PART I

TYROSINASE INDUCTION BY ANTIMETABOLITIES IN *NEUROSPORA*

PART II

AMINO ACID TRANSPORT IN *NEUROSPORA*

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Martin L. Pall

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ABSTRACT

Part I

A technique for inducing very high levels of tyrosinase activity with various antimetabolites is described. A modification of this technique was used for studying tyrosinase induction in Neurospora over periods of a few hours. An amount of the antimetabolite inducer sufficient to induce the enzyme is rapidly taken up into the mycelium. Following uptake, however, there is a lag period of about two hours before tyrosinase is synthesized. During the lag period, some active, energy requiring process prepares the mycelium for tyrosinase synthesis. Rapid synthesis of tyrosinase then ensues. High concentrations of cycloheximide, an inhibitor of protein synthesis, inhibit the development of any further enzyme activity when added to inducing cultures, indicating that the synthesis of tyrosinase is de novo.

Low concentrations of cycloheximide which had been previously shown to induce tyrosinase, partially inhibit general protein synthesis. Ethionine and parafluorophenylalanine appear to induce the enzyme by being incorporated into proteins in place of methionine and phenylalanine, thus lowering the functional activity of newly synthesized proteins. The partial inhibition of the synthesis of functional proteins, then, is sufficient, in some way, to induce tyrosinase.

Part II

Kinetic studies have revealed the existence of two transport systems for amino acids in Neurospora. Transport system I corresponds to

a system previously studied by Wiley and Matchett (24). Its activity is specifically missing in mtr mutant cultures previously described by Lester (26) and Stadler (25). It is capable of transporting most neutral L-amino acids. Amino acid transport system II has not been described previously. It has an affinity for a wide variety of amino acids. It transports amino acids with hydrophobic and hydrophilic side chains, both basic and neutral amino acids, and D- as well as L-amino acids. Transport system II has an affinity for both β- and α-amino acids.

Transport system I has high activity in young, rapidly growing cultures. Transport system II has little or no activity in young cultures. In older, carbon-starved cultures, however, it is more active than transport system I. This, together with the high affinities it shows for many amino acids, suggests that amino acid transport system II serves a scavenger function, removing from the medium traces of exogenous amino acids.

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INTRODUCTION

The problem of determining the molecular basis of differentiation has focussed, in recent years, on the problem of control of the synthesis of enzymes and other proteins. The greatest success in determining the mechanism of control of protein synthesis has been achieved in bacteria with the development of the operon theory (1) and the recent work confirming and clarifying it (2-7, 65, 66). The details of this well-known theory will not be repeated here.

The interest in the operon theory has led to attempts to ascertain its relevance to control of protein synthesis in eukaryotes and particularly in fungi where detailed genetic analysis is possible. Unlike bacteria, fungi do not, in general, have the structural genes for a metabolic pathway linked together in groups of genes (9-11). Thus, control of a group of genes as a unit or operon is, in most cases, impossible. Analysis of a few cases where two or more functions of a metabolic pathway do seem to have their structural information closely linked, have not yielded any clear cut examples of operon-like control (12-21).

Thus, although several good examples of genes affecting the regulation of enzymes in fungi exist (9-11), the molecular basis of control in fungi is still uncertain.

Whatever the relevance of operon-like control to protein synthesis in eukaryotes, it is clear that in some cases, at least, the control must involve more complex circuitry than that found in the original operon concept. That is, more complex control circuits must occur

than the operon theory describes in bacteria. This has been recognized both by classical embryologists (22) and by Monod and Jacob (8). Thus, examples of differentiation in eukaryotes have shown many degrees of reversibility, ranging from completely and easily reversible to completely irreversible. Jacob and Monod (8, 23) have constructed several model circuits whose regulatory elements are the same as those found in bacteria, e.g., operator loci, regulator genes, repressors, etc. These model circuits demonstrate the entire range of reversibility, from completely reversible to completely irreversible. They include a circuit which has two stable, steady state conditions, possibly corresponding to the control states in two different cell types. They also include a circuit that can be irreversibly turned on by an inducer from outside, possibly corresponding to the induction of classical embryology. These circuits all include feedback loops, both positive and negative, which give them varying degrees of stability and reversibility.

Other possible control levels not discussed above, serve to complicate further the consideration of control systems. Control of protein synthesis may also occur through the regulation of the translation of messenger RNA (69-73). This translational control, moreover, may not be completely separate from control at the DNA level (78). Furthermore, protein degradation may also be a site for regulation (73, 74). Thus, the sites for potential control and the possible circuit elements in control circuits may reach a high degree of flexibility and complexity.

These circuits and circuit elements and the considerations which support them serve to indicate the types of complexity which may be found in control systems in eukaryotes. No system has been verified where such a circuit exists. It is interesting to note, however, that sequential induction has been found in insects (68). Furthermore, the galactose system in yeast (12-14) with its several regulatory loci may be organized into a circuit. It is suggested that serious attempts to search for such circuits will be necessary for a thorough understanding of control in eukaryotes.

The control of tyrosinase synthesis in Neurospora is, in some ways, typical of enzyme control systems in eukaryotes. Although little or no enzyme is synthesized in rapidly growing vegetative cultures, it is present in cultures undergoing sexual differentiation (24). Tyrosinase can be induced in liquid cultures by starvation for sulfur, carbon, or nitrogen (25). It is useful to note that sexual fruiting is normally induced in a medium that is deficient in reduced nitrogen (26). In addition, various antimetabolites added to mycelial cultures suspended in a basal salts medium, induce the enzyme (25). Certain amino acid analogues and D-amino acids have been found to be especially effective for this induction.

Two recessive female sterile mutants, ty-1 and ty-2, are partially defective in the induction of tyrosinase. Although wild type cultures synthesize large amounts of tyrosinase on starvation in phosphate buffer, the ty-1 and ty-2 strains produce little or no enzyme under these conditions (25). The enzyme can be induced in these mutant

strains, however, by adding antimetabolites to the phosphate buffer.

Part I of this thesis is concerned with a number of basic physiological and biochemical questions about tyrosinase induction with antimetabolites. A method for studying tyrosinase induction with antimetabolites showing improved reproducibility is described. Using this method, various antimetabolite inducers show a lag period after the addition of the inducer during which no enzyme is synthesized. The nature of this lag is investigated. Evidence supporting the proposition that the tyrosinase synthesis is de novo is presented. In addition, the physiological mechanism by which at least some of the inducers act is investigated.

MATERIALS AND METHODS

Strains

Wild type strains of Neurospora crassa, 69-1113a and 4-137a, were used in most experiments. These strains were isolated by Horowitz and Fling (27) as producers of the thermostabile (T^S) and thermolabile (T^L) form of tyrosinase respectively. Unless otherwise stated, all experiments were performed on wild type strain 69-1113a. Other wild type strains used were 4A and 25a (67).

In addition, the tyrosinaseless strain ty-1 was used. This mutant, obtained from Dr. M. Westergaard, is female sterile. Unlike wild type, it fails, when starved on phosphate buffer, to produce significant quantities of tyrosinase (28). It can be induced to synthesize the enzyme by using other techniques (25,28). A recent isolate of ty-1, designated 131-2, was used. It had been obtained by outcrossing the original ty-1 strain four times to wild type strain 4-137a (29).

Chemicals

All chemicals used were of reagent grade quality except as noted below. Table sugar was used as sucrose in the medium. Commercial grade Triton X-100 was obtained from Rohm and Haas. Cycloheximide (actidione) was obtained from Nutritional Biochemicals Corp. DL-p-fluorophenylalanine-3-C14 was obtained from Calbiochem. L-ethionine-ethyl-1-H3, D-phenylalanine-1-C14, and uniformly labelled C14 L-lysine were obtained from New England Nuclear Corp. H3 labelled L-lysine was

obtained from Schwarz Bioresearch, Inc.

Maintenance and Growth of Neurospora Cultures

All Neurospora strains were maintained on slants of Horowitz complete medium (30). The liquid medium used was one-half strength Vogel's medium N salts (31) containing one-half per cent sucrose. 125 ml. Erlenmeyer flasks containing 20 ml. of liquid medium were inoculated with one drop of a conidial suspension.

High Level Induction Procedure

Flasks of liquid minimal medium, prepared and inoculated as described above, were grown for two days at 25° C. without shaking. An antimetabolite, dissolved in water (25) was added as an inducer of tyrosinase and the cultures were shaken gently on a reciprocal shaker for two additional days at 25° C. With any of several antimetabolites as inducers, the above procedure was found to regularly produce very high levels of tyrosinase activity.

Short Term Induction Procedure

Flasks of liquid minimal medium, prepared and inoculated as described above, were grown without shaking at 25° C. for two days. They were then gently shaken on a reciprocal shaker for one day. An antimetabolite inducer (25) dissolved in water was added to the medium and the cultures were further shaken for various lengths of time. This procedure was found to give significant tyrosinase induction in a few hours. It was found useful in the study of tyrosinase induction over relatively short periods of time.

Extraction of Tyrosinase

The Neurospora mycelium was washed with distilled water, blotted dry and weighed. It was then ground with sand with a mortar and pestle and diluted with ten volumes of .1M sodium phosphate buffer, pH 6.0. The mixture was spun in a centrifuge and the supernatant assayed for tyrosinase.

Assay For Tyrosinase Activity

Tyrosinase activity was measured on .01 to .1 ml. aliquots of the above extract. The assay was performed by measuring the conversion of the substrate, DL-DOPA, to dopachrome colorimetrically as described previously (28). The measured absorbance of dopachrome was converted to Enzyme Commission units (32) according to the conversion factor previously described (33). Tyrosinase activity is expressed here in Enzyme Commission units per gram wet weight of mycelium.

