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PEPTIDE UTILIZATION IN YEAST

STUDIES ON METHIONINE AND LYSINE AUXOTROPHS OF *SACCHAROMYCES CEREVISIAE*

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SUMMARY

A study was made of the growth response and cellular peptidase activity of several amino acid auxotrophs of *Saccharomyces cerevisiae* to peptides containing the required amino acid. A methionine-requiring auxotroph grew on, and contained intracellular peptidase activity toward Met–Met, Met–Met–Met, and Met–Gly–Met–Met. In contrast, Gly–Met–Gly did not support the growth of this mutant nor did three lysine-requiring strains utilize any lysine-containing peptides tested, although cell-free extracts from the respective mutants contained the necessary peptidase activity. The absence of a transport system of relatively high affinity for these peptides is suggested as the reason for their inability to satisfy the nutritional requirements of the cells.

INTRODUCTION

In recent years peptide utilization by bacteria has been studied extensively. A series of investigations carried out on *Escherichia coli* have resulted in the following generalizations with regard to the structural specificity required for peptide transport: (1) dipeptides as well as higher oligopeptides are transported into the bacterium by a different transport system than that utilized in the transport of amino acids[1,2]; (2) dipeptides use a separate transport system from that used by higher peptides[3,4]; (3) all oligopeptides are transported by the same system regardless of amino acid composition[1,3]; (4) there is a size restriction to oligopeptide transport which is related to the hydrodynamic volume of the peptide[5,6]; (5) a C-terminal carboxyl group is necessary for dipeptide transport whereas decarboxylated oligopeptides can be transported[4,7,8]. Until recently it was also believed that a free α-amino group was a prerequisite for oligopeptide transport[2,5]. Payne[9] has demonstrated, however, that N-methylation of glycyl peptides does not inhibit their ability to enter the cell.

Several reports have recently appeared to indicate that uptake of peptides also occurs in mammalian systems. In the process of absorption of protein digestion products it is now believed that peptides may be absorbed intact from the lumen of the gut, hydrolyzed within the mucosal cells, and then transported into the blood as amino acids[10]. Matthews and co-workers[11–13] observed that in several mammals the intestinal absorption of the glycine moiety from diglycine and triglycine was much

more rapid than when the free amino acid was used. A similar result was found for free methionine as compared to methionyl peptide absorption from rat intestine[14].

Yeast organisms are particularly favorable for the study of those aspects of molecular and cell biology that distinguish eucaryotic from procaryotic cells. They are amenable to laboratory manipulation yet contain much of the cellular complexity of higher eucaryotes. Mutants lacking the ability to synthesize individual amino acids provide a means to study the nutritional response to various peptides. A positive growth response to peptides containing the required amino acid is an indication of the utilization of these materials. In the absence of extracellular peptidases such utilization is evidence for the penetration of the peptide into the cell.

Yeast auxotrophs for methionine or lysine were examined for their growth response to some peptides containing methionine and lysine, respectively. The peptidase activity of cell-free extracts of these mutants was determined concurrently. It was found that the methionine mutant grew on L-methionyl–L-methionine (Met–Met), L-methionyl–L-methionyl–L-methionine (Met–Met–Met) and L-methionyl–glycyl–L-methionyl–L-methionine (Met–Gly–Met–Met). In contrast, glycyl–L-methionyl–glycine (Gly–Met–Gly) did not satisfy the nutritional requirement for methionine. All of these peptides, however, were hydrolyzed to their component amino acids by the cell-free extract. None of the three lysine mutants that we examined grew on lysine-containing peptides.

MATERIALS AND METHODS

Yeast strains and growth conditions

Saccharomyces cerevisiae G1333, a methionine auxotroph, originally from the culture collection of Dr R. K. Mortimer, University of California, Berkeley, was kindly supplied by Dr Simchen, The Hebrew University, Jerusalem, Israel. Several *S. cerevisiae* lysine auxotrophs (S288C-24, X3356-1B, and X3357-1C) lacking activities of the first, fourth, and tenth enzymes, respectively, of the lysine biosynthetic pathway were a gift of the Yeast Genetics Stock Center, University of California, Berkeley.

