to the requirement for addition of tweens and ergosterol to the medium for anaerobic growth. Two petite mutants (PM1 and PM2) transported $(Met)_3$ as well as the wild-type when these strains were grown on VGluYE. The reduced rate of transport on this medium may be due to ammonium ions (a known inhibitor of amino acid and peptide transport) which is present in the yeast extract.

As shown in Table 5, glucose grown cells transported $(Met)_3$ when glucose (100%) or mannose (58%) was present in the uptake buffer. D-mannose can be utilized both fermentatively and oxidatively. Therefore, the presence of D-mannose in the uptake buffer for 5 min could derepress the mitochondrial system enough to decrease the transport activity 42%. L-sorbose was a nonfermentable carbon source and provided no energy for the transport process. If the cells were grown on mannose and assayed for transport activity in

TABLE 5. Effect of D-glucose and D-mannose on the uptake of trimethionine.

	% Initial Rate	
	C source (Growth Medium)	
Uptake Buffer	D-glucose	D-mannose
D-glucose	100[a]	38
D-mannose	58	7
L-sorbose	0	3

Cells were grown on VGlu or Vogel's containing D-mannose. Cells were harvested and incubated in the uptake buffer (50 mM phosphate buffer, pH 5.5) containing 0.25% (w/v) of the sugar for 5 min at 37°C prior to the addition of trimethionine.

[a]Initial rate was 2.6 nmoles/min/mg dry wt cells.

the presence of glucose, only 38% of the initial activity was observed. Again, the presence of glucose for 5 min was probably enough time to repress mitochondrial function to some extent. If the cells were assayed in D-mannose, there was almost no transport of trimethionine (7%).

Inhibitors. Several glycolytic energy inhibitors were tested for their ability to inhibit $(Met)_3$ transport (Table 6). NaF (glycolytic inhibitor - enolase) had no inhibitory effect. However, arsenate (glycolytic inhibitor - glyceraldehydephosphate dehydrogenase) inhibited transport 80% and iodoacetic acid (glycolytic inhibitor - 6-phosphofructokinase) inhibited transport 100%.

TABLE 6. Effect of inhibitors on the uptake of trimethionine.

Uptake Conditions	% Initial Rate
50 mM Tris-citrate, 0.25% glucose, pH 5.5	100[a]
+ 10 mM Sodium arsenate	20
50 mM $NaPO_4$, pH 5.5, 0.25% glucose	100[b]
+ 10 mM Sodium fluoride	108
+ 10 mM Iodoacetate	0

Cells were grown in VGluYE, washed and resuspended in distilled water.

[a]Initial rate was 1.6 nmoles/min/mg dry wt cells.

[b]Initial rate was 1.7 nmoles/min/mg dry wt cells.

CHAPTER IV

DISCUSSION

The data indicate that glucose (or a fermentable substrate) is required for $(Met)_3$ transport. In early log phase cells (Table 1, p. 7), there was approximately a ten-fold difference in the transport rates in the buffers with and without glucose. As shown in Fig. 1, p. 8, there was approximately an eight-fold difference in transport rates; 2.62 nmoles/min/mg cells (0.25% glucose) versus 0.32 nmoles/min/mg cells (no glucose). The data in Fig. 2, p. 12, indicates a 7.5-fold difference in V_{max} (6.25 nmoles/min/mg cells (0.25% glucose) versus 0.83 nmoles/min/mg cells (no glucose)) with no observable change in the K_m. Also, cells, which had been depleted of their endogenous reserves by starvation in distilled water, lost all transport capabilities (uptakes in buffer without glucose) (data not shown). Thus, the low rates observed in the absence of glucose was probably due to the low endogenous energy levels available to the cell at the time of harvest.

The results in Table 4, p. 15, indicate that a functional mitochondrial system represses peptide uptake. First, cells grown on glucose, or glucose and chloramphenicol, transport $(Met)_3$ at maximum rates. Glucose, as the sole carbon source, is known to repress mitochondrial function ("Crabtree Effect") such as cytochrome oxidase, DPNH-Cyt C-Oxidoreductase, DPNH oxidase, Cyt $a+a_3$, and thus lower oxidative capacity (18-20). Chloramphenicol inhibits the synthesis of cytochromes $a+a_3$, b, and c (19). Cells grown on glucose in the

presence of chloramphenicol transport $(Met)_3$ at approximately 75% of the value in glucose grown cells. This 25% reduction in peptide uptake is probably due to effects other than mitochondrial repression since shloramphenicol (in growth medium) also reduced the $(Met)_3$ uptake rate in the petite mutant, PM1, by approximately 30% (data not shown). Second, cells grown anaerobically on glucose transported $(Met)_3$ at the same rate as cells grown aerobically in the same growth medium.. Cells grown anaerobically cannot form cytochromes (19). The reduced transport rate as compared to aerobic glucose grown cells may be due to the altered lipid composition caused by the added components required for anaerobic growth (21,22). Third, two petite mutants (PM1 and PM2), lacking a functional mitochondrial system, transported $(Met)_3$ to the same extent as the wild-type grown on VGluYE. Fourth, glycerol or acetate grown cells, in which the mitochondrion is maximally derepressed (18,19), demonstrated no ability to transport $(Met)_3$. Growth rate of the cells had no influence on the transport rate since cells grown on VGlu at 25°C (approximately same growth rate as VAc) transported $(Met)_3$ equally as well as cells grown at 30°C. Fifth, D-mannose grown cells transported $(Met)_3$ but at a much reduced rate (Table 5). DeDeken (20) reported that mannose grown cells derive their energy from both fermentation and oxidation. Thus, the reduced rate of transport in these cells was probably related to the oxidative capacities of the cell.