Fractionation of Neurospora Mycelia

The mycelial fractionation procedure described here was developed by Roberts et al. for Neurospora (34). It has proved useful in following the incorporation of radioactive compounds into various fractions of the mycelium. The washed mycelium is extracted for thirty minutes at 4^{o}.C. with ice-cold 5% trichloroacetic acid (TCA) in a centrifuge tube. The extract is removed and the mycelium extracted again for ten minutes at 4^{o} C. with 5% TCA. These two extracts, containing the free amino acids of the mycelium, are combined as the cold TCA soluble fraction. The mycelium is extracted next with 75% ethanol at 40-50^{o} C. for thirty minutes and the extracts removed. Then it is extracted with

ether-75% ethanol (1: 1) for thirty minutes at 40-50° C., and the two extracts combined. In Neurospora, this ethanol-soluble fraction contains mostly lipids. After drying for fifteen minutes at 60° C., 5% TCA is added and the tubes are placed in a water bath. The water bath is then brought slowly to boiling to avoid bumping. The mycelia are held in the 5% TCA by glass rods to insure effective extraction. The pads are extracted for seventy-five minutes at 90-100° C. The hot TCA-soluble extract contains mainly RNA and those amino acids attached to s-RNA. The pads are washed once with 75% ethanol and once with ether, and the washes are discarded. Then the pads are dried at 60° C. for thirty minutes. The proteins in the mycelium are extracted overnight at room temperature with 3% sodium hydroxide. The remainder of the mycelium is discarded. The above fractions can be adjusted to neutral pH and counted as described below.

Determination of Amino Acid Pool Size

The pool size of individual amino acids in the free pool can be determined as follows: The cold TCA fraction, isolated as described above, is extracted four times with an equal volume of ether. The remaining aqueous solution is dried overnight in a vacuum dessicator. The residue is resuspended in a small volume of distilled water. A small aliquot of this solution, equal to about one-tenth to one-twentieth of the free pool of a mycelial pad, is run on high-voltage electrophoresis as described below. Known amounts of the amino acid whose pool is to be measured are run simultaneously.

Hydrolysis of Neurospora Proteins

The sodium hydroxide fraction, isolated as described above, is chilled and precipitated with .2 volumes of cold 50% TCA. The precipitate is spun down, washed with cold acetone, and dried. It is dissolved in about .5 ml. of constant boiling HCl, and placed in a tube. The tube is flushed five times with nitrogen and sealed. The tube is heated to 105° C. for 24 hours, opened and dried in a dessicator. The hydrolysate is then resuspended in a small volume of distilled water and small aliquots run on high-voltage electrophoresis to separate the component amino acids.

High-Voltage Paper Electrophoresis of Amino Acids

The amino acid solutions were applied to the paper and run on high-voltage electrophoresis as described previously (36). 1.64M formic acid was used as a solvent. The amino acid pools were run for two hours at 4900 volts in a Gilson model D electrophorator. These amino acids were eluted and their quantities determined as described previously (36). For maximum separation of the amino acids of interest, the protein hydrolysates were run at 7900 volts for 2.5 hours on a Gilson model DW electrophorator. The paper was dried. The positions of the amino acids were determined by spraying with a ninhydrin solution made up of 50 mg. of ninhydrin in 75 ml. of absolute ethanol plus 25 ml. of 2N acetic acid. The amino acids were cut out and eluted twice with 4 ml. of methanol and the eluate dried. The residue was redissolved in water and counted as described below.

Counting of Radioactive Samples

All radioactive samples were counted in a Beckman CPM 200 scintil-

lation counter. A toluene based scintillation fluid using Triton X-100 as a solubilizer (35) was prepared as follows: A solution of toluene containing .4% PPO and .01% dimethyl POPOP was prepared. Two volumes of this solution was mixed with one volume of Triton X-100 to make the scintillation fluid. One to 1 1/2 volumes of radioactive aqueous solution were dissolved in ten volumes of scintillation fluid and counted. Nonaqueous samples were counted in the above toluene solution without solubilizer.

Removal of Tyrosine From the Culture Medium

The removal of added D-tyrosine from the medium by the mycelial pads was measured by taking half ml. aliquots from the medium. The D-tyrosine in these aliquots was measured by a colorimetric assay for tyrosine involving its reaction with nitrosonaphthol and nitric acid (37).

RESULTS

High Level Induction Procedure

The high level induction procedure, as described above, is a very effective method for inducing tyrosinase activity. Activities as high as 900 Enzyme Commission units per gram wet weight of mycelium have been obtained. It can also be used to induce the enzyme in large quantities of mycelium (38) for purification of the tyrosinase protein (33).

Typical levels of tyrosinase activity obtained are listed in table I. Most wild type strains of Neurospora are inducible by this method. The concentration of each inducer required to give optimal induction varies, however, from strain to strain. It is interesting to note that the ty-1 mutant is induced by this method, yielding levels of activity similar to those obtained with some wild type strains.

Induction of the enzyme by the above method with ethionine in the canavanine sensitive strain, 25a, is inhibited over 99% by 50 μg./ml. of canavanine sulfate.

The primary effect of canavanine in canavanine sensitive strains of Neurospora appears to be its incorporation into proteins in place of arginine, thus producing nonfunctional proteins (40). Therefore, the inhibition of induction by canavanine supports the proposition that tyrosinase induction involves de novo protein synthesis. This proposition is further supported by the finding that 20 μg./ml. of cycloheximide, an inhibitor of protein synthesis (41, 42), also inhibits

TABLE I HIGH LEVEL INDUCTION PROCEDURE

Strain	Inducer	Concentration of inducer (in µg./ml. of culture medium)	Tyrosinase activity (units/g.)
69-1113a	DL-ethionine	100 µg./ml.	450
	D-phenylalanine	150 µg./ml.	470
	D-tyrosine	200 µg./ml.	202
	DL-p-fluorophenylalanine	150 µg./ml.	248
	Actinomycin D	25 µg./ml.	122
	Cycloheximide*	.2 µg./ml.	272
4-137a	D-phenylalanine	200 µg./ml.	321
131-2 ty-1	DL-ethionine	100 µg./ml.	130
25a	DL-ethionine	200 µg./ml.	268
4A	DL-ethionine	200 µg./ml.	234

Cultures were grown and induced according to the high level induction procedure described above. They were harvested and assayed for tyrosinase.

*Cycloheximide induction performed by G. Horn (39).

the induction of tyrosinase. The inhibition of induction by cycloheximide is considered in more detail below.

Short Term Induction Procedure

The short term induction procedure is useful in the study of tyrosinase induction over periods of a few hours. Figure 1 shows a fairly typical response to the addition of an inducer. After the addition of the inducer, there is a lag period during which no detectable enzyme is synthesized. This is followed by a period of rapid synthesis. Finally, a cessation of tyrosinase synthesis occurs. Similar responses have been obtained with several different inducers.

Studies on the Lag Period

The lag period for tyrosinase induction with several different inducers has been measured. The results are listed in table II. The lag measured for all the inducers studied here is about two to three hours. That found for induction by the D-amino acids may be slightly shorter than that found using the amino acid analogues as inducers. It is not clear that the difference is significant.

The similar lag periods determined here suggest that they may be due, at least in part, to some critical process inside the mycelium which must precede tyrosinase induction. Another possibility is that the lag simply represents the time required for sufficient inducer to be taken up into the mycelium. It is, therefore, important to test this second possibility.

For this test, pads of 69-1113a were prepared according to the short term induction procedure. Inducers were added and the pads

Fig. 1. TYROSINASE INDUCTION WITH L-ETHIONINE. Pads of 69-1113a were prepared according to the short term induction procedure. L-ethionine was added to a concentration of 100 μg. per ml. of medium. Pads were harvested at various times after the addition of the inducer and assayed for tyrosinase. Each point represents the average of five determinations from five identically treated pads.

