JOURNAL OF BACTERIOLOGY, June 1975, p. 949-956
Copyright © 1975 American Society for Microbiology
Vol. 122, No. 3
Printed in U.S.A.

Size Restriction on Utilization of Peptides by Amino Acid Auxotrophs of *Neurospora crassa*

L. WOLFINBARGER, JR.,* AND GEORGE A. MARZLUF
Department of Biochemistry, Ohio State University, Columbus, Ohio 43210

Received for publication 17 January 1975

Growth of an amino acid auxotroph of *Neurospora crassa* on oligopeptides is shown to occur by extracellular hydrolysis, with subsequent utilization of monomer amino acid residues, and by transport of peptides. Peptides with a hydrodynamic volume greater than that of trileucine are not transported, and this lack of transport is shown to be due to restriction by the oligopeptide transport system rather than the cell wall.

Within the last few years, numerous studies have been published describing the transport and hydrolysis of peptides in both prokaryotic and eukaryotic organisms (1, 7, 9). The studies in bacteria have resulted in the characterization of transport systems for dipeptides and oligopeptides (2-4, 9). The specificities of these two systems for substrate molecules have been well characterized, and the reader is referred to the excellent review by Payne and Gilvarg (9) for further reading in this area.

Of particular interest were the studies describing the importance of the size of oligopeptides in relation to their ability to support growth of amino acid auxotrophs (8). Fractionation of a commercial enzymatic protein hydrolysate (Neopeptone) by using a Sephadex G-15 column resulted in the separation of the peptides as a function of their hydrodynamic volumes. It was found that oligopeptides above a given size would not support growth of auxotrophs, and this effect was attributed to the inability of these larger peptides to permeate through the bacterial cell wall (8).

Molecular sieving studies with *Neurospora crassa* cell walls (10, 11) suggested that *Neurospora* should not exhibit such growth restrictions on large oligopeptides since the average pore size was sufficient to admit molecules approaching a molecular weight of 4,750. Because we had already suggested (14) that *Neurospora* possesses an oligopeptide, but not a dipeptide, transport system, it seemed desirable to determine the upper size restriction of peptide for transport. The studies of Trevithick and Metzenberg (10, 11) on the pore size of cell walls suggested to us that an inability to transport a peptide would probably not be due to cell wall restrictions and, thus, failure of *Neurospora* auxotrophs to grow on a given oligopeptide would be indicative of a failure to be transported by some membrane system.

The data reported here do indeed indicate that a restrictive mechanism prevents growth on oligopeptides above a given hydrodynamic volume, and it is shown that the restriction is not due to exclusion by the cell wall or a failure of intracellular enzymatic digestion (proteolysis) of the peptides. Furthermore, a mutant defective in the oligopeptide transport system is described.

MATERIALS AND METHODS

Strains and growth conditions. The strains employed in this study were, with the exception of *tyr-s* (1) and *glt-r* (1), obtained from the Fungal Genetics Stock Center, Arcata, Calif. The wild type used was Tatum a(SY4f$_s$a). All strains were maintained on Vogel minimal medium N (12) supplemented with 2% sucrose, 2% agar, and amino acids or other supplements as required.

Growth assays. To determine the growth responses to a supplement, growth tests were run in 125-ml Erlenmeyer flasks containing 31 ml of liquid minimal medium supplemented with 2% sucrose and other supplements as indicated in the text. The flasks were inoculated with 1 to 2 drops of a nonturbid (approximately 2,000 to 3,000 conidia per ml) conidial suspension and incubated in stationary culture in continuous light at 30 C for 3 days. After this growth period, each mycelial mass was harvested, pressed dry of excess moisture, and dried in a drying oven (60 C) to a constant dry weight. The data were recorded as milligrams (dry weight) per mycelial pad.

Cell-free extracts. The cell-free extracts and peptidase assays were as described previously (14), except that the cells were ground in a mortar with an equal weight of acid-washed sand.

Column chromatography on Sephadex G-15: (i) Column calibration. A column of Sephadex G-15 was prepared essentially as described by Payne and Gilvarg (8). The dry gel (100 g) was allowed to swell in eluent (0.1 M sodium chloride in 0.01 M sodium

phosphate, pH 7) and was poured into a column (100 by 2.5 cm). This column was used in the fractionation of Neopeptone and was calibrated by using a homologous peptide series of both lysine and leucine.

The peptides of known sequence and size (used for calibration) were applied in 1-ml volumes at variable concentrations depending on whether the resultant fractions were to be assayed for support of growth of an auxotroph or spectrophotometrically. Fractions of 4 or 5 ml were collected. Blue dextran was used to determine the void volume (160 to 170 ml) that eluted in fractions 20 to 25. Because the column was repoured several times over the course of the study, the void volume was variable. Each column run was corrected by normalization to its void volume, i.e., that fraction at which blue dextran eluted.