The effect of inhibitors (Table 6) also indicates that glycolysis is essential for transport activity. Arsenate and iodoacetate, which affect early steps in glycolysis, inhibit

transport 80% and 100%, respectively. Blocking of metabolic steps by these compounds would lead to an energy drain on the cell as two ATPs are used in the metabolism of one molecule of glucose. However, fluoride has no inhibitory effect on transport. This inhibitor of glycolysis should lead to no net change in cellular ATP pools since it blocks glycolysis at a step after two ATPs are produced.

As shown in Table 7, glycolytic activity appears to be responsible for peptide transport. Cells grown on VGlu possess a repressed mitochondrial system and an active glycolytic system and exhibit a high capacity for $(Met)_3$ uptake. On the other hand, cells grown on VAc possess an active mitochondrial system, yet the peptide was not transported. With the use of inhibitors, glycolytic activity was inhibited in VGlu grown cells (no mitochondrial activity) and transport was inhibited. Kotyk (8) implicated the involvement of glycolytic energy for glycine uptake in S. cerevisiae and demonstrated a high molecular weight polyphosphate (probably from substrate phosphorylation) was required. Jayakumar (9) also proposed that glycolytic energy was required for proline uptake in C. albicans.

Previous work by Eddy and coworkers (2-5) on several strains of yeasts, primarily S. carlsbergensis, indicated that a H^+ ion gradient was responsible for amino acid uptake. Eddy and coworkers (2-5) studied amino acid uptake in energy depleted cells (antimycin and 2-deoxyglucose treatment) in a buffer containing no fermentable energy. In our cells, which had been starved in distilled water, trimethionine uptake was very low when glucose was present and

TABLE 7. Summary of results.

Conditions	$(Met)_3$ Transport Activity	Mitochondrial Function	Glycolytic Activity
VGlu	+	-	+
VAc	-	+	-
VGlu-Inhibitors (Arsenate, Iodoacetate)	-	-	+

undetectable in the uptake mixture when glucose was absent. Thus, any attempt to detect a H^+ ion flux for peptides in starved cells, as shown by Eddy (2-5) for amino acids, was not possible in the absence of glucose. The efflux of H^+ ions, observed on peptide uptake in the presence of glucose, was attributable to glucose induced H^+ flux; a H^+ influx due to peptide uptake was not observable by our techniques, because of the low capacity of the cells for peptide uptake (2-3 nmoles/min/mg cells) as compared to the great capacity for proton efflux (36 nmoles/min/mg cells) during glucose uptake (data not shown). This does not rule out that peptides do not enter cells by a mechanism similar to that reported by Eddy (5) for amino acids, but we are technically unable to demonstrate directly a H^+ influx accompanying peptide uptake.

In summary, the glycolytic energy (whether it be ATP or high molecular weight polyphosphate) required for peptide uptake could act directly on the transport protein or could drive the cytoplasmic membrane ATPase (which could be responsible for the H^+ ion gradient). This gradient would then be responsible for the uptake of $(Met)_3$. The inhibitory activity of the mitochondrion on peptide transport is currently investigated. The energy charge of the cell (high when mitochondrion is active) could be responsible. Also, if high molecular weight polyphosphate is responsible for peptide transport, this molecule may not be synthesized when the mitochondrion is operative.

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PART II

PEPTIDE TRANSPORT IN YEAST: NONINVOLVEMENT OF EXTERNAL PEPTIDASE IN THE TRANSPORT OF TRIMETHIONINE

CHAPTER I

INTRODUCTION

Recent reports have demonstrated compartmentalization of peptidases in the yeast Saccharomyces cerevisiae (1-4). Proteases A and B and carboxypeptidase Y are located in the vacuole with their respective inhibitors in the cytoplasm (1,2); aminopeptidase I and a dipeptidase were segregated into the vacuole and cytoplasm, respectively (3). Approximately one-half of the total amount of aminopeptidase II, which has some limited activity towards native dipeptides and greater activity on amino acid-p-nitroanilides, is located external to the cytoplasmic membrane (4).

The cellular distribution of peptidases is of great importance in our understanding of the mode of peptide-cell interaction and peptide transport (see ref. 5 for reviews of models of peptide transport). If peptidase is extracellular, the transport of intact peptides would not be necessary; external hydrolysis would result in amino acids which would be subsequently taken up by their respective transport systems. We have been studying the transport of trimethionine in S. cerevisiae 139 (6,7). Our results have led us to the conclusion that peptides are transported per se by this strain (8). This report addresses the question in detail, from another approach, as to whether or not an external peptidase is present in this strain and if so, its role in the transport of trimethionine.

CHAPTER II

MATERIALS AND METHODS

Yeast strain and maintenance. Saccharomyces cerevisiae Fleishman strain 139 (ATCC 9896) was employed. This strain was maintained on agar slants (YEPD) of 1% yeast extract, 2% bacto-peptone (Difco), and 2% glucose. Every month the organism was transferred to fresh medium, incubated 24 h at 30°C, and stored at 4°C.

Growth medium. The mineral growth medium used for experiments was Vogel's-N-Medium (9) modified by the addition of inositol (36 ug/ml), pantothenic acid (2 ug/ml), pyridoxine-HCl (2 ug/ml), thiamine (4 ug/ml), biotin (0.5 ng/ml) and proline (4 mg/ml) and by the elimination of NH_4NO_3. A sterile solution of glucose was added to a final concentration of 2% (w/v).

Growth conditions. For growth, the yeast was taken from a YEPD slant, inoculated into the growth medium, grown for 24 h at 30°C, and used directly or stored at 4°C for up to two weeks. A portion (2 ml) of the 24 h culture was inoculated into 100 ml of the medium and grown for 16 h at 30°C with stirring. The culture was then inoculated into 1 liter of the medium and grown at 30°C with stirring. Growth was determined by the increase in turbidity at 400-420 nm (blue filter) using a Klett-Summerson photoelectric colorimeter.