The mineral growth media used for all experiments was Vogel's N-Media modified by the addition of inositol (36 μg/ml), pantothenic acid (2 μg/ml), pyridoxine·HCl (2 μg/ml), thiamine (4 μg/ml), and biotin (0.5 ng/ml). The mineral media were supplemented by amino acids or peptides where indicated and autoclaved at 121 °C for 15 min. Electrophoresis of the media showed that no breakdown of the peptides occurred by this sterilization procedure. After autoclaving, a sterile solution of glucose was added to a final concentration of 2 % (w/v).

The yeast strains were maintained on agar slants composed of 1 % yeast extract, 2 % Bacto-peptone and 2 % glucose. For growth studies the yeast was inoculated into minimal media supplemented with the required amino acid (20 μg/ml) and grown for 24–48 h at 30 °C. The cell crop was harvested by centrifugation, washed twice with distilled water, and resuspended in distilled water. 1 ml of the washed cell suspension ($1 \cdot 10^7$–$5 \cdot 10^7$ cells) was inoculated into 50 ml of mineral media, supplemented with amino acid or peptide. The complete media, contained in a 500-ml erlenmeyer flask fitted with a side-arm, was placed in a reciprocating shaker water bath (110 strokes/min) kept at 30 °C. Growth was determined by the increase in turbidity at 400–420 nm (blue filter) with a Klett–Summerson photoelectric colorimeter.

Chemicals

The methionine-containing peptides Met–Met, Met–Met–Met, Met–Gly–Met–Met and Gly–Met–Gly used in this study were purchased from Schwarz-Mann, Orangeburg, N.Y. Lys–Gly, Gly–Lys and Lys–Lys were purchased from Miles Laboratories, Elkhart, Ind. The higher lysine peptides were the gift of Dr A. Yaron, Weizmann Institute, Rehovot, Israel. The other chemicals used were analytical reagent grade or the purest commercially available. The peptides were homogeneous as judged by paper electrophoresis and amino acid analysis.

Cell-free extract

Cells were harvested at late logarithmic phase of growth (150–200 Klett units), washed twice with distilled water, and resuspended to 1 mg/ml dry weight ($5 \cdot 10^7$ cells/ml) in 0.1 M Tris–HCl buffer (pH 7), containing 50 μg/ml of chloramphenicol or in 0.05 M potassium phosphate buffer (pH 4). Part of the cell suspension (10 ml) and glass beads (20 g; Minnesota-Mining and Manufacturing, 50–70 μm diameter) were added to the 50-ml size Omnimixer stainless-steel cup (Ivan Sorvall Co.). The suspension was kept on ice and homogenized at top speed for 5 min. The glass beads were removed by filtration through a sintered-glass funnel. The unbroken cells and cell debris were removed by centrifugation (600 $\times g$, 5 min). The resulting supernatant solution was used as the cell-free extract.

Peptidase assay

A portion of cell-free extract (usually 100 μl) was incubated at 30 °C with an equal volume of various peptide solutions (2 mg/ml in distilled water). At various time intervals aliquots of the reaction mixture were removed, placed on ice, and applied to paper for electrophoresis. Similar peptidase activities were obtained with cell-free extracts prepared in both phosphate and Tris–HCl buffers.

Osmotic shock

The technique used was a modification of the method developed by Schwencke, *et al*[16]. Logarithmic-phase cells were suspended in a medium of high osmotic pressure containing 1.5 M sorbitol, 1 mM 2-mercaptoethanol, 0.5 mM EDTA and 100 mM Tris–HCl buffer (pH 8). The suspension was stirred for 15 min at 30 °C, the cells harvested by centrifugation, and resuspended in ice-cold distilled water containing 0.5 mM $MgCl_2$. After stirring on ice for 10 min the suspension was centrifuged (600 $\times g$, 5 min) and the supernatant was saved as the shock fluid.