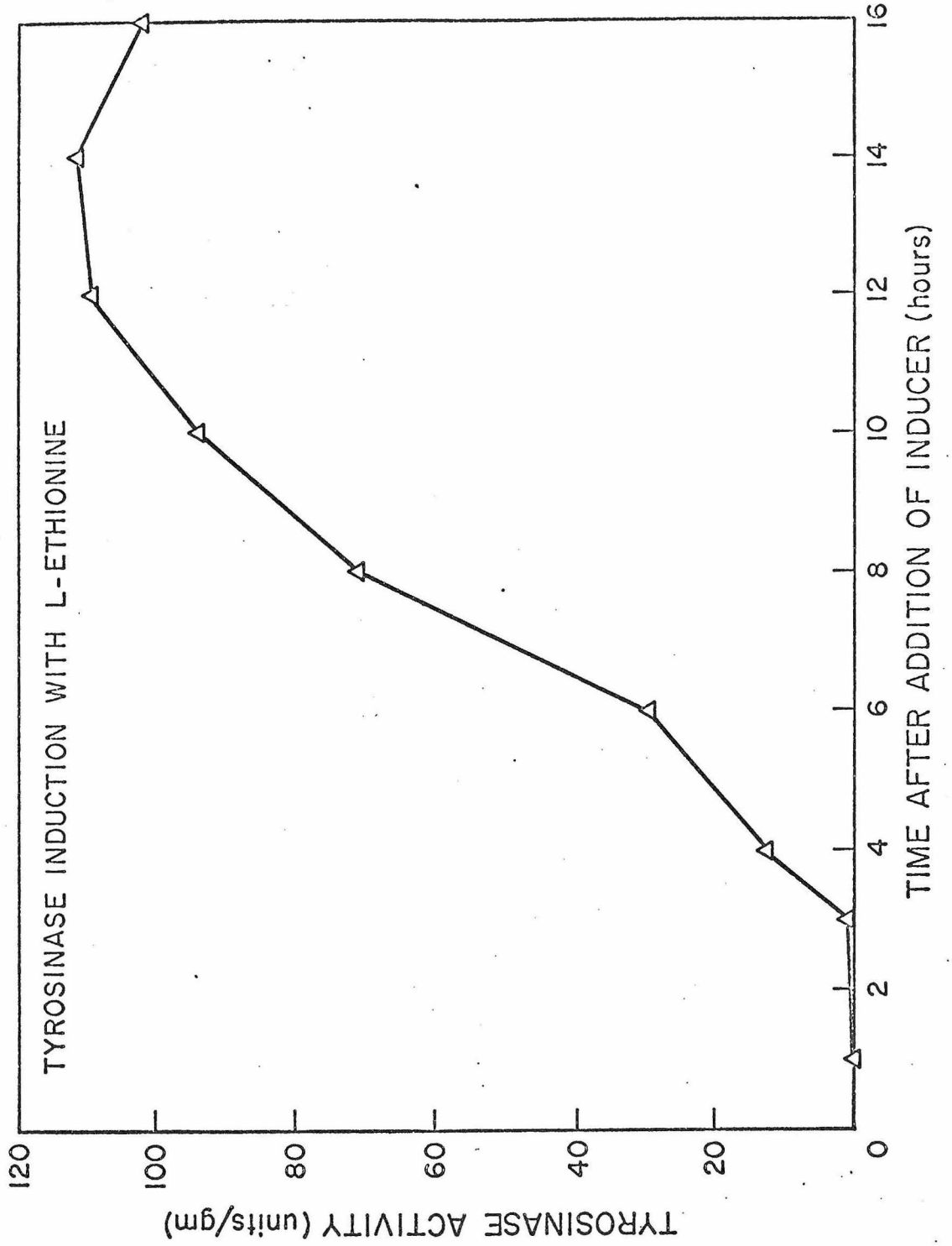

TYROSINASE INDUCTION WITH L-ETHIONINE
TYROSINASE ACTIVITY (units/gm)
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
TIME AFTER ADDITION OF INDUCER (hours)

TABLE II

LAG PERIODS FOR DIFFERENT INDUCERS IN STRAIN 69-1113a

Inducer	Concentration of inducer (final concentration on addition to culture medium)	Lag period (after addition of inducer)
DL-ethionine	100 ug./ml.	2-3 hours
DL-p-fluorophenylalanine	150 ug./ml.	2-3 hours
DL-selenomethionine	10 ug./ml.	2-3 hours
D-phenylalanine	100 ug./ml.	2 hours
D-tyrosine	150 ug./ml.	2 hours

Flasks containing mycelial pads of strain 69-1113a were prepared according to the short term induction procedure. An inducer dissolved in water was added to the culture medium to the final concentration listed above. Mycelial pads were harvested and assayed for tyrosinase at various times after the addition of the inducer. The lag period is the time after the addition of the inducer, during which no detectable tyrosinase activity has appeared.

shaken. After various lengths of time in the presence of inducer, each pad was washed twice with 20 ml. of distilled water and placed in 20 ml. 1/2 strength Vogel's medium N salts. In this way, all the inducer except that already taken up into the mycelium was removed. The mycelial pads were then shaken until five hours had elapsed following the addition of inducer, harvested, and assayed.

Control pads treated as above, but where no inducer was added, gave little or no tyrosinase activity. Pads exposed to DL-ethionine (100 ug./ml.) or DL-paraflourophenylalanine (150 µg./ml.) gave good induction when they had been shaken with inducer for five to ten minutes. Therefore, inducing amounts of ethionine and parafluorophenylalanine are taken up in five to ten minutes. D-phenylalanine (100 µg./ml.) requires 15 minutes of uptake to give significant induction and about one hour to give good induction. Thus, most of the lag period is not due to the time required for uptake of the inducer, but rather appears to be due to some process occurring inside the mycelium.

Since the lag period involves some process inside the mycelium, it is desirable to find out if this process requires metabolic energy. Specifically, can metabolic inhibitors inhibit progress through the lag period?

The metabolic inhibitors cyanide and azide were used to help answer this question. A set of mycelial pads was divided into two groups. The first group had an inducer added. Then, after ten minutes, potassium cyanide was added to a final concentration of 3×10^{-3}M. The pads were shaken for three hours. Then they were washed three times with 20 ml. of distilled water, shaking the pads briefly with each

TABLE III PROGRESS THROUGH THE LAG PERIOD IN THE PRESENCE OF KCN OR NA AZIDE

Time after washing away of metabolic inhibitor	Experiment 1 a. Inducer added before KCN	Experiment 1 b. Inducer added after KCN was washed away	Experiment 2 a. Inducer added before Na Azide	Experiment 2 b. Inducer added after Na Azide was washed away
2 hours	0 units/gm.	0 units/gm.	0 units/gm.	0 units/gm.
3 hours	0 units/gm.	--	.9 units/gm.	2.7 units/gm.
4 hours	1.1 units/gm.	1.6 units/gm.	4.0 units/gm.	6.8 units/gm.

In experiment 1a, pads of 69-1113a, prepared according to the short term induction procedure, had DL-ethionine added to a final concentration of 100 ug./ml. in the medium. Ten minutes later, KCN was added to a final concentration of 3×10^{-3}M. After shaking for three hours, the pads were washed three times, as described in the text, and resuspended in 1/2 strength Vogel's medium N salts. Experiment 1b pads were treated identically to experiment 1a pads except that the ethionine was added after the washing rather than before the KCN was added. Experiment 2 was performed identically to Experiment 1 except that 5×10^{-3}M sodium azide was used in place of 3×10^{-3}M KCN.

The values in the table are for tyrosinase activity expressed as units per gram wet weight. Each value listed is the average of two determinations from two independently treated pads.

wash water. After washing, they were suspended in 20 ml. of 1/2 strength Vogel's medium N salts and shaken for various lengths of time until harvesting. They were then assayed for tyrosinase. The second set of pads was treated as above except that the inducer was added after the washes rather than before the cyanide was added. It should be noted that although the washing is effective in removing the cyanide from the mycelial pads, the ethionine used here as an inducer is fairly efficiently retained.

If cyanide does not prevent all progress through the lag period, the first set of pads should induce sooner, after the cyanide has been washed out, than the second set. The results, shown in table III, indicate that there is no progress through the lag in the presence of cyanide. Similar results were obtained using 5×10^{-3}M sodium azide as a metabolic inhibitor. Thus, progress through the lag appears to be an energy requiring process.

These results are in agreement with previous results using phosphate buffer starvation to induce tyrosinase (43). In phosphate buffer induction, progress through the lag period is stopped in the absence of oxygen. Thus, regardless of the method of induction, progress through the lag period requires metabolic energy.

Inhibition of Tyrosinase Induction by Cycloheximide

Previous experiments on the induction of tyrosinase in ty-1, where the enzyme was labelled with C14 valine, indicated that most, if not all, of the enzyme induced involved de novo synthesis of the tyrosinase protein (25). The results cited above further support this conclusion

in wild type. They showed that the induction of tyrosinase is over 99% inhibitable by canavanine in a canavanine sensitive strain. In addition, the inhibitor of protein synthesis, cycloheximide, was also shown to inhibit tyrosinase induction. It should be noted, however, that experiments using antimetabolites to inhibit induction are subject to certain criticisms. One of these is that the inhibition of synthesis by the antimetabolite may be a secondary effect rather than an immediate and primary effect of the antimetabolite, e.g., the culture may have been too debilitated by the drug to synthesize much tyrosinase. The experiment described below is designed to distinguish between these possibilities.

A set of pads was divided into two groups. Group 1 was induced with ethionine, harvested, and assayed at various times. Group 2 pads were induced with ethionine. Cycloheximide (20 µg./ml.) was added to these pads at various times after the addition of the ethionine. All pads in group 2 were assayed for tyrosinase at seven hours after the addition of the ethionine. If the inhibition of the development of tyrosinase activity is a direct and immediate effect of the cycloheximide, the activity of the pads of group 1 harvested at a particular time should equal the activity of the pads of group 2 which received cycloheximide at that time. The results of this experiment are shown in table IV. The predicted result is, indeed, found. The cycloheximide immediately stops the development of further tyrosinase activity.

The most straightforward interpretation of this experiment is that tyrosinase induction involves <u>de novo</u> synthesis of the tyrosinase protein and that no large pool of protyrosinase is present in the mycelium

TABLE IV

INHIBITION OF TYROSINASE INDUCTION BY CYCLOHEXIMIDE

Group 1		Group 2	
Time of assay for tyrosinase (after addition of inducer)	Tyrosinase activity (Units/gm.) at time listed	Time of addition of (20 µg./ml.) cycloheximide (after addition of inducer)	Tyrosinase activity at seven hours
1 hr.	0	1 hr.	0
2 hrs.	0	2 hrs.	0
3 hrs.	4.2	3 hrs.	4.7
4 hrs.	35.7	4 hrs.	30.7
5 hrs.	79.1		

Pads of 69-1113 were prepared according to the short term induction procedure. The pads in group 1 had DL-ethionine added to a final concentration of 100 µg./ml. of medium and were harvested and assayed at the times listed. Pads of group 2 also had DL-ethionine added to a final concentration of 100 µg./ml. Cycloheximide (20 µg./ml. of medium) was added at the times listed. All group 2 pads were harvested and assayed at seven hours after the addition of ethionine.

at any time during the induction procedure. This interpretation depends on the assumption that the activation of a hypothetical protyrosinase would not be inhibited by the cycloheximide. The activation of proenzymes commonly involves proteolytic activity. Consequently, it is necessary to determine if proteolytic activity is generally dependent on protein synthesis, the known site of action of cycloheximide.