Preparation of cells for transport, enzyme analysis, and permeabilization. Cells at 100-150 Klett units (approx. 4-5 h) were

harvested by centrifugation at 6300 x g for 10 min at 5°C and washed twice in distilled water. The cells were resuspended in distilled water so that a 1:10 dilution of the resuspended cells (1-1.2 mg dry wt/ml) gave a Klett reading of 40-46. These freshly harvested cells were termed intact cells in the enzyme analyses.

Transport studies. For uptake studies, 1.5 ml cells were added to 1.5 ml 0.1 M phosphate buffer, pH 5.5, containing 0.5% glucose and incubated for 5 min at 37°C. Uptake was initiated by the addition of 0.03 ml ^{14}C-$(Met)_3$ (1 mg/ml, 1 mCi/mmole) or 0.02 ml ^{14}C-Met (1 mg/ml, 2 mCi/mmole) to the cell suspension. The mixture was vortexed and at approximately 15 sec, 30 sec, 1 min, 1.5 min, and 2 min, 0.5 ml samples were removed and applied to 0.45 um Amicon filters (25 mm diameter, Amicon Corp., Lexington, MA) supported by a Yeda manifold (Yeda Scientific Instr., Rehovot, Israel). After filtration, the cells were washey twice with 2 ml distilled water. The filters were removed from the manifold, placed in 5 ml Bray's solution (Research Products Intern. Corp., Elk Grove Village, IL) and counted in a Beckman Liquid Scintillation counter, Model LS7000. Initial velocities in cpm were converted to nmole/min/mg dry wt cells by taking into account specific activity (1 mCi/mmole for $(Met)_3$), counting efficiency (80%), and the weight of cells sampled (0.25 mg dry wt).

Cell permeabilization. Permeabilization was carried out basically by the procedure described by Miozzari, et. al. (10). Intact cells (see above) were harvested and resuspended in 0.1 M Tris-HCl,

0.05% Triton X-100, pH 7.6, at a concentration of 50 mg dry wt cells/ml and frozen at -20°C for 16 h, at which time the cells were thawed at room temperature and ice cold distilled water was added to bring the concentration to 1-1.2 mg dry wt cells/ml (permeabilized cells).

Cell fractionation. Permeabilized cells were centrifuged at 6000 x g and the cell pellet was resuspended at a concentration of 15 mg dry wt/ml in distilled water. One volume of 0.45-0.5 mm glass beads (Bronwill Scientific, Rochester, NY) was added, and the cells were broken in a Braun homogenizer (Bronwill Scientific, Rochester, NY) for one minute under a CO_2 stream. This procedure was 99% effective in cell breakage as judged by microscopy. The beads were removed by filtration through Whatman #1 filter paper and the filtrate was centrifuged at 1000 x g for 10 min to remove unbroken cells and large particulate matter. The supernatant was termed the soluble fraction and the membrane pellet was resuspended in a known volume of distilled water. All fractions were frozen at -20°C prior to analysis.

Enzyme analyses. Activity for α-glucosidase was determined by adding 3 ml cell suspension (intact or permeabilized cells, 1-1.2 mg dry wt/ml) to 3 ml buffer (0.1 M phosphate buffer, pH 7.0) and adding 0.2 ml 0.01 M PNPG (p-nitrophenyl-α-D-glucoside). Three milliliters of the reaction mixture was filtered immediately through a 0.45 um Amicon filter for a zero time determination while the remaining 3 ml was incubated at 37°C for 30 min or 1 h and then filtered. Absorbance of the filtrates was determined on a Gilford

spectrophotometer, Model 250, at 400 nm.

Aminopeptidase activity was measured using several amino acid-p-nitroanilides. Gly-PNA (Glycine-p-nitroanilide), Ala-PNA (Alanine-p-nitroanilide), Lys-PNA (Lysine-p-nitroanilide), and Leu-PNA (Leucine-p-nitroanilide) were all kept in 4 mM stocks in 5 mM H_2SO_4. These stocks were diluted in phosphate buffer immediately prior to assay. For assay, 3 ml cells (intact or permeabilized) was added to 3 ml 0.1 M phosphate buffer, pH 7.0, containing the appropriate concentration of amino acid-p-nitroanilide (usually 1.06×10^{-3}M). Three milliters of the reaction mixture was filtered immediately while the remaining portion was incubated at 37°C for 30 min or 1 h and then filtered. Absorbance of the filtrates was measured at 405 nm.

$(Met)_3$ peptidase activity was measured in intact cells by adding 1.5 ml cells to 1.5 ml phosphate buffer (pH 7.0 or 5.5) containing 6.4×10^{-4}M $(Met)_3$. Incubation was carried out at 37°C. At 0 time and 1 h, 1.5 ml of the reaction mixture was filtered, boiled 3 min in a boiling water bath to stop the reaction, and 0.5 ml analyzed for free methionine by the use of a coupled enzyme assay described by Fujita, et al. (11). To the 0.5 ml sample, 2 ml of the L-amino acid oxidase, o-dianisidine, peroxidase reagent was added, incubated at room temperature for 1 h, and the absorbance determined at 440 nm. A standard curve was linear between 0-300 nmoles methionine and the presence of $(Met)_3$ caused no deviation from linearity. Peptidase activity for $(Met)_3$ was measured in permeabilized cells by adding 0.5 ml cells to 1.0 ml distilled water and 1.5 ml phosphate buffer containing $(Met)_3$. Incubation was also at 37°C and 1.5 ml samples

were removed and filtered at 0 time and 15 min. Further analysis was then carried out as described previously for intact cells. The increase in absorbance was linear for one hour for all enzymes assayed.

Chemicals. The peptide L-Met-L-Met-L-Met was purchased from Bachem (Marina Del Ray, CA). L-Met-L-Met-L-(^{14}C)Met (1 mCi/mmole) was prepared by a previously described procedure (6). Radioactive methionine (L-(methyl-^{14}C)-methionine, 50 mCi/mmole) was purchased from Amersham (Arlington Heights, IL). p-Nitrophenyl-α-D-glucoside, L-Alanine-p-nitroanilide-HCl, Glycine-p-nitroanilide, L-Leucine-p-nitroanilide-HCl, L-Lysine-p-nitroanilide-DiHBr, L-amino acid oxidase (Type I), peroxidase (Horseradish, Type VI, salt free), o-dianisidine were purchased from Sigma (St. Louis, MO). Triton X-100 was purchased from BioRad (San Diego, CA). All other chemicals were the purest commercially available.