Electrophoresis

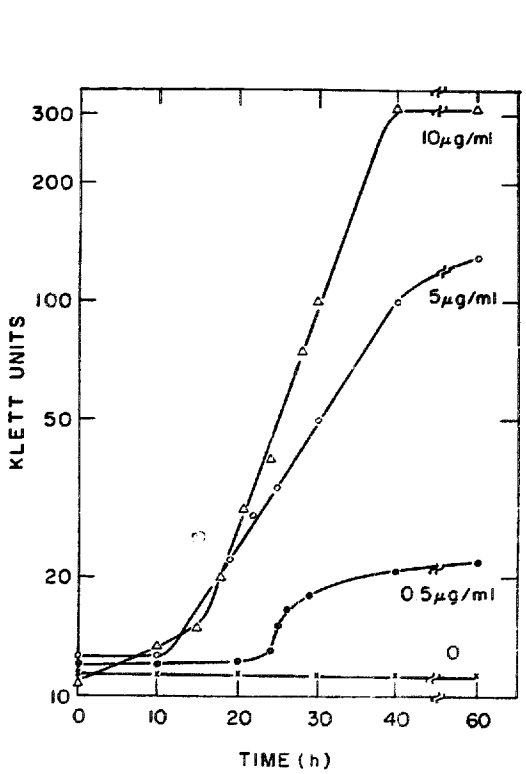
Electrophoresis was carried out in a Model LT-36 electrophoresis tank, E.C. 123 coolant, and an HV-5000 power supply (Savant Instruments). Pyridine acetate buffer (pH 3.5) was prepared from glacial acetic acid–pyridine–water (10:1:89, by vol.). Samples were applied to Whatman No. 3 MM paper and run at a gradient of 50 V/cm for 1–2 h. After electrophoresis the paper was dried, dipped in a solution of ninhydrin (0.5 %, w/v) in 95 % aqueous acetone and developed by heating in a well-ventilated oven.

RESULTS AND DISCUSSION

Growth of S. cerevisiae G1333 on methionine and methionine-containing peptides

The growth response of the methionine mutant to various amounts of L-methionine is represented in Fig. 1. The minimum amount of amino acid yielding maximum growth was 10 μg/ml ($6.7 \cdot 10^{-5}$ M). When the medium was supplemented with more than 10 μg/ml no decrease in generation time or increase in cell crop was obtained. The complete lack of growth without L-methionine supplementation shows the absolute requirement of this mutant strain for this amino acid.

Various peptides were tested to see if they could satisfy the nutritional requirement for methionine. The data in Fig. 2 represent the responses to Met–Met, Gly–Met–Gly, Met–Met–Met and Met–Gly–Met–Met. The media was supplemented with concentrations of peptides which gave the same amount ($6.7 \cdot 10^{-5}$ M) of methionine residues in each growth flask. No response was obtained on Gly–Met–Gly even after incubation for 7 days. This observation is examined in more detail in subsequent sections. The total cell crops were the same for all peptides investigated. However, different generation times were observed for growth on Met (4 h), Met–Met (6 h), Met–Met–Met (7 h), Met–Gly–Met–Met (7 h). The different rates of increase in cell mass found with the peptides as compared with methionine may indicate either differences in the rates of peptide absorption from the medium, or differences in the rates of enzymic hydrolysis.



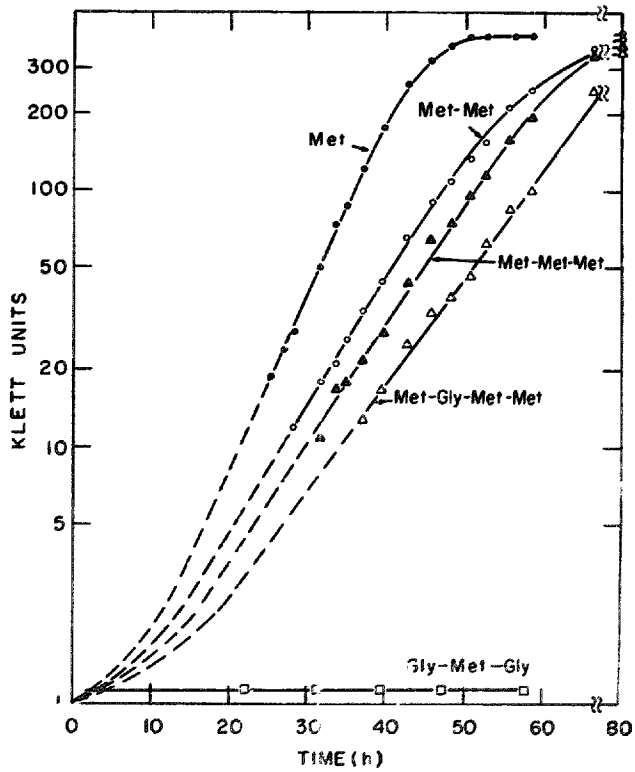


Fig. 1. Growth of methionine auxotroph G1333 in mineral salts medium *plus* L-methionine. ×—×, 0.0 μg/ml; ●--●, 0.5 μg/ml; ○—○, 5.0 μg/ml; △—△, 10 μg/ml.