(ii) Fractionation of Neopeptone. Neopeptone (1 g) was dissolved in 5 ml of eluent and centrifuged to remove insoluble oligopeptides. The clear, amber-colored supernatant was applied to the column and eluted in 4-ml (200-drop) fractions. The fractions were stored frozen until used. One-milliliter volumes of these fractions were used in the growth assays. The concentration of peptides in each fraction varied some between column runs, but was on the average 1 to 2 mM as measured by the trinitrobenzene-sulfonic acid (TNBS) assay of free amino groups (5).

Hydrolysis of Neopeptone fractions. Samples (1 ml) were removed from fractions beginning with the void volume and added to an equal volume of concentrated (12 N) HCl in constricted test tubes. The tubes were flushed with nitrogen, sealed, and autoclaved overnight (18 h) at 15 lb/in^2. The hydrolysates were taken to dryness under reduced pressure in the presence of $CaCl_2$ and NaOH. The residues were redissolved in buffer (0.1 M NaCl in 0.01 M sodium phosphate, pH 7.0) and stored frozen until used.

TNBS assays. The TNBS assay of free amino groups (5) was performed by adding 10 μl of each fraction (both before and after acid hydrolysis) to 1 ml of buffer (0.1 M 2-dimethylaminoethanol, pH 9.0) and 1 ml of 0.1% TNBS. This solution was allowed to react for 30 min at room temperature, and then 1 ml of 10% sodium dodecyl sulfate and 0.5 ml of 1 N HCl were added. The absorbance, at 340 nm, of each sample was determined immediately in a spectrophotometer. The blank consisted of 10 μl of distilled water treated as the samples were treated. The molar concentrations of amino groups were determined by using an extinction coefficient at 340 nm of 14,000 (5). The presence of α-amino groups of lysine was considered to remain constant in both the acid-hydrolyzed and non-acid-hydrolyzed samples and thus was insignificant in subsequent determinations of ratios of amino groups before and after hydrolysis of the oligopeptides.

Protein assays. Protein concentrations were determined by the method of Lowry et al. (6).

RESULTS

Column chromatography of Neopeptone. Because the results reported here depend on the nature of the peptides occurring within a given fraction, we feel it essential to briefly discuss certain aspects of the procedure. The procedure employed has been carefully described by Payne and Gilvarg (8), who used it in a study with bacteria similar to that undertaken here with *Neurospora*.

The high molar concentration of salt (0.1 M sodium chloride in 0.01 M sodium phosphate buffer, pH 7.0) was reported to negate interactions of charged peptides with free carboxyl groups introduced into the gel during its manufacture (see reference 8). Aromatic amino acid-containing peptides will be retarded on the column, i.e., some larger peptides might be shifted from early to later fractions. However, this does not alter the interpretations of the data, since the primary concern is with the upper size of a peptide and the presence of aromatic groups would not shift small peptides into the earlier fractions.

Although peptides of a homologous series elute from the column in a linear relation of molecular weight versus fraction number, the column basically separates heterogeneous mixtures of peptides on the basis of their hydrodynamic volume. Thus, L-lysyl-L-lysine will be found to elute along with hexaglycine or near to L-leucyl-L-leucyl-L-leucine. This will be an important point to consider when interpreting the data obtained using fractionated Neopeptone.

Fractionation of Neopeptone. To follow the elution of Neopeptone from the column, 0.1-ml samples were removed from each fraction, beginning with the void volume, and assayed for peptide content. Figure 1 shows an initial high concentration of peptides occurring in the fractions near the void volume and declining slowly thereafter.

Growth of leu-2 on Neopeptone fractions. The various fractions were next tested for their ability to support growth of a leucine auxotroph, *leu-2* by transferring 1 ml of each fraction into Vogel minimal medium N (30 ml) supplemented with 2% sucrose, autoclaving, and inoculating with conidia of *leu-2*. Figure 2a shows that the early fractions (fractions 21 through 29), which contain larger peptides, do not support growth of *leu-2*, whereas the later fractions (fractions 30 through 44) support growth comparable to free leucine supplied at approximately 0.15 mM. None of these fractions inhibits growth of the wild-type strain. Fractions 39, 40, and 41 provided for greater growth than fractions 30 through 38; this may be explained by the finding that amino acid analyzer assays of fraction 40 revealed a large amount of neutral aliphatic amino acids, with leucine itself con-