CHAPTER III

RESULTS

Aminopeptidase activity. The ability of intact (freshly harvested) cells or permeabilized cells to cleave amino acid-p-nitroanilides and $(Met)_3$ was determined. ∝-glucosidase activity (internal marker) was also determined (Table 1). Intact cells had activity toward the amino acid-p-nitroanilides. Assuming that the substrates did not enter the cell (see below and ref. 4), this indicates that there was indeed an external aminopeptidase present in these cells. However, there was no $(Met)_3$ peptidase activity or ∝-glucosidase activity associated with the intact cells. In permeabilized cells, aminopeptidase activity was usually greater (especially when using Leu-PNA as a substrate) and $(Met)_3$ peptidase activity and ∝-glucosidase activity was detected, indicating that appearance of $(Met)_3$ peptidase activity coincided with the appearance of the internal marker.

pH profiles and competition experiments. Leucine and lysine aminopeptidase activity was determined in intact cells at various pH values (Fig. 1). This activity profile was similar to that reported by Frey and Rohm (4) for intact cells as well as isolated aminopeptidase II. Also, the presence of 10 mM nitrilotriacetate (a nonpermeable Zn^{++} chelator) did not affect the aminopeptidase activities of intact cells (data not shown). This is in agreement with the results reported by Frey and Rohm (3) who demonstrated the external enzyme,

TABLE 1. Detection of aminopeptidase activity in intact and permeabilized cells.

Substrate	Cell Condition	
	Intact	Permeabilized
PNPG	0	0.99
Ala-PNA	1.38	1.47
Gly-PNA	0.56	0.30
Lys-PNA	0.37	2.32
Leu-PNA	3.28	5.81
$(Met)_3$	0	101

The final concentrations of the substrates were PNPG, 3.3×10^{-4}M; Ala-PNA, Gly-PNA, Lys-PNA, and Leu-PNA, 5.3×10^{-4}M; and $(Met)_3$, 3.2×10^{-4}M. The values given for PNPG and the amino acid-p-nitroanilides are expressed as units/mg dry wt cells. One unit was defined as the change of 0.1 O.D./h. The units for $(Met)_3$ activity are nmoles Met released/min/mg dry wt cells. Glucose, which was required for $(Met)_3$ uptake in intact cells, was not included in the incubation buffers.

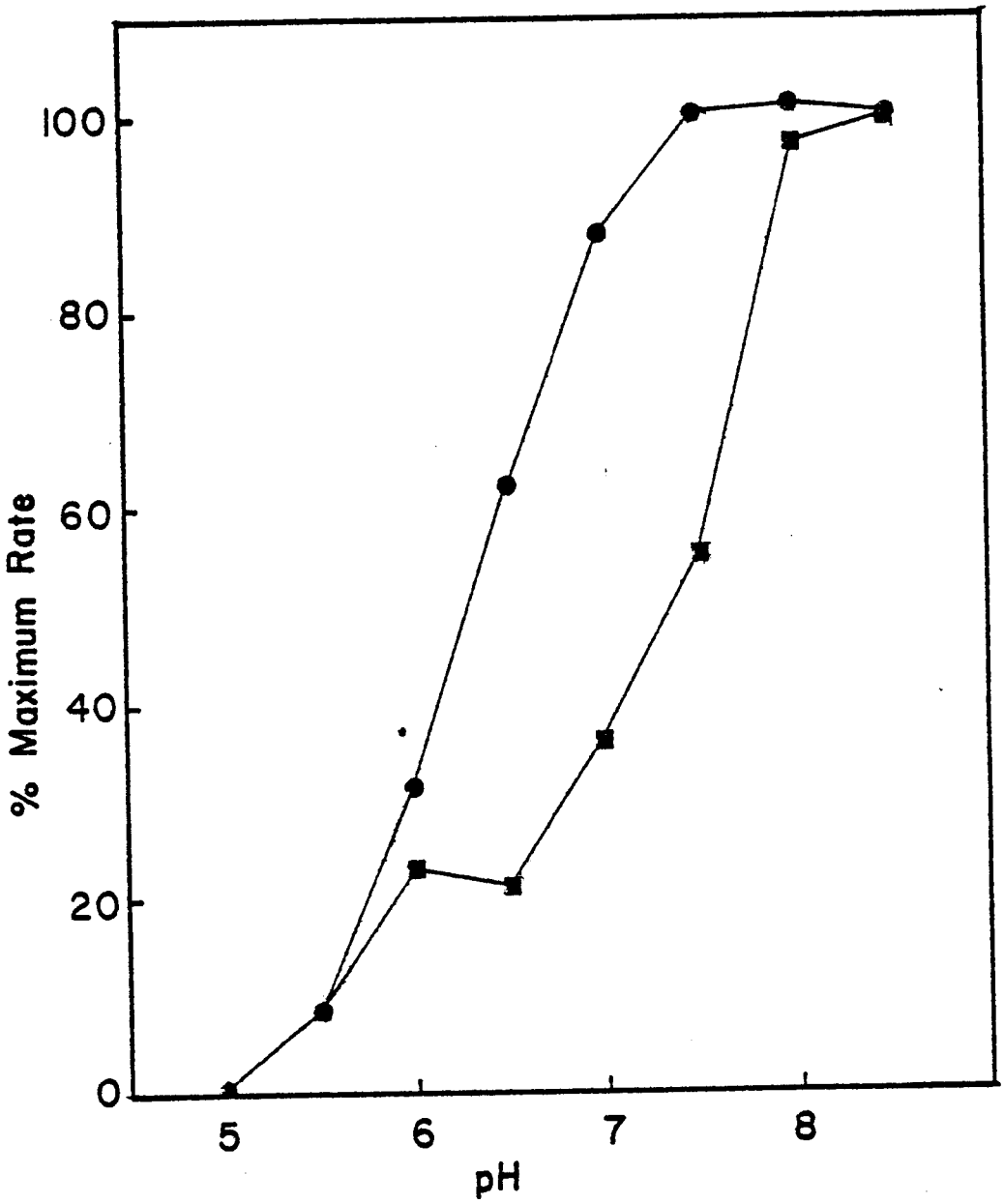


Figure 1. pH profile of leucine and lysine aminopeptidase activity in intact (fresh cells).