Fig. 2. Growth of methionine auxotroph G1333 in mineral salts medium *plus* Gly–Met–Gly (□—□), Met–Gly–Met–Met (△—△), Met–Met–Met (▲--▲), Met–Met (○—○), or L-methionine (●—●). Each flask contained the equivalent of $6.7 \cdot 10^{-5}$ M in methionine.

Peptidase activity toward methionine-containing peptides

In order for the yeast mutant to utilize a methionine-containing peptide, the organism must be able to hydrolyze the peptide, thereby releasing free methionine. To test for the presence of peptidase activity toward the methionyl peptides a cell-free extract of strain G1333 was prepared using the technique described in Materials and Methods. The hydrolytic activity toward the three peptides which supported the growth of the mutant is summarized in Table I. The data show that this auxotroph contains peptidases capable of splitting all of the peptides to their component amino acids, although slight differences in rate were detected.

The lack of growth on Gly–Met–Gly may have been due to the inability of the cell to hydrolyze this tripeptide. We examined this possibility by checking the hydrolytic acitivity of the cell-free extract from strain G1333 toward Gly–Met–Gly. This was done in comparison to the hydrolysis of Met–Met–Met, a growth-supporting tripeptide. The standard electrophoresis procedure employed, *i.e.* electrophoresis at pH 3.5 for 60 min, results in good separation between the free amino acids liberated during hydrolysis and Gly–Met–Gly or Met–Met–Met. No satisfactory separation was observed, however, between the tripeptides and the expected dipeptides. The latter could be separated from the tripeptides by carrying out the electrophoresis under slightly modified conditions (*e.g.* pH 1.9 for 120 min). Under the modified conditions the incubation mixture of Met–Met—Met with the cell-free extract was found to contain methionine and Met–Met, as well as the starting tripeptide. Since the purpose of our experiment was to determine the relative rates of release of free methionine from Met–Met–Met and Gly–Met–Gly, we give in Fig. 3 the electrophoretic analysis of the corresponding digestion mixtures, carried out at pH 3.5. The results presented show that free methionine is released from both tripeptides at approximately equal

TABLE I

HYDROLYSIS OF GROWTH-SUPPORTING PEPTIDES

The peptides and the cell-free extract were incubated at 30 °C for the times specified in the table. Hydrolysis products were detected as ninhydrin-positive spots on Whatman No. 3 MM after electrophoresis at pH 3.5.

Peptide	*Products after incubation with cell-free extract*		
Incubation time:	*2 h*	*4 h*	*18 h*
Met–Met	Met–Met Met	Met	Met
Met–Met–Met	Met–Met–Met Met–Met * Met	Met–Met–Met Met–Met * Met	Met
Met–Gly–Met–Met	Peptide mixture ** Gly *** Met ***	—	Gly Met Peptide mixture **, ***

* Met–Met could be separated electrophoretically from Met–Met–Met only at pH 1.9 using Whatman No. 1 paper.

** Undetermined mixtures of methionine- and glycine-containing peptides. We were not able to resolve the various possible products by our electrophoretic system.