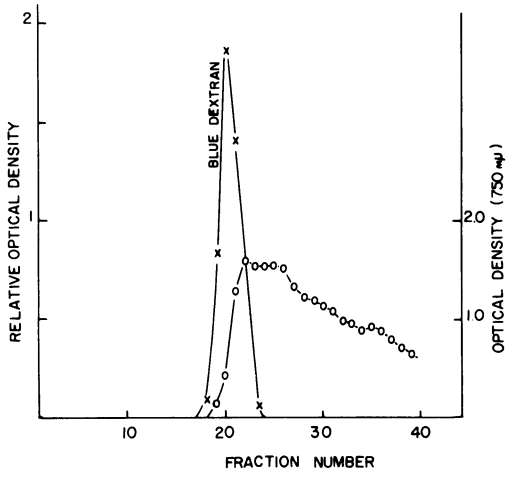

FIG. 1. *Optical density at 750 nm of fractions of fractionated Neopeptone assayed by the method of Lowry et al (6). Fraction volumes as indicated in text. The values indicated in the figure as "blue dextran" are included to indicate the void volume of the column run. Blue dextran was used only in calibration runs and was not included in the column runs used in fractionating the Neopeptone.*

stituting 39% of the total ninhydrin-positive material (see Fig. 7a).

To test for the possibility that peptides of the early fractions may not contain leucine, they were acid hydrolyzed (1-ml aliquots) and then added to similar growth assays. Figure 2b shows that after acid hydrolysis fractions 21 through 29 supported significant levels of growth of *leu-2*. The absence of leucine in peptides of the early fractions cannot, therefore, be the reason for their inability to support growth of *leu-2*. There are several other possible reasons why the early fractions will not support growth. First, intracellular peptidase activity may not be able to hydrolyze the larger peptides; second, the larger peptides (above a given hydrodynamic volume) may not be able to traverse the cell wall and/or membrane. A third possibility is that none of the peptides (in any of the fractions) can enter the cell, and growth on fractions 30 through 44 is the result of extracellular peptidase activity that liberates leucine from smaller external peptides into the medium.

Intracellular peptidase activity. Assays of cell extracts from the wild type (grown on minimal medium) and *leu-2* (grown on minimal medium plus Neopeptone) revealed the capability of hydrolyzing peptides contained in all fractions (21 through 40). Indeed, two-dimensional chromatography (14) of the acid-hydrolyzed and intracellular peptidase hydrolysis products of fraction 26 revealed a similar pattern of 18 ninhydrin-positive spots where no such spots existed in the unhydrolyzed fraction.

The third possibility mentioned above was examined by concentrating the medium, using Amicon filtration (14), in which *leu-2* had grown. The concentrated fluid was dialyzed overnight against tris(hydroxymethyl)aminomethane-hydrochloride buffer (14) before use in peptidase assays. These assays revealed only a small amount of extracellular peptidase activity. Furthermore, this activity appeared to liberate equivalent amino acids (or small peptides) from all fractions of the fractionated Neopeptone. However, as shown below, this extracellular peptidase does play a role in growth on exogenous peptides. To further test the possibity that growth on the later fractions might be due to extracellularly liberated leucine,

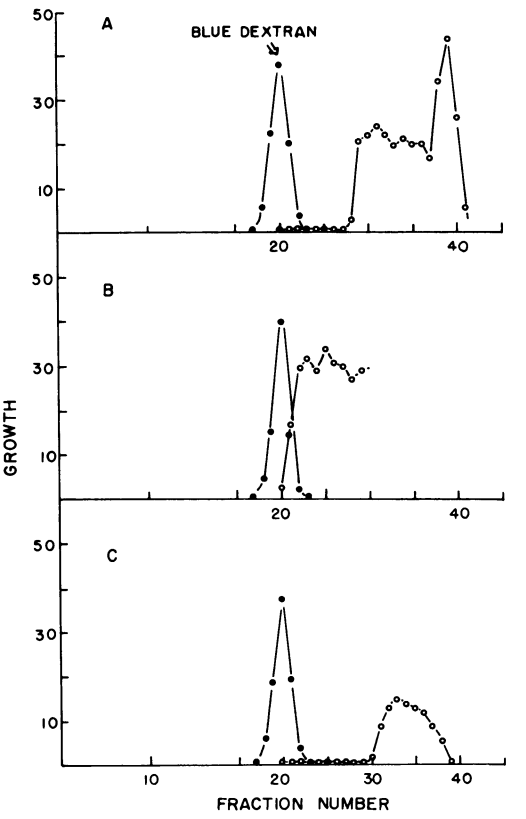

FIG. 2. *Growth (milligrams [dry weight] of mycelial pad) of leu-2 on 1-ml aliquots of the various fractions of fractionated Neopeptone (A), on acid-hydrolyzed fractions of fractionated Neopeptone (B), and on the fractions of fractionated Neopeptone plus 3.3 mM L-phenylalanine (C). The "peaks" indicated as blue dextran were included to indicate the void volume as it related to subsequent fractions of the Neopeptone.*