Leucine aminopeptidase activity, ●, 100% value at pH 8.5, 3.15 units/mg dry wt cells. Lysine aminopeptidase activity, ■, 100% value at pH 8.5, 2.23 units/mg dry wt cells. Final concentration of both Leu-PNA and Lys-PNA was 5.3×10^{-4}M

aminopeptidase II, was not inhibited by this chelator. Frey and Rohm (3,4) also reported that in crude extracts, only aminopeptidase I (internal peptidase) was affected by this chelator since Zn^{++} was required for the activity of this enzyme. The results indicate that strain 139 has an external aminopeptidase similar to that peptidase of the yeast examined by Rohm (4).

A pH activity profile was also determined in permeabilized cells for both leucine aminopeptidase and $(Met)_3$ peptidase (Fig. 2). Although leucine aminopeptidase activity had a pH optimum at 7.5 while that of $(Met)_3$ peptidase was approximately 7.0, the shape of the activity profile was markedly different indicating different peptidases may be involved in Leu-PNA breakdown and $(Met)_3$ breakdown.

Competition experiments using $(Met)_3$ at 5-fold molar concentrations to that of Leu-PNA demonstrated that the presence of excess $(Met)_3$ had no inhibitory effect on Leu-PNA breakdown. Conversely, competition experiments using Leu-PNA at 6-fold molar concentrations in excess of the $(Met)_3$ concentration demonstrated that the presence of excess Leu-PNA had no inhibitory effect on $(Met)_3$ breakdown at either pH 5.5 or 7.0. These data also indicate that the enzymes involved in Leu-PNA breakdown and $(Met)_3$ breakdown are probably different.

It was thought that if $(Met)_3$ was cleaved extracellularly followed by the cellular uptake of the methionine products, then no external peptidase activity would be detected in intact cells because the products would be transported into the cells. To counter this argument, $(Met)_3$ peptidase activity was determined in intact

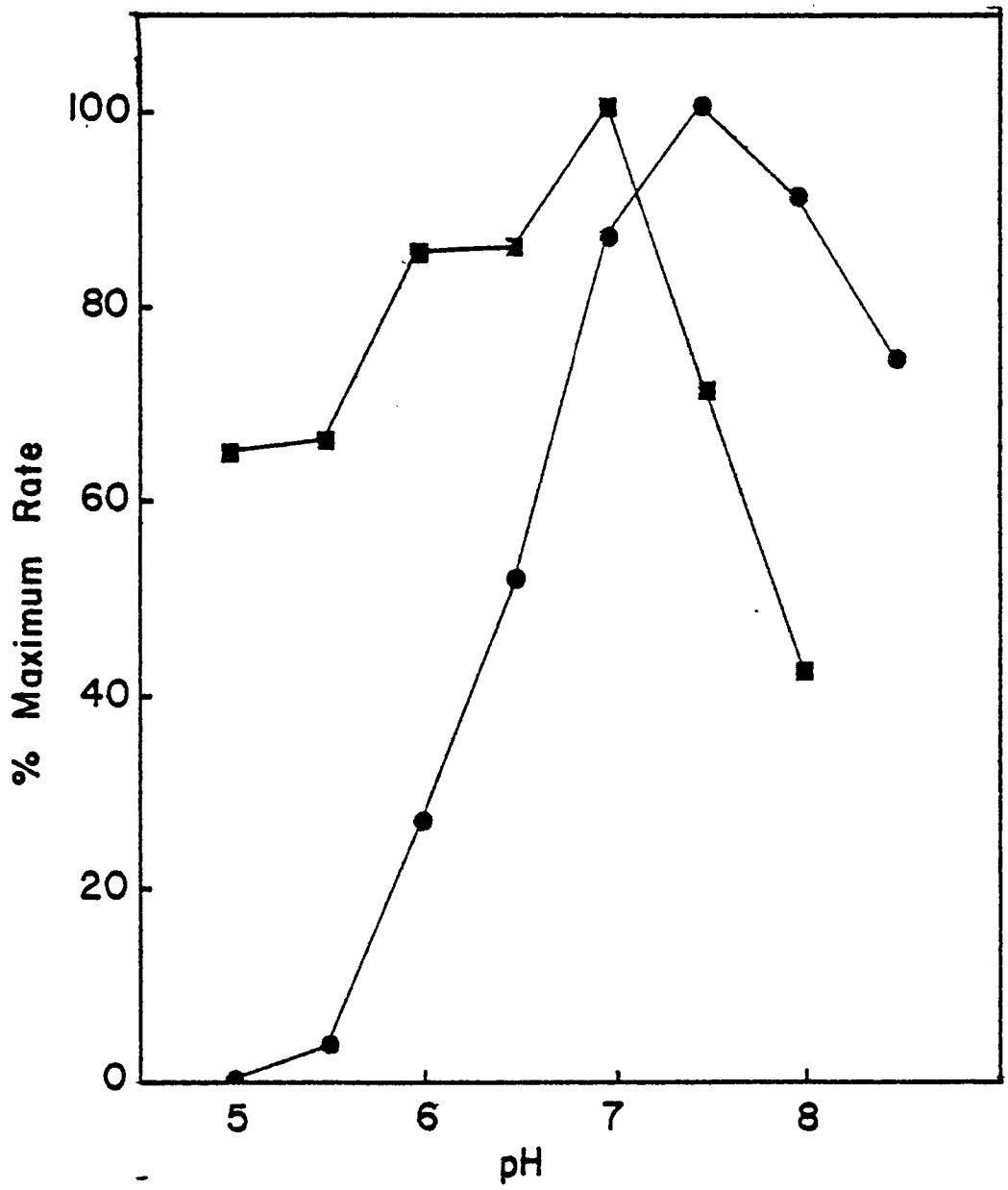


Figure 2. pH profile of leucine aminopeptidase and $(Met)_3$ peptidase in permeabilized cells.