*** Trace amounts.

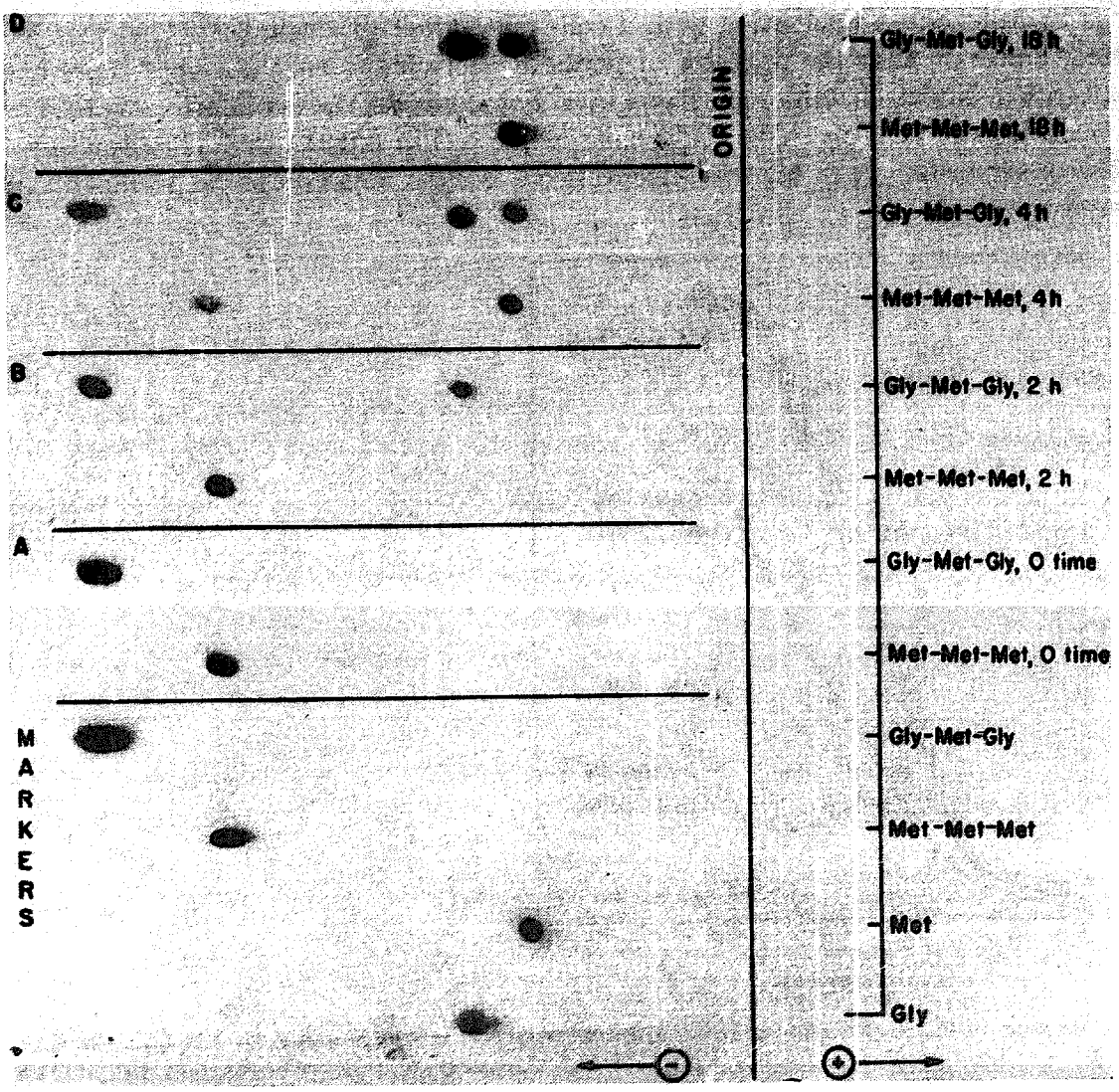


Fig. 3. Paper electrophoresis at pH 3.5 of $(Met)_3$ and Gly–Met–Gly at various time intervals after incubation with a cell-free extract from the methionine auxotroph. Panels A, B, C and D represent incubation for 0, 2, 4 and 18 h, respectively. The panel at the bottom contains the markers only.

rates. Thus, lack of growth on Gly–Met–Gly cannot be ascribed to the absence of peptidase activity toward this peptide.

Growth of strain G1333 on Gly–Met–Gly at various concentrations

Another possible explanation for the absence of growth on Gly–Met–Gly is that the yeast does not have a system of relatively high affinity to transport the peptide to the site of hydrolytic activity. One way to test this possibility is to force the peptide into the cell by creation of a large concentration difference between extracellular and intracellular peptide. This is easily accomplished by supplementing the medium with large amounts of Gly–Met–Gly ($3.3 \cdot 10^{-4} - 6.7 \cdot 10^{-3}$ M). The results of this experiment show that the higher concentrations of the tripeptide support growth of the mutant (Fig. 4). In addition, although several studies have indicated that some peptides inhibit bacterial growth[17,18], the response of the yeast to high Gly–Met–Gly concentrations ruled out the possible toxic effects of this peptide. It was shown above that Gly–Met–

Gly is hydrolyzed on incubation with a cell-free extract of strain G1333. It may be argued, however, that the presence of peptidase activity *iv vitro* does not represent necessarily a similar enzymic activity *in vivo*. For example, the peptidases involved in the hydrolysis of Gly–Met–Gly could exist in the cell in latent form. The growth response of strain G1333 to higher concentrations of Gly–Met–Gly seems to make this possibility remote.