the growth assays of all fractions were repeated but in the presence of 3.3 mM L-phenylalanine. It has been reported previously that excess L-phenylalanine will block growth of leucine auxotrophs on L-leucine and on leucine derived from the extracellular hydrolysis of peptides (14). Our data (Fig. 2c) show that in the presence of L-phenylalanine only fractions 32 through 40 support growth of *leu-2* and to a smaller extent than observed in the absence of phenylalanine. Thus, the upper size limit for transport of peptides occurs at fraction 32. These data suggest that the growth of *leu-2* on fractions 30 through 44 derived from Neopeptone is due both to liberation of leucine by extracellular peptidases and to direct entry of peptides into the cell.

Why does the apparent extracellular liberation of free leucine from peptides begin abruptly at fraction 30? We feel it may be due to a limited, but significant, ability of the spores to initiate growth on the smaller peptides of the later fractions, with a subsequent release of extracellular peptidase(s). The larger peptides may not enter the cell at all and, consequently, the leucine auxotroph may not even germinate in their presence. If this hypothesis is correct, the inclusion of very small amounts of free leucine, in addition to aliquots of the fractions of Neopeptone, should permit growth of *leu-2* on all fractions. Inclusion of 1 μmol of L-leucine in each flask (this amount of leucine alone allows 7 to 9 mg [dry weight] of growth) enables *leu-2* to grow on all fractions of Neopeptone (Fig. 3). Those fractions of Neopeptone that alone supported growth of *leu-2* (i.e., fractions 30 through 44) exhibit a simple additive growth effect to that of the added free leucine. However, the early fractions (21 through 30), inactive alone, clearly supported significant growth above that provided by just the added leucine. That the fractions (21 through 30) permit more growth as the peptides in a given fraction become shorter may be correlated with an apparent greater rate of enzymatic (intracellular peptidases) hydrolysis as the size (mean number of residues) of the peptide decreases (Wolfinbarger, unpublished data). It thus appears that growth of *leu-2* on smaller peptides can occur both by direct uptake of the peptides followed by their intracellular hydrolysis and by free leucine derived from extracellular peptide hydrolysis by external peptidases. The later mechanism can only occur with larger peptides. Thus, growth of *leu-2* on larger peptides cannot occur unless there is sufficient initial growth for synthesis and release of extracellular peptidase(s). It seems clear that growth is restricted on peptides above a certain size and that this restriction occurs at the level of entry of the peptide into the cell.

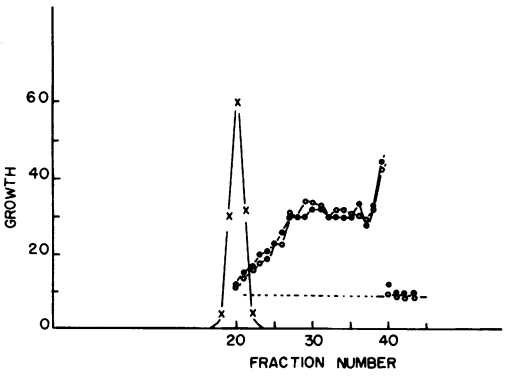

FIG. 3. *Growth (milligrams [dry weight] of mycelial pad) of leu-2 (O) and leu-2, glt-r (●) on 1-ml aliquots of the various fractions of fractionated Neopeptone plus 1-μmol of L-leucine per 31 ml of medium. Those growth values in fractions 40 through 44 indicate the level of growth obtained solely on the leucine (also indicated by the dashed line).*

Before examining the restrictive mechanism for peptide utilization, we should perhaps take a closer look at the peptides in the fractions themselves. The growth data indicate that support of growth ceases with peptides of a greater hydrodynamic volume than that of trileucine. What is the average number of amino acid residues in the peptides eluting in a given fraction? This may be found by quantitation of free amino groups present before and after acid hydrolysis (5, 8). Figure 4 shows the concentration of "free" amino groups in each fraction before and after acid hydrolysis. The concentration in the non-acid-hydrolyzed fractions was fairly constant at 1 to 2 mM, whereas the concentration of free amino groups, i.e., amino acids, in the acid-hydrolyzed fractions jumped dramatically to 14 mM in fraction 23 and declined slowly to 2 mM by fraction 44. The ratios of these values (acid hydrolyzed/non-acid hydrolyzed) are plotted in Fig. 5 and reveal that fractionation of Neopeptone by the Sephadex G-15 column resulted in separation of peptide molecules into fractions in which the mean number of amino acid residues varies from 30 (at the void volume) to 2 (latest fractions collected). The point at which support of growth ceased is indicated in Fig. 5 and is seen to occur at a mean number of residues equal to four to five. This does not mean that all peptides larger than four to five residues cannot support growth, since the physical parameter(s) of the peptides responsible for separation was their hydrodynamic volume. However, on the aver-