In bacteria, inhibitors of protein synthesis have no immediate effect on protein degradation (75). In mammalian systems, inhibitors of general metabolism and protein synthesis do inhibit protein degradation *in vivo*, but this effect is only partial (76, 77).

The degradation of several enzymes in rat liver has been studied in the presence of inhibitors of protein synthesis. Tyrosine transaminase degradation is inhibited by inhibitors of protein synthesis (80). The degradation of other enzymes, however, is not affected (73,81). The basis for the particular behavior of tyrosine transaminase is not clear.

In most cases, then, proteolytic activity is not closely linked to protein synthesis. Thus, the most straightforward interpretation of the above experiment is the most likely, i.e., tyrosinase induction involves *de novo* synthesis of the tyrosinase protein.

Inhibition of Protein Synthesis by Cycloheximide

The experiments cited above showed that high concentrations of cycloheximide inhibit the synthesis of tyrosinase. Furthermore, lower concentrations of cycloheximide induce the enzyme. The interpretation of these results depends on the assumption that the primary mode of action of cycloheximide is the inhibition of protein synthesis. The

following experiment suggests that this assumption is warranted in Neurospora.

Various amounts of cycloheximide (actidione) were added to flasks containing pads of 69-1113a. The cultures were shaken for one hour. At the end of the hour, .15 μc. of C14 labelled L-lysine were added and the pads shaken for ten additional minutes. The pads were then harvested and fractionated as described above in the methods section. The pools of free lysine in the mycelial pads were also measured as described above. The results are shown in table V.

At .2 μg./ml. of medium, cycloheximide inhibits protein synthesis about 50%. At higher concentrations, the inhibition of protein synthesis approaches 99%.

The above results show that cycloheximide is an effective inhibitor of protein synthesis in Neurospora. Similar results have been obtained in previous experiments on mammalian cells (42), algae (44), and yeast (41). The inhibition of protein synthesis at the ribosome level has been concluded to be the direct and primary effect of cycloheximide in yeast. It seems likely that the inhibition of protein synthesis is also the primary effect in Neurospora; however, other effects of the antibiotic have not been eliminated.

Aromatic D-Amino Acid Induction of Tyrosinase and Its Antagonism by L-Amino Acids

Macleod (29) has found that a mixture of D- and L-phenylalanine was less effective in inducing tyrosinase than the D-stereoisomer alone. Similar results were found using DL-tyrosine as an inducer as compared

TABLE V

INHIBITION OF PROTEIN SYNTHESIS BY CYCLOHEXIMIDE

Conc. of Cycloheximide in medium	Per cent of counts taken up into pad incorporated into protein	Free lysine pool size (millimicromoles per pad)	Per cent Inhibition of protein synthesis
0	64.0 %	386	--
.2 µg./ml.	33.9%	380	47.7%
1 µg./ml.	5.34%	498	89.2%
10 µg./ml.	.463%	564	98.9%

Mycelial pads of 69-1113a were prepared according to the short term induction procedure. Cycloheximide was added and the pads shaken for one hour. .15 µc. of C14 L-lysine (220 mc./mm.) was added and the pads shaken for ten additional minutes. The pads were then fractionated according to the procedure of Roberts et al (34) described above. The free lysine pools were determined as described above, from similar pads treated for one hour with the various concentrations of cycloheximide. The rate of protein synthesis was taken to be proportional to the incorporation of lysine into protein and inversely proportional to the pool size of lysine.

with D-tyrosine alone. The L-stereoisomer, then, appears to inhibit induction by the D-stereoisomer.

The findings of Calendar and Berg (57) were of interest in this regard. They showed that the activation of L-tyrosine was inhibited by D-tyrosine. This suggested a possible specific inhibition of the activation of an L-amino acid by the corresponding D-stereoisomer. The foregoing considerations led to a study of the induction of tyrosinase by D-phenylalanine and D-tyrosine and the inhibition of their induction by various L-amino acids. The results of this study are shown in table VI. Of the twenty amino acids commonly found in proteins, seventeen are capable of inhibiting the induction of tyrosinase by both D-phenylalanine and D-tyrosine. The remaining three amino acids do not inhibit induction by either of these aromatic D-amino acids.

Both D- and L-amino acids were added to the medium at the same time in the above experiments. Consequently, the inhibition of induction could be due to an inhibition of the uptake of the D-amino acid by the L-amino acid used. Furthermore, the wide variety of effective L-amino acids found here is similar to the wide variety of amino acids found effective in inhibiting the uptake of L-phenylalanine and L-arginine in Neurospora conidia (58, 59).

A series of experiments was performed to determine if the above inhibition of induction occurred through an inhibition of uptake of the inducer. The uptake of D-phenylalanine or D-tyrosine was determined by following its removal from the medium as described in the methods section. Uptake in the presence or absence of various L-amino acids was followed. With the exception of L-glutamine, the concentration of each

TABLE VI

INDUCTION OF TYROSINASE BY D-AMINO ACIDS IN THE PRESENCE OF VARIOUS L-AMINO ACIDS

L-amino acid added (1 mg./ml.)	D-phenylalanine (100 μg./ml.) as an inducer	D-tyrosine (150 μg./ml.) as an inducer
None	Induction	Induction
Alanine	No induction	No induction
Arginine	No induction	No induction
Asparagine	No induction	No induction
Aspartic acid	No induction	No induction
Cystine	No induction	No induction
Glutamic acid	Induction	Induction
Glutamine	Induction	Induction
Glycine	No induction	No induction
Histidine	No induction	No induction
Isoleucine	No induction	No induction
Leucine	No induction	No induction
Lysine	No induction	No induction
Methionine	No induction	No induction
Phenylalanine	No induction	No induction
Proline	Induction	Induction
Serine	No induction	No induction

TABLE VI (Cont.)

L-amino acid added (1 mg./ml.)	D-phenylalanine (100 µg./ml.) as an inducer	D-tyrosine (150 µg./ml.) as an inducer
Theonine	No induction	No induction
Tryptophan	No induction	No induction
Tyrosine	No induction	No induction
Valine	No induction	No induction

Mycelial pads of 69-1113a were prepared according to the short term induction procedure. D-phenylalanine (100 µg./ml.) or D-tyrosine (150 µg./ml.) were added as inducers. Simultaneously, an L-amino acid was added to the medium to a final concentration of 1 mg./ml. of medium. The pads were harvested and assayed for tyrosinase five hours after the addition of the amino acids. Pads with over four units of tyrosinase per gram wet weight were scored as induced. Those with less than one unit per gram wet weight were scored as non-induced.

L-amino acid used was the lowest concentration of that amino acid found effective in completely inhibiting tyrosinase induction by the D-amino acids.

The results are shown in figure 2. The five L-amino acids which inhibit induction also inhibit uptake of the inducers. L-glutamine, the one amino acid investigated which does not inhibit induction, has no effect on uptake of the inducer. Thus, a broad range of L-amino acids appear to inhibit induction by the aromatic D-amino acids by inhibiting their uptake. These results, together with those previously found in conidia (58, 59), raise the question as to how such a broad range of amino acids can inhibit the uptake of a particular amino acid.

Do these results imply the existence of an amino acid transport system with a very broad range of substrates? If so, the various amino acids would show competitive inhibition for uptake. Alternatively, can other types of inhibition be taking place? Such questions have received further attention and are to be discussed further (56).

Possible Sites of Action of the Aromatic D-Amino Acids

Although the above considerations serve to elucidate the uptake of the aromatic D-amino acids, they yield little information on the site of action of the D-amino acids in the induction of tyrosinase. Consequently, this matter should be considered further.

All the known inducers of tyrosinase are also growth inhibitors (25). The aromatic D-amino acids are well established as inhibitors of growth (60, 25). It appears likely, then, that the site of action of the aromatic D-amino acids in induction is identical to their site of

Fig. 2. INHIBITION OF UPTAKE OF THE AROMATIC D-AMINO ACIDS BY L-AMINO ACIDS. Pads of 69-1113a were prepared according to the short term induction procedure. Various L-amino acids were added simultaneously with the aromatic D-amino acids to the medium. Glycine (200 μg./ml.), L-lysine (200 μg./ml.) or L-arginine (100 μg./ml.) were added together with D-tyrosine (150 μg./ml.) L-phenylalanine (100 μg./ml.), L-methionine (100 μg./ml.), or L-glutamine (100 μg./ml.) were added together with C14 labelled D-phenylalanine (100 μg./ml.). The uptake of the D-tyrosine was followed colorimetrically and the uptake of the D-phenylalanine was followed isotopically as described in the methods section. Each value listed was the average of two determinations measured from two identically treated pads.

The L-amino acids added with D-tyrosine were added at the minimum concentration previously found to inhibit induction by D-tyrosine. L-phenylalanine and L-methionine were added at the minimum concentration found to inhibit induction by D-phenylalanine. L-glutamine, as noted above, is ineffective in inhibiting induction by the D-amino acids.