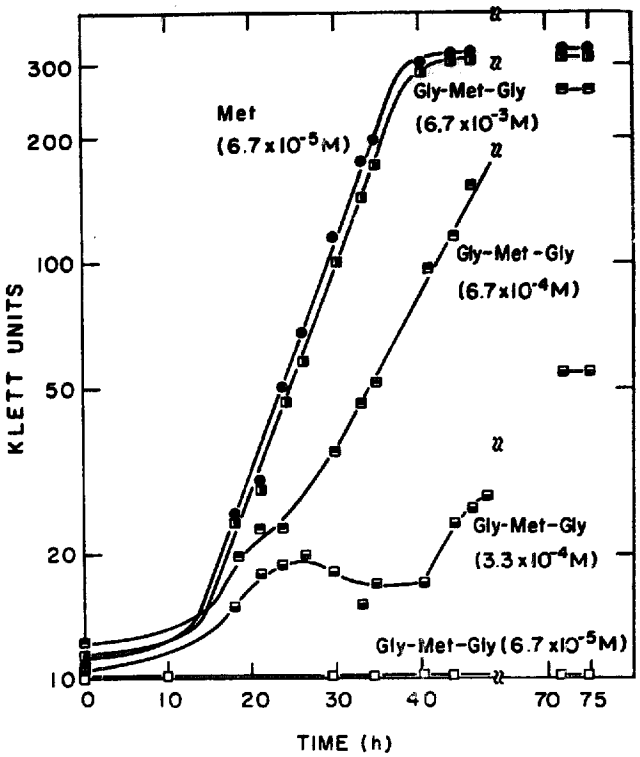


Fig. 4. Growth of the methionine mutant in mineral salts medium *plus* $6.7 \cdot 10^{-5}$ M L-methionine (●—●), or $6.7 \cdot 10^{-5}$ M (□—□), $3.3 \cdot 10^{-4}$ M (▣—▣), $6.7 \cdot 10^{-4}$ M (▤—▤) and $6.7 \cdot 10^{-3}$ M (▮—▮) Gly–Met–Gly.

The data presented in Fig. 4 indicate that the lack of growth on Gly–Met–Gly at low concentrations is due to the absence of a transport system with high affinity for this peptide. However, the report that peptidase activity is sometimes localized in lysosomal-like vacuoles in yeast[19] may suggest that a more complex set of events is taking place. Thus, it is possible that the pathway the peptide takes, en route to hydrolysis, involves permeation of at least two membrane-barriers: the periplasmic membrane and the membrane of the vacuole. In addition, the rate of passage through these barriers is likely to be different making the interpretation of these results all the more difficult. It is interesting to point out that our findings differ from those reported from a similar investigation using an *E. coli* methionine auxotroph. The bacterial mutant was able to utilize Gly–Met–Gly in addition to the other methionine-containing peptides mentioned above[20].

Location of peptidase activity

In the absence of extracellular peptidase activity the growth response of an amino acid auxotroph to a peptide containing the requisite amino acid is an indication of its passage across the cell membrane. One should consider, however, the possibility