Leucine aminopeptidase activity, ●, 100% value at pH 7.5, 4.72 units/mg dry wt cells. $(Met)_3$ peptidase activity, ■, 100% value at pH 7.0, 113 nmoles Met released/min/mg dry wt cells. Final concentration of substrates was Leu-PNA, 5.3×10^{-4}M; and $(Met)_3$, 3.2×10^{-4}M.

cells in the presence of a 6-fold molar excess glycine, a known inhibitor of methionine transport (12,13). Again, no hydrolysis of $(Met)_3$ was detected.

Peptidase localization. Permeabilized cells were fractionated, and α-glucosidase and $(Met)_3$ peptidase activities were determined (Table 2). As shown, all of the internal marker was recovered in the soluble fraction demonstrating that the permeabilization procedures employed were effective in determining intracellular enzyme activity. Also, 99% of the $(Met)_3$ peptidase activity was recovered in the soluble fraction indicating that the peptidases involved in $(Met)_3$ breakdown are intracellular.

Leu-PNA inhibition of $(Met)_3$ transport. The effect of Leu-PNA on the transport of $(Met)_3$ and methionine was examined (Table 3). As shown, excess Leu-PNA inhibited the transport of $(Met)_3$ by 86% but had no effect on the transport of methionine. At pH 7.0 (the pH optimum for leucine aminopeptidase activity), there was no transport of either substrate indicating that aminopeptidase was not involved in $(Met)_3$ transport. The inhibitory effect of Leu-PNA on $(Met)_3$ transport was further examined in competition experiments. It was determined that the Leu-PNA was a reversible inhibitor of $(Met)_3$ transport and that this inhibition was of a competitive nature (Fig. 3). This Leu-PNA inhibition of $(Met)_3$ transport is also indicative of no extracellular hydrolysis of $(Met)_3$ prior to transport. If $(Met)_3$ were cleaved extracellularly followed by the cellular transport of methionine, then the presence of Leu-PNA should have

TABLE 2. Enzyme activity in fractionated cells.

Fraction	Substrate			
	PNPG		$(Met)_3$	
	Sp. Act.	Total Act.	Sp. Act.	Total Act.
Intact cells	0		0	
Permeabilized cells	0.99	321	101	32724
Low speed pellet	ND		2.5	810
Soluble	1.05	341	99	32130
Membrane pellet	0	0	1.1	360

All assays were performed at pH 7.0. Units of activity are described in Table 1. Final concentrations of substrates were PNPG, 3.3×10^{-4}M; and $(Met)_3$, 3.2×10^{-4}M.

TABLE 3. Effect of Leu-PNA on the transport of $(Met)_3$ and methionine.

Uptake conditions	Substrate	
	$(Met)_3$	Met
Control (pH 5.5, 0.25% glucose)	4.2	16.8
+ Leu-PNA	0.6	16.1
Control (pH 7.0, 0.25% glucose)	0	0
+ Leu-PNA	0	0

Final concentrations of $(Met)_3$, Met, and Leu-PNA were 2.4×10^{-5}M, 4.5×10^{-5}M, and 8×10^{-5}M, respectively. Data is expressed as nmoles substrate transported/min/mg dry wt cells.

Figure 3. Competitive inhibition of $(Met)_3$ transport by Leu-PNA.

Final concentration of Leu-PNA was 2.7×10^{-5}M.