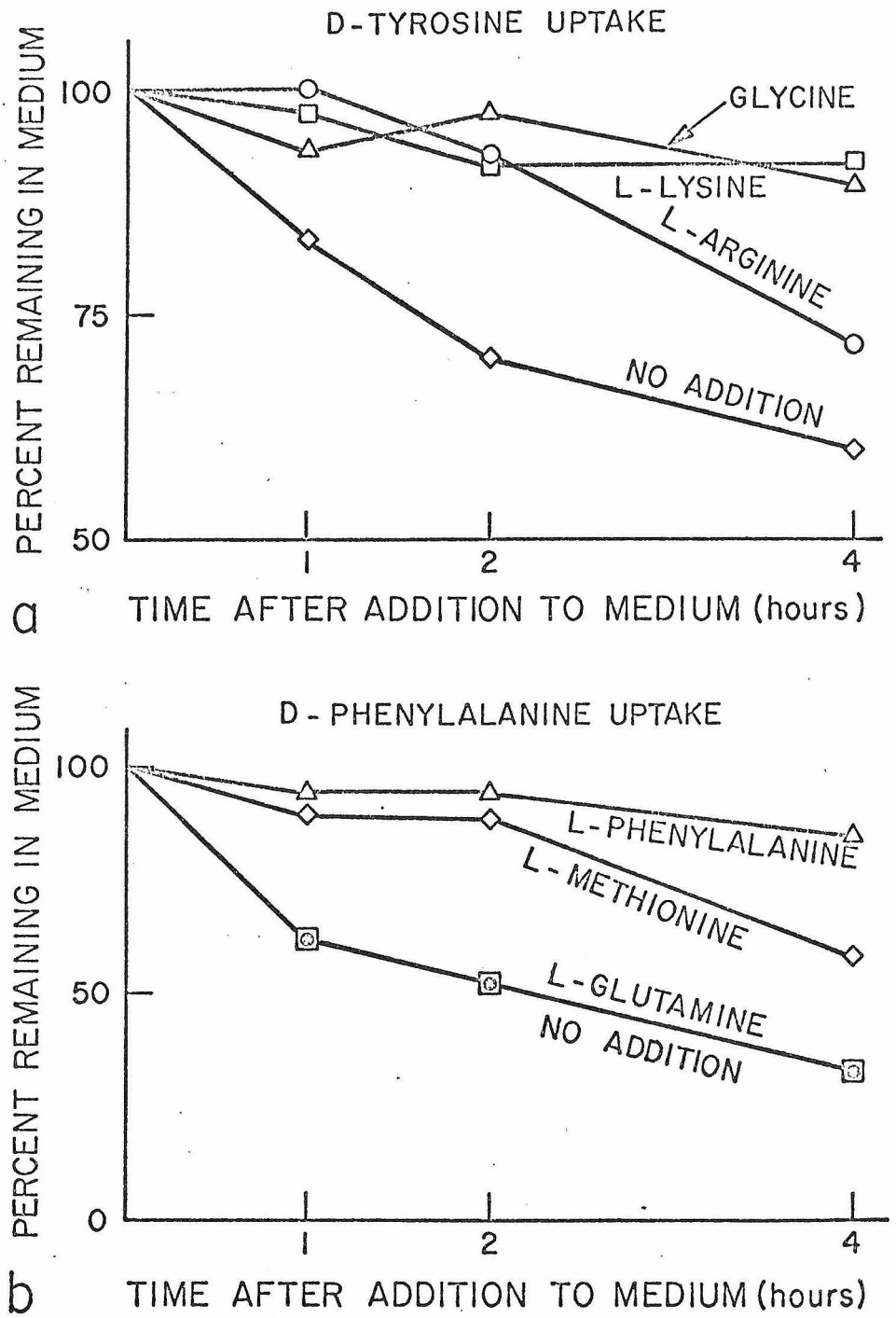

D-TYROSINE UPTAKE
PERCENT REMAINING IN MEDIUM
100
75
50
GLYCINE
L-LYSINE
L-ARGININE
NO ADDITION
1
2
4
a
TIME AFTER ADDITION TO MEDIUM (hours)
D-PHENYLALANINE UPTAKE
PERCENT REMAINING IN MEDIUM
100
50
0
L-PHENYLALANINE
L-METHIONINE
L-GLUTAMINE
NO ADDITION
1
2
4
b
TIME AFTER ADDITION TO MEDIUM (hours)

Figure 2

action in inhibiting growth.

Several examples have been cited where D-amino acids demonstrate some antimetabolic activity (61). In most cases, the sites of action of the D-amino acids have not been determined. Three more definitive examples have, however, been characterized. D-cycloserine inhibits the utilization of D-alanine for cell wall synthesis in bacteria (62, 63). D-serine inhibits the synthesis of β-alanine and its utilization in the synthesis of pantothenic acid (64). Also, as noted above, D-tyrosine inhibits L-tyrosine activation (57). This last observation suggests a specific antagonism between D- and L-stereoisomers of an amino acid at the level of amino acid activation. It is not known whether any of these effects occur in Neurospora.

Experiments were performed to ascertain if any of the above sites of activity could be involved in the induction of tyrosinase by D-phenylalanine. D-alanine, L-phenylalanine, β-alanine, and pantothenic acid were each tested for their effect on tyrosinase induction by D-phenylalanine. If D-phenylalanine acts by competitively inhibiting the utilization of one of these compounds, then that compound should inhibit the induction of tyrosinase by D-phenylalanine.

One hour was allotted for D-phenylalanine to be taken up into the mycelial pads before the addition of the other compounds to the medium. The pads were harvested and assayed for tyrosinase at five or seven hours after the addition of D-phenylalanine. The results are shown in table VII. None of the compounds tested were effective in inhibiting induction by D-phenylalanine. Therefore, none of the sites of action suggested here can be involved in induction by D-phenylalanine. Thus,

TABLE VII EFFECTS OF SEVERAL COMPOUNDS ON TYROSINASE INDUCTION BY D-PHENYLALANINE

Compound added (One hour after addition of D-phenylalanine)	Experiment 1 (Harvested and assayed at five hours after addition of D-phenylalanine)	Experiment 2 (Harvested and assayed at seven hours after addition of D-phenylalanine)
None	71.6 units/gm.	160 units/gm.
β-alanine	76.6 units/gm.	
Pantothenic acid	59.5 units/gm.	
β-alanine and Pantothenic acid	74.3 units/gm.	
L-phenylalanine		153 units/gm.
D-alanine		153 units/gm.

Mycelial pads of 69-1113a were prepared according to the short term induction procedure. D-phenylalanine (100 µg./ml. of medium) was added to the medium and the pads were shaken for one hour. Then the compounds listed above were added to the medium and the cultures shaken until harvesting. The following concentrations were used in the medium: β-alanine, 50 µg./ml. Calcium pantothenate, 10 µg./ml. L-phenylalanine, 1 mg./ml. D-alanine, .5 mg./ml. The values listed are the average of the tyrosinase activities determined for two identically treated mycelial pads.

although several possibilities have been eliminated, the site of action of the aromatic D-amino acids in the induction of tyrosinase remains undetermined.

A Possible Site of Action of Amino Acid Analogues in Induction

The inhibitor of protein synthesis, cycloheximide, was found to be an inducer of tyrosinase as noted above. In addition, a number of amino acid analogues have been found to be effective inducers of the enzyme (25). The fact that the enzyme is induced by a known inhibitor of protein synthesis and by amino acid analogues, which may be intimately connected with protein synthesis, suggests a site of action for these inducers. Partial inhibition of the synthesis of functional proteins may be sufficient to induce tyrosinase (43), after some process has occurred in the mycelium.

A likely mechanism by which an amino acid analogue might inhibit the synthesis of functional proteins can be suggested. An analogue could induce through its incorporation into proteins in place of a natural amino acid (45). Some of the newly synthesized proteins would be completely or partially nonfunctional due to the incorporation of the analogue into their structure. In this way, the synthesis of functional proteins would be partially inhibited.

Several examples have been reported where the amino acid analogues, ethionine and parafluorophenylalanine, are incorporated into protein. Ethionine can be incorporated into the proteins of rats (46), Tetrahymena (47), B. subtilis (48, 49), and Neurospora (54). Parafluorophenylalanine can be incorporated into the proteins of E. coli (50), B. subtilis (51), chicken (52), rabbit, and Neurospora (55). In several

cases, the incorporation of the analogue was found to lower the activity of specific enzymes.

The above considerations have led to a series of experiments analyzing the role of ethionine and parafluorophenylalanine in the induction of tyrosinase. These experiments show a strong correlation between the incorporation of the analogues into protein and their induction of tyrosinase.

Ethionine and Parafluorophenylalanine as True Analogues in Induction

If ethionine and parafluorophenylalanine are acting as true analogues in the induction of tyrosinase, then their inductive effect should be specifically inhibited by their natural counterparts, methionine and phenylalanine. To determine if this specific inhibition occurs, L-ethionine (25 μg./ml.) or DL-parafluorophenylalanine (150 μg./ml.) was added to several cultures. After ten minutes of shaking, one of the twenty common amino acids was added to each culture and the cultures were then shaken for six hours, whereupon they were harvested and assayed for tyrosinase. The results are shown in table VIII.

Of the twenty amino acids normally incorporated into protein, only methionine inhibits ethionine induction and only phenylalanine inhibits parafluorophenylalanine induction. It was found above that less than ten minutes is required for inducing amounts of analogue to be taken up into the mycelium. Since the natural amino acids were added ten minutes after the analogues, the observed inhibition cannot be due to the inhibition of uptake of the analogues. Furthermore, the broad specificities observed for amino acid transport in Neurospora (56) make such specific antagonisms at the level of uptake very unlikely.

TABLE VIII EFFECTS OF VARIOUS L-AMINO ACIDS ON INDUCTION BY ETHIONINE OR PARAFLUOROPHENYLALANINE

L-amino acid added	Ethionine as an inducer	Parafluorophenylalanine as an inducer
None	Induction	Induction
Alanine	Induction	Induction
Arginine	Induction	Induction
Asparagine	Induction	Induction
Aspartic Acid	Induction	Induction
Cystine	Induction	Induction
Glutamic Acid	Induction	Induction
Glutamine	Induction	Induction
Glycine	Induction	Induction
Histidine	Induction	Induction
Isoleucine	Induction	Induction
Leucine	Induction	Induction
Lysine	Induction	Induction
Methionine	No induction	Induction
Phenylalanine	Induction	No induction
Proline	Induction	Induction
Serine	Induction	Induction
Threonine	Induction	Induction
Tryptophan	Induction	Induction
Tyrosine	Induction	Induction
Valine	Induction	Induction

TABLE VIII (Cont.)