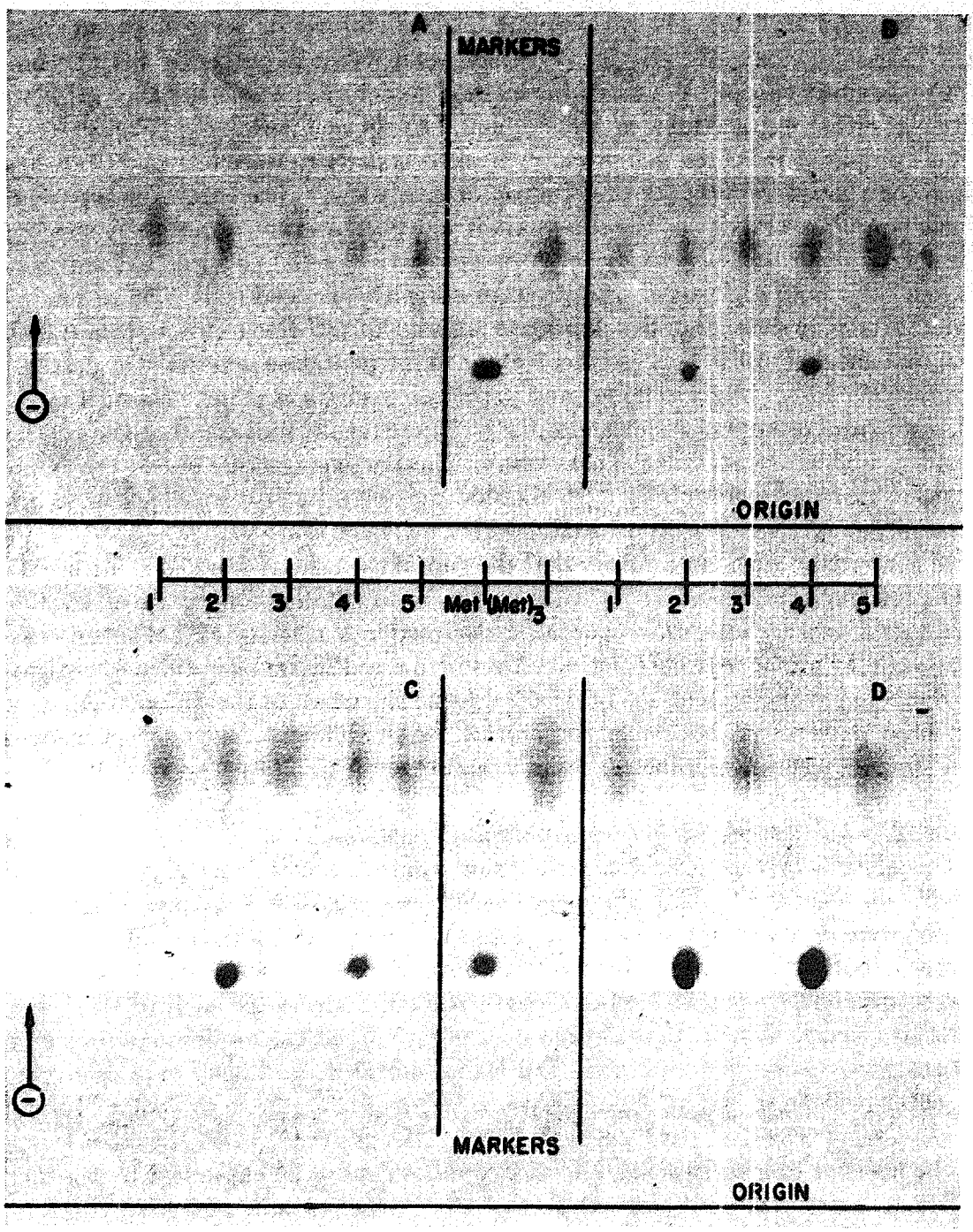


Fig. 5. Paper electrophoresis at pH 3.5 of $(Met)_3$ incubated with whole cells (1); cell-free extract from whole cells (2); supernatant from shocked cells (3); cell-free extract from shocked cells (4); and distilled water (5). Panels A, B, C and D represent incubation times of 0, 2, 4 and 24 h, respectively. The markers are represented in the middle panel.

that some peptidase activity is located within the cell envelope leading to peptide hydrolysis at the cell surface. Although such a possibility seems rather remote in view of the absence of growth on Gly–Met–Gly, an attempt was made to determine in somewhat greater detail the location of the relevant peptidase activity.