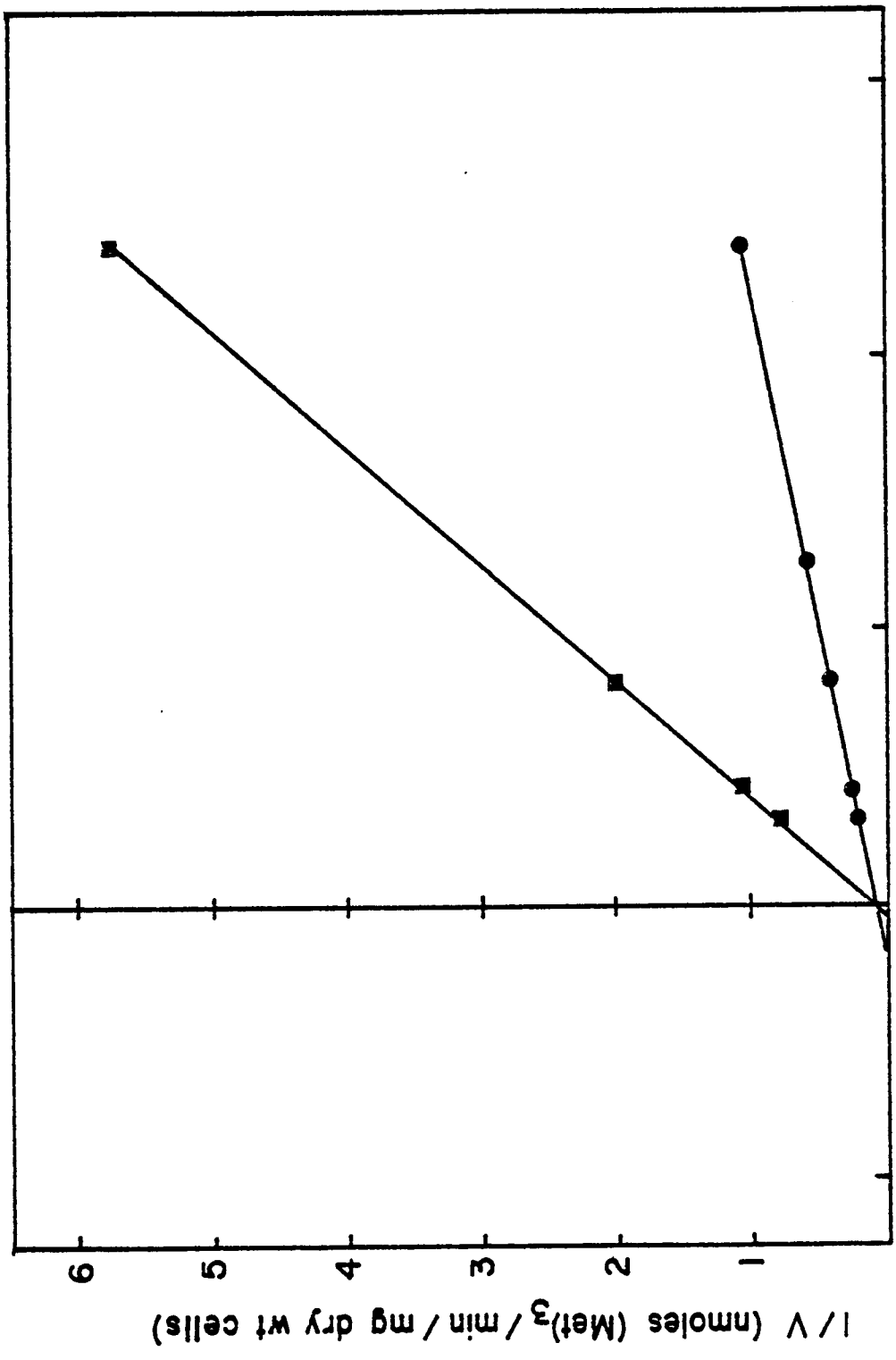


Figure 3.

had no inhibitory effect on the observed transport of $(Met)_3$.

Total peptidase activity. Examination of the kinetic parameters of total peptidase activity led us to conclude that the transport process was the rate limiting step in peptide transport. As shown in Fig. 4, the V_{max} of $(Met)_3$ peptidase activity was 120 nmoles methionine released/min/mg dry wt cells versus a V_{max} of transport of 8.3 nmoles $(Met)_3$ transported/min/mg dry wt cells (Fig. 3). This 14-fold faster rate of hydrolysis versus transport suggested that if $(Met)_3$ hydrolysis occurs extracellularly, the methionine products should have been detected under our assay conditions. Also, these data indicate why we have never recovered intact $(Met)_3$ in cells transporting $(Met)_3$ (6).

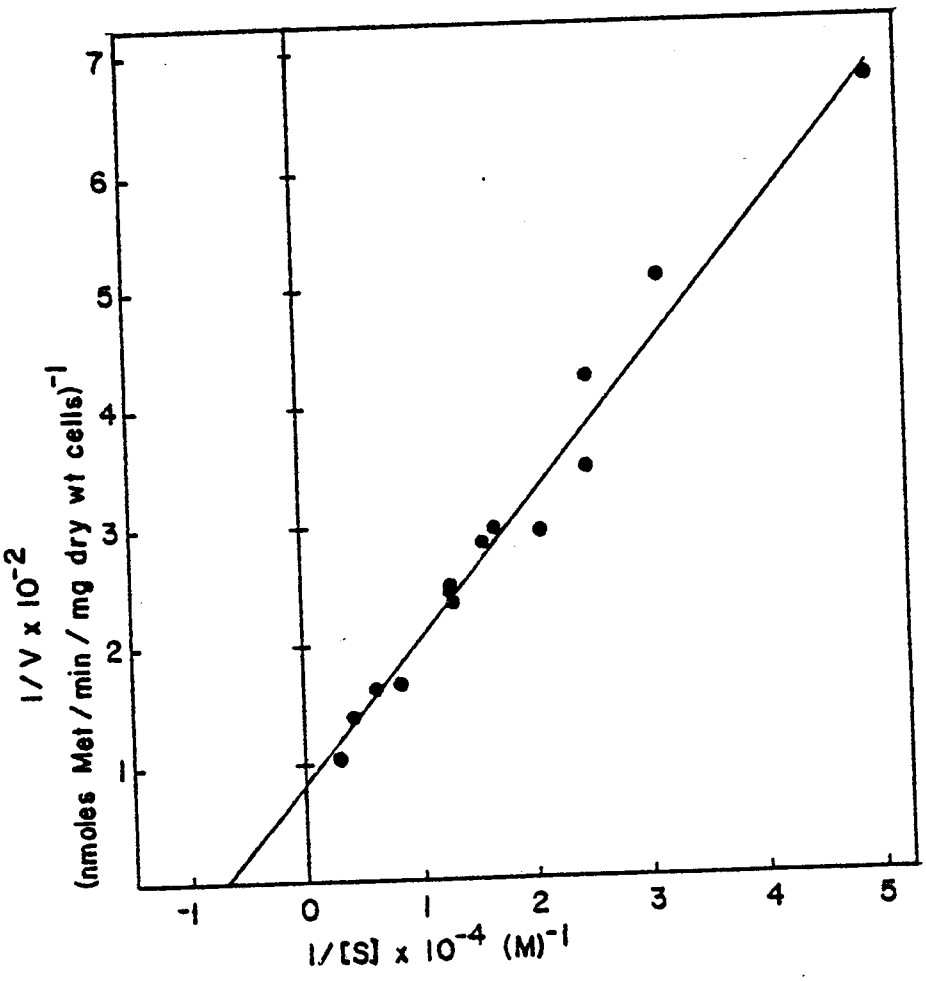


Figure 4. Lineweaver-Burke plot of total $(Met)_3$ peptidase activity in permeabilized cells.