Mycelial pads of 69-1113a were prepared according to the short term induction procedure. L-ethionine was added to a concentration of 25 µg. per ml. of medium or DL-parafluorophenylalanine was added to a concentration of 150 µg. per ml. of medium. The other amino acids were added ten minutes after the addition of the analogues and were added to a concentration of .5 mg. per ml. of medium. The mycelial pads were harvested and assayed six hours after the addition of the analogues. Pads with less than one unit of tyrosinase per gram wet weight were scored as non-induced. Pads with more than five units per gram wet weight were scored as induced.

Thus, a specific relationship exists in the mycelium between ethionine and methionine and also between parafluorophenylalanine and phenylalanine. This is probably due to ethionine and parafluorophenylalanine acting as true analogues in the induction of tyrosinase.

Incorporation of Ethionine and Parafluorophenylalanine into Neurospora Proteins

As was noted above, there is evidence that ethionine and parafluorophenylalanine are incorporated into Neurospora proteins (54, 55). Preliminary experiments showed that isotopically labelled proteins are synthesized by mycelia incubated with labelled ethionine or parafluorophenylalanine under conditions where these analogues induce tyrosinase. It is necessary to determine if the label incorporated into protein remained in the form of the analogue or was resynthesized into other amino acids. The results described below show that most of the label represents actual analogue incorporation into protein.

Isotopically labelled analogue was diluted with unlabelled analogue and added to cultures at inducing concentrations. The cultures were shaken for two hours. They were then fractionated according to the procedure of Roberts et al. (34). The proteins were precipitated, washed, redissolved, and hydrolyzed as described in the section on methods. The amino acids in the hydrolysate were separated on high voltage paper electrophoresis. Authentic, isotopically labelled analogue was also run on electrophoresis as a standard with each hydrolysate. The results are shown in table IX.

Parafluorophenylalanine migrates at a position between phenylalanine and aspartic acid. It was well resolved from both phenylalanine

TABLE IX ELECTROPHORESIS OF ETHIONINE AND PARAFLUOROPHENYLALANINE LABELLED PROTEIN HYDROLYSATES

Sample	Counts per minute applied to paper	Counts per minute recovered from paper at the position of authentic analogue	Percent of counts recovered from paper at position of the analogue	Percent of counts in protein hydrolysate due to incorporation of the analogue*
Ethionine labelled protein hydrolysate	3040	1060	34.9%	67.2%
Authentic ethionine	2935	1523	51.9%	--
Parafluorophenylalanine labelled hydrolysate	2278	1079	47.4%	103%
Authentic parafluorophenylalanine	49,266	22,612	45.9%	--

Mycelial pads of 69-1113a were prepared according to the short term induction procedure. Each pad was incubated with either .4 μc. of H3 L-ethionine, diluted with .5 mg. of unlabelled L-ethionine or with .15 μc. of C14 labelled DL-parafluorophenylalanine diluted with 3 mg. of unlabelled DL-parafluorophenylalanine. The pads were in shaker with the analogues for two hours. The pads were fractionated and protein hydrolysates prepared as described in the text.

*As shown here, a fraction of the counts of the authentic analogue in the standards are recovered from the paper at the proper position

after electrophoresis. In like manner, a fraction of the counts of authentic analogue in a protein hydrolysate will be recovered from the paper. It is assumed that the standard of the analogue will have the same percent recovery as that same analogue in the hydrolysate. The figure here is corrected for this percent recovery.

and tyrosine in a hydrolysate from parafluorophenylalanine labelled pads. When the recovery at the position of parafluorophenylalanine is normalized using the percent recovery of the standard, 100% of the counts applied in the hydrolysate were from parafluorophenylalanine. Thus, all the counts incorporated into protein remained as parafluorophenylalanine.

Similarly, ethionine was well resolved from methionine, migrating close to the position of glutamic acid. About two-thirds of the counts in a hydrolysate were found to be from ethionine when normalized for the percent recovery of counts from the paper found in the standard. It is not clear whether the remaining third of the counts was present as oxidation products of ethionine, formed during the hydrolysis or as completely different amino acids. In either case, most of the counts incorporated into protein from both ethionine and parafluorophenylalanine labelled cultures remained as ethionine and parafluorophenylalanine respectively. It can be concluded, therefore, that both analogues can be incorporated unchanged into Neurospora proteins.

Further studies were made to try to correlate the incorporation of the analogues into protein with their induction of tyrosinase. Various concentrations of methionine and phenylalanine were used to inhibit the induction of tyrosinase by ethionine and parafluorophenylalanine respectively. The range of concentrations of these natural amino acids required to substantially inhibit induction was determined. The tyrosinase activity obtained in the presence and absence of these concentrations of methionine and phenylalanine is shown in table X and table XI. The incorporation of the analogues was also studied under these same

conditions, using the natural amino acids, methionine and phenylalanine, to inhibit incorporation as well as induction. In both the incorporation and induction experiments, the analogues were added ten minutes before the addition of the natural amino acids. The results are shown in table X and table XI.

The results show that the incorporation of parafluorophenylalanine into protein is inhibited by phenylalanine. Furthermore, the concentration range of phenylalanine previously found to give effective inhibition of induction is the same concentration range required to give effective inhibition of incorporation. Similarly, the incorporation of ethionine into protein is inhibited by methionine. The minimum concentration of methionine required to inhibit ethionine induction of tyrosinase is approximately the same as the minimum concentration of methionine required to substantially inhibit ethionine incorporation. The small amount of inhibition of incorporation of ethionine by phenylalanine is due to the inhibition of uptake of the ethionine remaining in the medium at the time of addition of the phenylalanine. Only 75% as much ethionine is taken up in the phenylalanine treated pads as in pads with no second amino acid added.

Thus, parafluorophenylalanine is incorporated into protein in place of phenylalanine and ethionine is incorporated in place of methionine. Furthermore, the incorporation of the analogues into protein is correlated with their induction of tyrosinase. These results, together with the above described induction of tyrosinase by the inhibitor of protein synthesis, cycloheximide, provide excellent support for the proposed site of action of these compounds during induction: That is, that

TABLE X INCORPORATION OF ETHIONINE INTO PROTEIN AND THE INDUCTION OF TYROSINASE

Second amino acid added (concentration per ml. of medium)	Incorporation into protein (millimicromoles of ethionine per pad)	Incorporation into protein (percent of amount with no second amino acid added	Tyrosinase activity (units per gram wet weight)
None	41.6	(100%)	31.5
4 µg./ml. L-methionine	19.6	47.1%	1.5
8 µg./ml. L-methionine	7.2	17.3%	.5
8 µg./ml. L-phenylalanine	31.9	76.6%	--

Pads of 69-113a were prepared according to the short term induction procedure. .4µc. of H3 L-ethionine was diluted with .5 mg. of cold L-ethionine and added to the medium of each culture. When a second amino acid was added, it was added ten minutes after the addition of the ethionine. Two hours after the addition of the ethionine, the pads were fractionated according to the procedure of Roberts et al. (34) as described in the text. The amount of ethionine incorporated into protein was calculated from the specific activity of the ethionine added and the number of counts found in the protein fraction. It was assumed that all counts incorporated represent ethionine.

The tyrosinase activity was determined from mycelial pads treated identically to those in which the protein labelling was measured except that no H3 labelled ethionine was added. The pads were harvested and assayed for tyrosinase five hours after the addition of the ethionine.

Each value listed is an average determined from two identically treated mycelial pads.

TABLE XI

INCORPORATION OF PARAFLUORO-PHENYLALANINE INTO PROTEIN AND THE INDUCTION OF TYROSINASE

Second amino acid added (concentration per ml. of medium)	Incorporation into protein (millimicromoles of parafluoro-phenylalanine per pad)	Incorporation into protein (per cent of amount with no second amino acid added)	Tyrosinase activity (units per gram wet weight)
None	389	(100%)	31.0
50 μg./ml. L-phenylalanine	165.5	42.6%	3.7
100 μg./ml. L-phenylalanine	109.2	28.0%	0
100 μg./ml. L-methionine	390	100%	--

Pads of 69-1113a were prepared according to the short term induction procedure. .3 μc. of C14 DL-parafluorophenylalanine was diluted with 3 mg. of cold DL-parafluorophenylalanine and added to the medium of each culture. Where a second amino acid was added, it was added ten minutes after the addition of the parafluorophenylalanine. Two hours after the addition of the parafluorophenylalanine, the pads were fractionated according to the procedure of Roberts et al. (34) as described in the text. The amount of parafluorophenylalanine incorporated into protein was calculated from the specific activity of the parafluorophenylalanine added and the number of counts found in the protein fraction. It was assumed that all counts incorporated represent parafluorophenylalanine.

The tyrosinase activity was determined from mycelial pads treated identically to those in which the protein labelling was measured except that only unlabelled parafluorophenylalanine was added. The pads were harvested and assayed for tyrosinase five hours after the addition of the parafluorophenylalanine.

Each value listed was an average determined from two identically treated mycelial pads.

these inducers act by partially inhibiting the synthesis of functional proteins.