The preparation of spheroplasts of the mutant was attempted in order to compare total peptidase activity of a cell-free extract to a spheroplast extract. The cells,

however, were refractory to several techniques of spheroplasting using snail gut enzyme. The release of extracellularly located enzymes in yeast has recently been accomplished by osmotic shock[16]. We used this method to determine whether the peptidase activity was either extracellular or loosely bound to the cell periphery. A cell-free extract was prepared from the osmotically shocked cells (see Materials and Methods) by the same procedure used for preparation of a cell-free extract from intact whole cells. The peptidase activity of the supernatant solution from the osmotically shocked cells was compared to the activity in: untreated whole cells; cell-free extract from unshocked cells; and cell-free extract from osmotically shocked cells. The results represented in Fig. 5 show that the peptidase activity of cell-free extracts from whole cells (2) and shocked cells (4) is identical. Only slight peptidase activity was detected after 24 h with whole intact cells (1) and peptidase activity was not observed in the supernatant from osmotically shocked cells (3). These results indicate that the peptidase activity is not extracellular. To ascertain whether our osmotic shock procedure released enzymes we determined that alkaline pyrophosphatase was present in the supernatant, in agreement with the findings of Schwencke *et al.*[16].

In connection with our findings that the peptidase activity studied is intracellularly located in *S. cerevisiae* G1333, it is pertinent to recall the findings of Matile[21] concerning the extracellular aminopeptidase detected in *S. cerevisiae* ETH strain 1022. No extracellular aminopeptidase activity toward the methionine peptides investigated was detected in the present study. This might be the result of the different enzymic topographic patterns of both microorganisms, or of different enzymic specificities toward leucine- and methionine-containing peptides.

Response of lysine auxotrophs to lysine-containing peptides

The growth of the three different lysine mutants (S288C-24, X3356-1B, and X3357-1C) on a series of lysine oligopeptides was investigated. The three lysine mutants used were deficient in the first, fourth and last enzymatic activities of the lysine biosynthetic pathway in yeast. None of the mutants grew on minimal media unless they were supplemented with lysine. Growth response curves for each of the lysine mutants to L-lysine showed that 10 μg/ml ($6.8 \cdot 10^{-5}$ M) of the amino acid yielded a maximum growth rate and cell crop. The lysine mutants, however, were unable to grow on the following lysine oligopeptides supplemented at $6.8 \cdot 10^{-5}$ M in lysine: $(Lys)_2$, Lys–Gly, Gly–Lys, $(Lys)_3$, $(Lys)_4$, $(Lys)_6$, $(Lys)_7$, $(Lys)_8$ and $(Lys)_n$ ($n = 95$).

The absence of growth on lysine oligopeptides cannot be explained by the lack of peptidase activity as cell-free extracts of the lysine mutants showed hydrolytic activity towards the oligopeptide series as determined using paper elelctrophoresis. Neither trilysine nor polylysine at $6.8 \cdot 10^{-5}$ M (lysine equivalent)inhibited the growth of strain X3356-1B supplemented with lysine. The lack of growth on lysine oligopeptides is apparently the result of the inability of the cells to take up these compounds.

CONCLUSION

The extensive investigations of peptide utilization in bacteria have uncovered information about the structural specificity of the peptide transport system. *E. coli* and some other bacterial amino acid auxotrophs generally contain peptide transport systems through which short oligopeptides, regardless of amino acid composition, enter

the cell. It is important to ascertain whether such findings can be generalized to eucaryotic cells as the uptake of peptides in higher organisms seems to be of physiological significance. For example, in the syndrome known as Hartnup Disease, a condition in which there is a defective intestinal absorption of neutral amino acids, nutrition is dependent on the uptake of peptides containing these amino acids[22,23].

Our results demonstrate that in strain G1333 peptides are absorbed, broken down by intracellular peptidases, and used nutritionally. These findings are similar to those reported in analogous studies with *E. coli*. It is pertinent to note, however, that the nature of peptide transport in *S. cerevisiae* seems to differ from that in *E. coli*. Thus, we observed that the utilization of methionine peptides by strain G1333 depends to some extent on the chemical structure of the peptide.

The inability of the yeast mutant to utilize Gly–Met–Gly was shown to be due to the lack of an appropriate transport system. Thus suggesting for yeast, a different specificity for the transport of the methionine-containing peptides than that recorded for a methionine auxotroph of *E. coli*[20]. The finding that three lysine auxotrophs were unable to utilize lysine-containing peptides further indicates that the nature of peptide transport in *S. cerevisiae* may be very different from that in bacteria. We are currently continuing these studies to determine the nature of the structural requirements of the peptide transport system in strain G1333 as compared to that in *E. coli*.

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