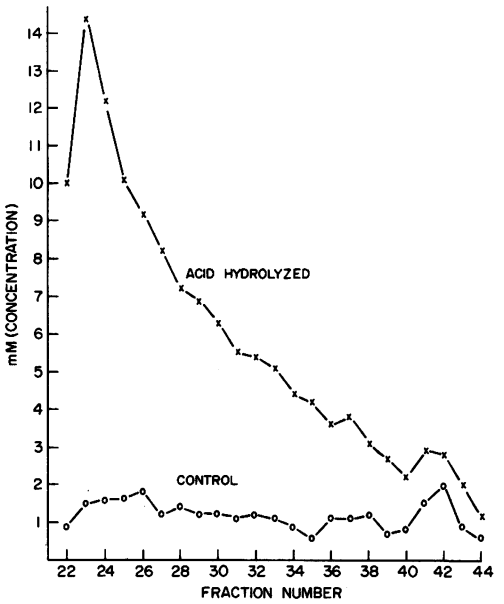

FIG. 4. *Concentration (millimolar) of amino groups, i.e., peptides and/or amino acids, in each fraction of the fractionated Neopeptone* (O) *(control) and acid-hydrolyzed fraction* (x) *of Neopeptone.*

age, peptides containing more than five amino acids would not be expected to support growth of *Neurospora*.

Characterization of the restrictive mechanism. As shown above, *N. crassa* (*leu-2* specifically) is incapable of growing on peptides that do not inhibit growth and that it is capable of enzymatically hydrolyzing inside the cell. It would appear, therefore, that this organism cannot transport peptides above a given hydrodynamic volume. Three possible explanations for this apparent lack of permeation across the peripheral cell barrier are as follows. (i) The cell wall acts as a permeability barrier by physically preventing peptides larger than the available pore size in the cell wall from traversing through to a membrane oligopeptide transport system. (ii) The oligopeptide transport system does not "recognize" (or will not handle) peptides above a given "size" as substrate molecules. (iii) The close proximity of the cell wall and membrane prevents proper alignment of larger peptides with the binding site of the transport system, i.e., steric hindrance.

The possibility that the cell wall of *N. crassa* is acting as a permeability barrier by restricting access of oligopeptides to a peptide transport system might be tested by use of a *leu-2* auxotroph with larger pores in its cell wall. The mutant *os-1* has been reported by Trevithick and Metzenberg (10, 11) to be altered in the properties of its cell wall such that the pores of this mutant are approximately four times larger than comparable pores in the cell wall of the wild type. For example, the "exclusion threshold" (11) of *os-1* (osmotic) and the wild type occur at molecular weights corresponding to 18,500 and 4,750, respectively.

The double mutant *leu-2;os-1* was constructed as a leucine auxotroph unable to grow on leucine-supplemented minimal medium in the presence of 4% NaCl, and used in growth assays on the fractions of fractionated Neopeptone. The *leu-2;os-1* double mutant is virtually equivalent to the *leu-2* parent for those fractions which will and will not support growth either in the presence or absence of excess L-phenylalanine (Fig. 6).

Therefore, contrary to that reported for bacteria (8), the cell wall of *Neurospora* does not appear to act as a permeability barrier to oligopeptide molecules containing more than four to five residues but less than a molecular weight of approximately 4,750 (10, 11). Also, the possibility that the close proximity of the cell membrane and wall sterically hinders access of the peptides to the peptide transport system becomes more remote. It might be expected that the increased porosity of the cell walls

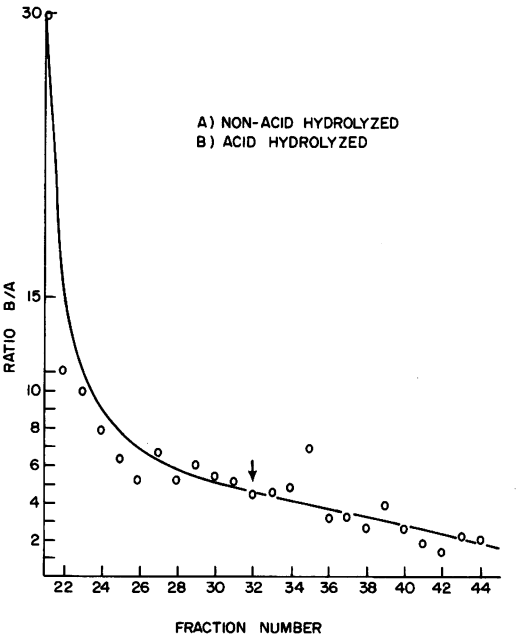

FIG. 5. *Ratio of millimolar concentrations of amino groups in acid-hydrolyzed fractions of Neopeptone (see Fig. 4) over the concentrations of amino groups of non-acid-hydrolyzed fractions of Neopeptone. Fractions 21 through 32 do not alone support growth of leu-2 (see Fig. 2).*