DISCUSSION AND CONCLUSIONS

Uptake of the D-Amino Acids

The induction of tyrosinase by the aromatic D-amino acids is antagonized by a wide variety of L-amino acids. The L-amino acids act by inhibiting the uptake of the aromatic D-amino acids. These results may be due to the existence of an amino acid transport system with broad specificity, capable of transporting both D- and L-amino acids. If so, the L-amino acids should be competitive inhibitors of the uptake of the D-amino acids. Alternatively, the inhibition of uptake of the D-amino acids could be due to some other effect of the L-amino acids, such as an allosteric inhibition of the D-amino acid transport system. Clearly, these problems require further attention. They are dealt with in more detail in the second part of this thesis.

Site of Action of the Inducers of Tyrosinase

The mechanisms by which some of the inducers of tyrosinase act have become quite clear. Cycloheximide, ethionine, and parafluorophenylalanine all act by partially inhibiting the synthesis of functional proteins. Cycloheximide, at levels which partially inhibit general protein synthesis, is an effective inducer of tyrosinase. Ethionine and parafluorophenylalanine become incorporated into newly synthesized proteins. They are capable, in this way, of partially inhibiting the functional activity of the newly synthesized proteins. Thus, these three inducers, at least, act in physiologically similar ways. A partial inhibition of the synthesis of functional proteins is sufficient, in some way, to induce tyrosinase synthesis.

The site of action of the aromatic D-amino acids is much less evident. Those possible sites which have been tested above, have not been implicated in the induction of tyrosinase. Although the aromatic D-amino acids do inhibit general protein synthesis to some extent (55), it is not clear that the inhibition is a direct effect of the D-amino acids or that the low level of inhibition is sufficient to explain the efficient induction of tyrosinase. More work is needed in this area.

The Lag Period

Most tyrosinase inducers, when added to a Neurospora culture, are taken up rapidly into the mycelium. Following uptake, however, there is a lag period during which no tyrosinase synthesis occurs. During the lag period, some active, energy requiring, process must occur. This process must precede the induction of tyrosinase. The lag period is followed by the de novo synthesis of the tyrosinase protein. At no time during the induction process is there any large pool of a protyrosinase enzyme precursor present.

Control of Tyrosinase Induction

Thus, the state of knowledge of tyrosinase induction by antimetabolites can be summarized briefly. A partial inhibition of the synthesis of functional proteins is followed by a lag period. During the lag period, some active process prepares the mycelium for tyrosinase synthesis. Following the lag period, rapid synthesis of the tyrosinase protein ensues.

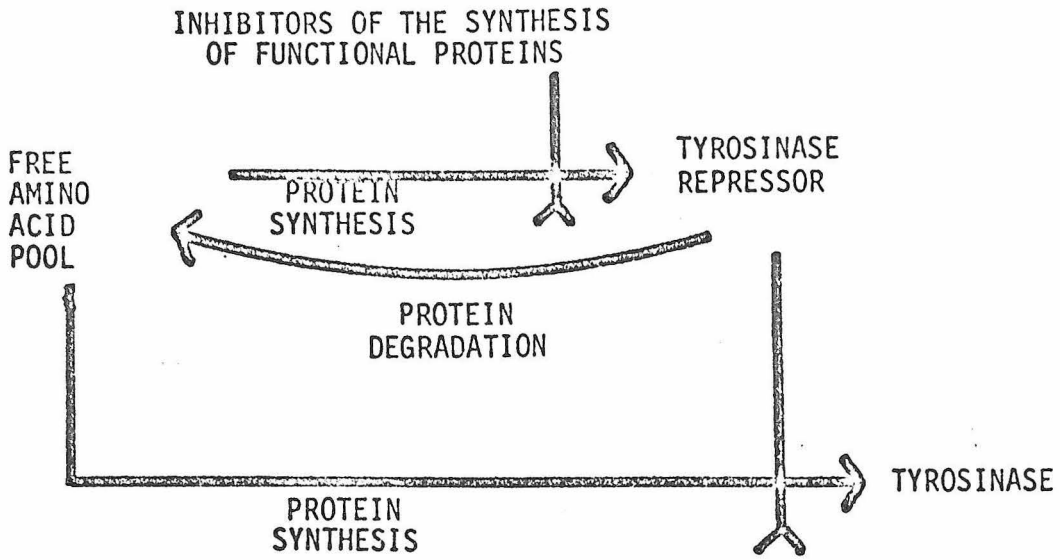
Evidently, the most critical biochemical studies on tyrosinase induction to be approached in the future must involve the elucidation

of the processes occurring during the lag period. Current work in this laboratory, for instance, is determining whether changes in transfer RNA accompany the induction of tyrosinase. Transfer RNA changes have been suggested to be involved in the control of protein synthesis (78). Another unresolved question is whether the synthesis of new tyrosinase messenger RNA must precede the induction of tyrosinase. The partial inhibition of tyrosinase induction by actinomycin D is suggestive in this regard (43). However, as discussed above, many ambiguities occur in interpreting experiments with antimetabolites. Perhaps, more efficient inhibitors of RNA synthesis in Neurospora will lead to more definitive information on tyrosinase messenger RNA synthesis.

Other studies of changes during and immediately following the lag period suggest themselves. However, the development of major new techniques may be required. Are histones involved in the control of tyrosinase synthesis? Their implication in other control systems (79) suggests that they are. Studies in this area require a knowledge of the various histone fractions in Neurospora and the properties of these fractions. In addition, an in vitro system for the synthesis of tyrosinase would be useful.

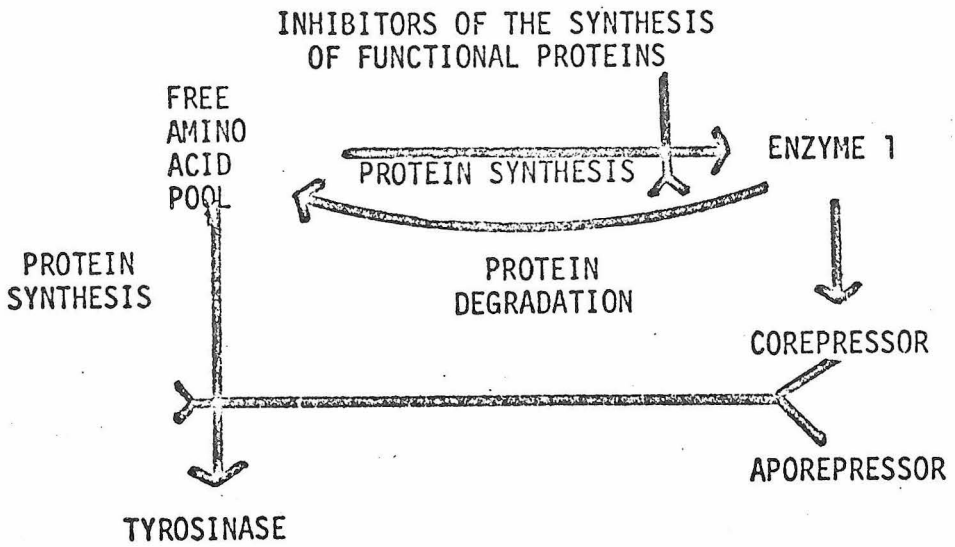
Possibly the most important and most difficult studies on the control of tyrosinase synthesis are suggested by the consideration of control circuits in the introduction. If control circuits are involved in tyrosinase control then the synthesis of a series of proteins and possibly other molecules must precede the synthesis of tyrosinase in tyrosinase induction. Since the proteins or other molecules involved would probably be synthesized only in minute quantities, they might be

MODEL 1.



a.

MODEL 2.



b.

Figure 3.

difficult to detect by the usual techniques. Perhaps, genetic rather than biochemical techniques may prove more effective in establishing an understanding of control circuitry. Such possibilities are discussed further below.

Models of Tyrosinase Control

The above considerations suggest a number of possible models for the control of tyrosinase synthesis. Figure 3a contains a diagrammatic representation of the rapidly turning over repressor model previously proposed by Dr. N. H. Horowitz (43). In this model, the tyrosinase repressor, which is responsible for the repression of tyrosinase synthesis, is both rapidly synthesized and destroyed. Normally, during vegetative growth, a sufficiently high level of repressor is maintained to repress tyrosinase synthesis. During starvation or upon the addition of inhibitors of functional protein synthesis, the synthesis of the repressor is diminished. The continued rapid destruction of the repressor then produces a lowering of repressor level. When the repressor concentration falls below some critical point, rapid tyrosinase synthesis begins.

The rapidly turning over repressor model does not specify whether the control of tyrosinase synthesis occurs at the DNA or RNA level. It must, however, assume that the destruction of the repressor is, in some way, coupled to the utilization or synthesis of metabolic energy. This last conclusion is a consequence of the finding that progress through the lag period requires metabolic energy.

The model presented in figure 3b is quite similar to the rapidly

MODEL 3.