CHAPTER IV

DISCUSSION

In previous studies, on the basis of several different kinds of experiments, we have concluded that peptides are not hydrolyzed extracellularly prior to transport into yeast: amino acids did not compete for peptide transport (6,14), some non-transported peptides were hydrolyzed by cell extracts (5,16), and a peptide transport mutant retained peptidase activity (17). Nevertheless, a recent report of an external aminopeptidase, aminopeptidase II, which possesses some activity towards native dipeptides (e.g. Leu-Gly, Lys-Gly) and a tripeptide (Leu-Gly-Gly) (4) could conceivably cleave peptides externally followed by the cellular transport of the cleaved amino acids. Should this be the case, the accumulation of radioactivity from labelled amino acids would be misinterpreted as peptide uptake where actually amino acid transport was occurring. Therefore it was necessary for us to investigate the involvement of aminopeptidase II in peptide transport in yeast.

The results presented in Table 1, p. 36, demonstrating peptidase activity in intact cells, were the first indication that there was an external aminopeptidase present in S. cerevisiae 139. The activity profile reported for intact cells (Fig. 1, p. 37) is similar to that reported by Frey and Rohm (4) for intact cells as well as for the isolated aminopeptidase II. The sharp drop in activity from pH 7.5 to 5.5 was indicative of an external location of the enzyme according to the following argument. In this pH range, intracellular pH would

not change more than 0.5 pH units and the activity of the enzyme would reflect the intracellular pH, not the pH of the medium in which the cell is suspended. However, the enzyme activity in intact cells directly correlated with the pH of the medium. Thus the enzyme was located extracellularly, or exposed to the medium pH, in intact cells. Other evidence of the similarity of the aminopeptidase activity in S. cerevisiae 139 and aminopeptidase II, as reported by Frey and Rohm (4), includes the insensitivity to nitrilotriacetate, a potent Zn^{++} chelator, and its sensitivity to Cu^{++}.

On the other hand, $(Met)_3$ peptidase activity appears to be localized entirely intracellularly (Table 1, p. 36, Table 2, p. 41). $(Met)_3$ peptidase activity was never detected in intact cells even after a one hour incubation with substrate. As shown in a previous report (7), glucose (or a fermentable energy source) was required for uptake or $(Met)_3$. In the experiments reported in this paper, the assays of $(Met)_3$ peptidase activity were always performed in buffers not containing glucose, which was required for $(Met)_3$ uptake. Thus, the tripeptide substrate remained outside the cells optimizing extracellular hydrolysis conditions. In some experiments (data not shown), glucose was included in the assay buffer. Under these conditions, no free methionine was ever detected extracellularly indicating that once the peptide was transported, methionine was not leaking into the extracellular fluid. As shown in Table 2. p. 41, the location of $(Met)_3$ peptidase was found almost entirely in the soluble fraction (as was the case with -glucosidase). Perhaps the strongest evidence for the intracellular location of the $(Met)_3$ peptidases was

that Leu-PNA inhibited $(Met)_3$ uptake while not affecting methionine uptake. The presence of Leu-PNA should have no effect on the apparent transport of $(Met)_3$ if extracellular hydrolysis was taking place.

The aminopeptidases in yeast and the peptidases responsible for $(Met)_3$ hydrolysis appear to be different enzymes. As stated previously, the location of these enzymes appear to be different with aminopeptidase II being partially external (4) and $(Met)_3$ peptidases being entirely intracellular. In addition, the pH profile of $(Met)_3$ peptidase activity and leucine aminopeptidase activity (Fig. 2, p. 39) suggests that the enzymes are different due to the difference in pH optima and the general shape of the profiles. Also, competition experiments were performed which indicated that the enzymes were different. That is, excess $(Met)_3$ did not inhibit Leu-PNA breakdown while excess Leu-PNA did not inhibit $(Met)_3$ hydrolysis.

The data presented demonstrated that there is no extracellular breakdown of the tripeptide prior to transport. First, the $(Met)_3$ peptidases are located intracellularly (Table 2, p. 41), and $(Met)_3$ does not appear to be a substrate for the aminopeptidase which is located partially externally. Second, in a previous report (7), it was determined that acetate grown cells did not transport $(Met)_3$, but did transport dectable amounts of methionine. These cells contain an extracellular aminopeptidase (data not shown). Therefore, extracellular hydrolysis should have led to measurable $(Met)_3$ uptake. Third, in intact cells, the presence of excess glycine did not cause the detection of any external $(Met)_3$ peptidase activity. The argument in this case is: if $(Met)_3$ were hydrolyzed extracellularly followed by

the cellular transport of methionine, the presence of excess glycine should compete with the methionine transport. Under these conditions, no external methionine was detected indicating no external hydrolysis of $(Met)_3$. Fourth, an examination of the kinetic parameters of peptide transport versus peptidase activity leads us to conclude that for $(Met)_3$ uptake in S. cerevisiae 139 the rate limiting step is transport. The 14-fold difference in V_{max} (120 for peptidase activity versus 8.3 for $(Met)_3$ transport) suggests if extracellular peptide hydrolysis occurs, methionine should be detected under our assay conditions using glycine as an inhibitor of methionine transport. Finally, Leu-PNA inhibits $(Met)_3$ uptake while not affecting methionine transport (see previous discussion).

In conclusion, we have presented evidence showing the rate limiting step in trimethionine transport appears to be that of the transport process itself rather than the internal peptidase activity. We have also demonstrated conclusively, the noninvolvement of external aminopeptidase activity with $(Met)_3$ transport in S. cerevisiae 139. Finally, there is no extracellular hydrolysis of the tripeptide prior to transport by any peptidase since these peptidases appear to be located in the soluble interior of the cell (no membrane association).

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