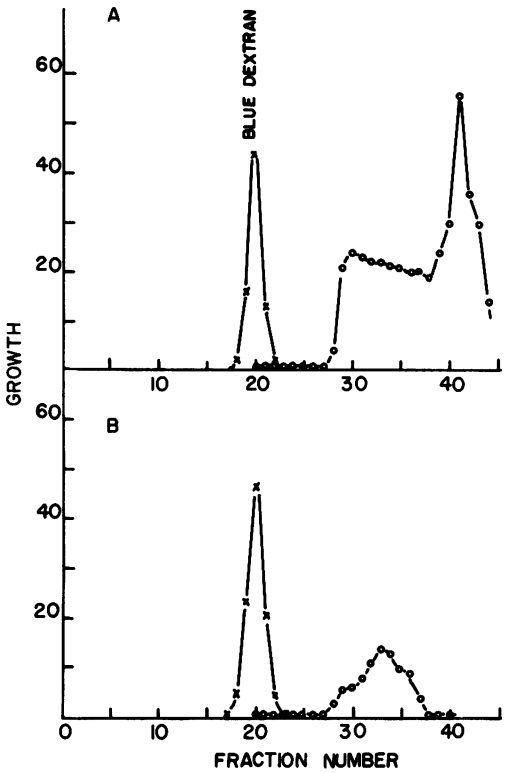

FIG. 6. *Growth (milligrams [dry weight] of mycelial pad) of leu-2,os-1 on 1-ml aliquots of the various fractions of Neopeptone (A) and on 1-ml aliquots of fractionated Neopeptone in the presence of 3.3 mM* L-*phenylalanine (B). The "peaks" indicated as blue dextran were included to indicate the void volume as it related to subsequent fractions of the Neopeptone.*

ingerent in *leu-2;os-1* would tend to decrease any effects due to steric hindrance. It thus appears that *Neurospora* possesses a peptide transport system that is incapable of handling oligopeptides above a certain size, on the average a pentapeptide.

Peptide transport mutant. Given the presence of a peptide transport system, it should be possible to isolate a mutant strain unable to transport peptides of known sequence, e.g., glycyl-L-leucyl-L-tyrosine, or those peptides occurring in fractions 32 through 40 of the Neopeptone. Initially we attempted to isolate a mutant strain of *leu-3* unable to grow on glycyl-L-leucyl-L-tyrosine as a source of L-leucine. This particular tripeptide was chosen because previous studies had shown that it was not hydrolyzed by extracellular peptidase activity (14). The one such mutant isolated surprisingly turned out to be sensitive to the L-tyrosine liberated by the intracellular peptidase activity (Wolfinbarger and Marzluf, manuscript in preparation) and has been designated *tyr-s*(1). The extreme sensitivity (80% inhibition of growth by as little as 0.07 mM L-tyrosine) of *tyr-s*(1) to either L-tyrosine or the tyrosine liberated from glycyl-L-leucyl-L-tyrosine prompted the use of this strain (separated from the *leu-3* background) for isolation of a mutant resistant to glycl-L-leucyl-L-tyrosine but still sensitive to L-tyrosine. Several such mutants, designated *glt-r*, were isolated, crossed into *leu-2* backgrounds, and tested for growth on peptides of known sequence as well as on the peptides present in Neopeptone. We should note here that various *tyr-s;glt-r* double mutants isolated demonstrated varying degrees of resistance to the tripeptide, and it was at first thought that those strains exhibiting the greatest resistance might be peptidase-defective mutants whereas those exhibiting a lesser resistance might represent transport-defective strains. However, on testing (14) of the cell extracts of representatives of the isolated strains, they were all found to hydrolyze a variety of di- and tripeptides equivalent to their *tyr-s* parent and the wild type.

The *leu-2;glt-r* progeny obtained by crossing the most resistant *tyr-s,glt-r* strain with *leu-2* were chosen for a study of their ability to grow on various leucine-containing peptides as a source of leucine. Unlike *leu-2*, the double mutant *leu-2;glt-4*(1) will not grow on glycyl-L-leucyl-L-tyrosine or glycyl-L-alanyl-L-leucine as sources of leucine. Growth of this strain still occurs on L-leucyl-L-leucyl-L-leucine but can be dramatically reduced by the simultaneous addition of excess L-phenylalanine. The data (Fig. 7b) reveal that, with the exception of fractions 39 and 40, none of those fractions that supported growth of *leu-2* (in the presence of excess L-phenylalanine) supported growth of *leu-2;glt-r*(1). In the absence of excess L-phenylalanine, only fractions 36 through 45 support growth of this double mutant (Fig. 7). These fractions, in all probability, contain some free leucine. Indeed, inclusion of L-[^{14}C]leucine with Neopeptone prior to fractionation yields a peak of radioactivity correlating with those fractions supporting growth. Thus, growth of *leu-2;glt-r*(1) apparently occurs only on those fractions of Neopeptone that contain sufficient free leucine to initiate minimal levels of growth. Referral back to Fig. 3 reveals that, when a small amount of free leucine is provided, all of the fractions of Neopeptone support growth of *leu-2; glt-r*(1) identical to *leu-2* (under equivalent conditions). This suggests that *leu-2;glt-r*(1) lacks

the capacity to transport short-chain oligopeptides but can still produce and secrete extracellular peptidase activity. As shown before, L-leucine enters the cell by two genetically distinct amino acid transport systems, both of which are subject to competitive inhibition by L-phenylalanine (13).

The *leu-2;glt-r* mutant was tested and found to be equivalent to *leu-2* (and wild type) in intracellular peptidase activity.

DISCUSSION

The evidence presented in this study supports the conclusions that *N. crassa* is capable of discriminating between peptides, for support of growth, and that peptides with hydrodynamic volumes larger than trileucine are not used. Contrary to that found for *Escherichia coli* (8), however, the cell wall proper does not appear to function in that discrimination. Furthermore, the isolation of a single mutant unable to grow (in the presence of L-phenylalanine) on the majority of fractions of Neopeptone, as well as on various peptides of known sequence, suggests that there may be only one peptide transport system in this fungus. It is suggested that it is at this transport system that size discrimination for entry of peptides into the cell occurs. It appears that, on the average, the peptide transport system cannot handle oligopeptides larger than a pentapeptide (with a hydrodynamic volume larger than that of trileucine).

Although a heterogeneous mixture of peptides was employed in this study, Payne and Gilvarg (8) (and this study) have shown quite adequately that: (i) the use of a buffered solution of NaCl as eluent appears to minimize errors arising from electrostatic interactions; (ii) the presence of aromatic groups in the peptides should not alter the elution of peptides such that error would arise in calculation of the greatest hydrodynamic volume which ceases to be transported; (iii) that no abrupt change in conformation of peptide structure occurs within the peptide sizes studied, i.e., random chains should predominate; and (iv) the hydrodynamic volume of those peptides within a given fraction may be equated to the hydrodynamic volume of known peptides for direct comparison.

We do not know at present exactly why peptides of hydrodynamic volume greater than that of trileucine are not transported by the suggested peptide transport system. We are, however, planning to conduct kinetic studies of transport of radioactively labeled peptides. It was considered that in the solubilization of Neopeptone we were selecting for peptides containing charged amino acids, as opposed to neutral amino acids, in the larger oligopeptides. Payne and Gilvarg (8) noted that positively charged amino acids were more concentrated in the early fractions whereas neutral amino acids predominated in the later fractions. If this were true, then one might consider the possibility that a peptide transport system might not accept as substrate any peptide containing a charged (acidic or basic) amino acid. Such is not probable, however, because the cut-off point for peptide utilization occurs at a peptide size far smaller than required to make a homopolymer of neutral amino acids, e.g., leucine, insoluble.

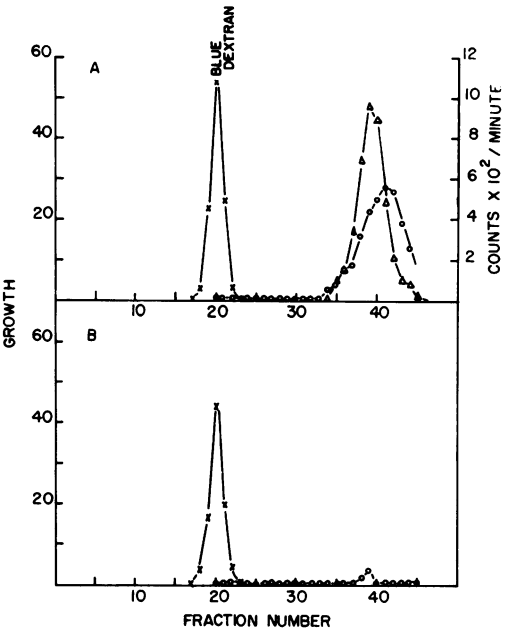

FIG. 7. *Growth (milligrams [dry weight] of mycelial pad) of leu-2,glt-r on 1-ml aliquots of the various fractions of Neopeptone (A) and on 1-ml qliquots of fractionated Neopeptone in the presence of 3.3 mM L-phenylalanine (B). The "peak" of radioactive leucine occurred in fractions of Neopeptone to which the [^{14}C]leucine was added prior to fractionation over the Sephadex G-15 column (not used in growth tests) (Δ).*

A possibility that must be considered is a potential compartmentalization of peptidases. Although we found peptidase activity in cell extracts quite capable of complete hydrolysis of the peptides in all fractions of Neopeptone, it is possible, although unlikely, that this activity was released by the extraction procedure and is not normally available to hydrolyze oligopeptides entering the cell.

The larger (excluded) peptides do not appear

to affect growth of *leu-2* on known tripeptides, e.g., glycyl-L-leucyl-L-tyrosine. Since the cell wall, in this case, does not restrict larger oligopeptides from interacting with the peptide transport system, it cannot be assumed (8) that every peptide has the potential to bind at the transport system.

Germination and growth of *leu-2* conidia using exogenous peptides as a leucine source can apparently occur by two different mechanisms in the case of small oligopeptides: (i) direct entry of the peptides via the transport system followed by their intracellular hydrolysis, and (ii) uptake of free leucine liberated from the exogenous peptides by extracellular peptidase activity. Larger peptides that can still traverse the cell wall cannot be transported and cannot by themselves provide for growth. However, if a small amount of growth occurs by virtue of some other leucine source, the synthesis and secretion of extracellular peptidase(s) apparently permit subsequent utilization of the larger peptides. It should be noted that these studies were carried out under conditions in which leucine was the sole growth factor that needed to be provided for by peptide utilization. Whether additional peptide transport systems may exist and function under different conditions, such as during nitrogen limitation, is an interesting question that we cannot currently address.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant 5RO1 GM-18642 from the National Institute of General Medical Services.

We would like to thank A. Gib Debusk for the use of his facilities and amino acid analyzer during a portion of these studies, and T. K. Rao and M. Saunders for their assistance.

LITERATURE CITED

1. Becker, J. M., F. Naider, and E. Katchalski. 1973. Peptide utilization in yeast. Studies on methionine and lysine auxotrophs of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **291**:388-397.
2. Cascieri, T., Jr., and M. F. Mallette. 1974. New method for study of peptide transport in bacteria. Appl. Microbiol. **27**:457-463.
3. De Felice, M., J. Guardiola, A. Lamberti, and M. Jaccarino. 1973. *Escherichia coli* K-12 mutants altered in the transport systems for oligo- and dipeptides. J. Bacteriol. **116**:751-756.
4. Fickel, T. E., and C. Gilvarg. 1973. Transport of impermeant substances in *E. coli* by way of oligopeptide permease. Nature (London) **241**:161-163.
5. Habeeb, A. F. S. A. 1966. Determination of free amino groups in proteins by trinitrobenzene-sulfonic acid. Anal. Biochem. **14**:328-336.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
7. Naider, F., J. M. Becker, and E. Katzir-Katchalski. 1973. Utilization of methionine-containing peptides and their derivatives by a methionine-requiring auxotroph of *Saccharomyces cerevisiae*. J. Biol. Chem. **249**:9-20.
8. Payne, J. W., and C. Gilvarg. 1968. Size restriction on peptide utilization in *Escherichia coli*. J. Biol. Chem. **243**:6291-6299.
9. Payne, J. W., and C. Gilvarg. 1971. Peptide transport. Adv. Enzymol. **35**:187-244.
10. Trevithick, J. R., and R. L. Metzenberg. 1966. Molecular sieving by *Neurospora* cell walls during secretion of invertase isozymes. J. Bacteriol. **92**:1010-1015.
11. Trevithick, J. R., and R. L. Metzenberg. 1966. Genetic alteration of pore size and other properties of the *Neurospora* cell wall. J. Bacteriol. **92**:1016-1021.
12. Vogel, H. J. 1956. A convenient growth medium for *Neurospora* (medium N). Microbiol. Genet. Bull. **13**:42-43.
13. Wolfinbarger, L., Jr., and A. Gib Debusk. 1971. Molecular transport. I. *In vivo* studies of transport mutants of *Neurospora crassa* with altered amino acid competition patterns. Arch. Biochem. Biophys. **144**:503-511.
14. Wolfinbarger, L., Jr., and G. A. Marzluf. 1974. Peptide utilization by amino acid auxotrophs of *Neurospora crassa*. J. Bacteriol. **119**:371-378.