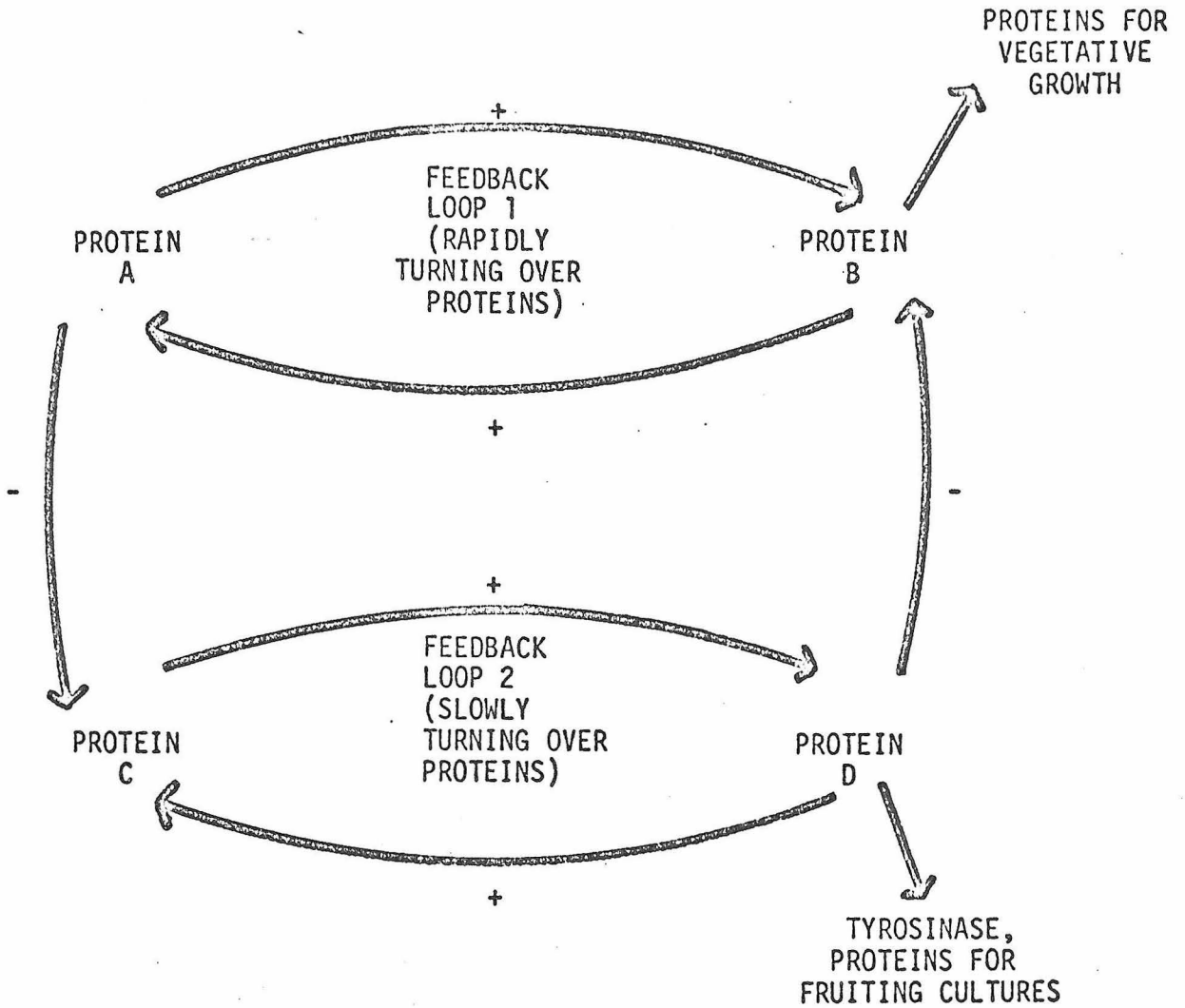


Figure 4.

turning over repressor model. In it, however, a rapidly turning over enzyme shows the critical dependence on protein synthesis. Such an enzyme would catalyze the synthesis of a corepressor involved in tyrosinase control.

Both of the above described models are presented with the terminology and concepts of the operon theory. As discussed in the introduction, it is not clear whether operon-like control occurs in eukaryotes. It is not possible, therefore, to determine that these model descriptions are strictly relevant. If another biochemical theory of control arises, however, these model descriptions may prove to be modifiable in terms of the new concepts.

A third model for the control of tyrosinase synthesis is presented in figure 4. It differs from the above two models in not being presented in the terminology of the operon theory. It is presented in terms of the concepts of information transfer and, therefore, is not a true biochemical model. The biochemical basis of the interactions described in the model is not specified.

The synthesis of two proteins, protein A and protein B, are related to each other in a positive feedback loop. The existence of protein A in the mycelium causes an increase in the synthesis of protein B. Protein B causes an increase in the synthesis of protein A. These, then, comprise a positive feedback loop, loop 1. Similarly, protein C causes an increase in the synthesis of protein D and protein D causes an increase in the synthesis of protein C. Thus, these comprise positive feedback loop 2. Protein D, however, inhibits the synthesis of protein B and protein A inhibits the synthesis of protein C.

Consequently, when the proteins in loop 1 are present in high concentrations, those in loop 2 are present in low concentration and vice versa. Proteins A and B in loop 1 are rapidly turning over and proteins C and D in loop 2 are more slowly turning over. Finally, it is assumed that all the interactions are such that in rapidly growing, vegetative cultures, the proteins in loop 1 are present in much higher concentrations than those in loop 2.

A partial inhibition of protein synthesis will lead to a much larger decrease in the initial level of the loop 1 proteins than the loop 2 proteins since the loop 1 proteins are more rapidly turning over. Moreover, a decrease in the loop 1 proteins will lead to an increase in loop 2 proteins which will further decrease loop 1 proteins, etc. These responses occur as a direct consequence of the above described interactions. Through further operation of the feedback loops outlined, a small inhibition in protein synthesis will lead to a large increase in the levels of the proteins in loop 2. Finally, since one of the loop 2 proteins, protein D, induces tyrosinase synthesis, the induction of tyrosinase by inhibitors of functional protein synthesis is explained.

Model 3 differs from the previous two models in that the exogenous inducers act not by inhibiting the synthesis of a single protein, but rather by influencing the synthesis of several proteins. The effect of this inhibition is magnified through the action of positive feedback loops. Model 3 serves as a prototype for models containing several macromolecules interacting in such a way as to form a control circuit. These circuits, as discussed in the introduction, are likely to be involved in some control systems and may be constructed in quite a variety

of ways (8, 23). Model 3, then, is a representative control circuit that explains tyrosinase induction.

The discussion of the above models raises a number of questions about tyrosinase control. Do the inhibitors of protein synthesis act on one or several proteins in inducing tyrosinase? How many proteins and other macromolecules are involved in tyrosinase control? Finally, how are these organized?

As discussed above, the biochemical techniques available are inadequate to answer these questions. Perhaps genetic techniques are the only ones available that may answer such questions. It is hoped that a thorough search for and study of mutants that affect tyrosinase induction may lead to an understanding of the number of genetic loci involved in control and their respective roles in this function.

Techniques for the isolation of mutants increasingly closer to the actual site of control have been applied in the galactose system in yeast (12-14) and may be applicable to the tyrosinase system in Neurospora. The characterization of such Neurospora mutants as to their dominance and behavior in strains carrying several markers affecting tyrosinase control should be useful in answering at least some of the questions raised above.

Perhaps the most enlightening perspective on the tyrosinase control system may be obtained from a consideration of the experience with the β-galactosidase system in E. coli. Here, the physiological and biochemical studies of Monod and coworkers (82) preceded the genetic analysis of the system. The physiological and biochemical studies reported here and elsewhere (25, 28, 43) may, then, correspond to the early studies by Monod.

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INTRODUCTION

The movement of substances through membranes is one of the most important phenomena of life. Studies on this phenomenon led to the finding that such movement often did not follow the laws of simple diffusion, but rather showed many of the properties of enzyme reactions. Transport through biological membranes showed, for instance, saturation kinetics, competition between chemically similar substances, and high temperature coefficients. In addition, a dependence on energy metabolism was often demonstrated. About fifteen years ago, the concept of active transport was developed in accordance with the above findings (1). The term permease (2) was later coined to describe the enzyme-like proteins involved in transporting compounds through membranes. A particular permease was able to catalyze the transport of a group of structurally related compounds through the membrane.

A large number of models have been proposed to explain the phenomenon of active transport. In many models, a protein functions as a mobile carrier of the transported substance, conveying it through the membrane (2-5). Often the protein carrier is identical to the permease which has the specific binding site for the substance transported. In other models, the permease may undergo an allosteric transition which changes the access of the bound substance to the two sides of the membrane (6).

Much recent work has concentrated on determining the validity of the various models and ascertaining, in general, the mechanisms involved in active transport. This area of research has concentrated on the

isolation of the proteins involved in active transport and the study of their properties (7-12). An understanding of the molecular biology of transport will most likely be derived from these studies.

This section is concerned with delimiting some of the transport systems* involved in amino acid transport in Neurospora. Two techniques have been commonly used to delimit the range of activities of particular transport systems and to distinguish them from other transport systems which may also be active in the same organism.

Genetic studies have centered on the isolation and characterization of mutants deficient in the transport of one or more compounds. In bacteria, these mutants, often termed permeaseless, are generally deficient in the uptake of a small group of structurally related substrates (2, 13-15). The mutants often do appear to be deficient in a specific protein or permease that binds the substrate and catalyzes its transport. The determination of the transport deficiencies of such mutants is of considerable value in delimiting the range of substrate specificity of the missing transport system.

The study of permeaseless mutants is complicated, however, by the existence of other types of mutants which have their active transport affected in more general ways. Several mutants of Neurospora, for example, have their amino acid transport activity altered through non-specific changes in membrane structure (16, 17) or through altered control

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*The term "transport system" rather than "permease" is frequently used in this thesis. The term is defined as one or a small group of membrane proteins capable of transporting a group of structurally related compounds. Transport systems usually, but not always, act independently of one another (20).

of the synthesis of the transport proteins (18). Only in the case of the mtr mutant, whose properties are described below, does it appear likely that a mutant strain of Neurospora, deficient in amino acid transport, is defective due to a permeaseless type of deficiency (25, 26). The use of mutants to delimit transport systems is restricted, then, by the ability to distinguish permeaseless from mutants unable to transport particular substances due to other types of lesions. This problem can be solved, in part, by kinetic studies on transport.

One of the original observations that led to the concept of active transport was that transport of substances across membranes often showed saturation at high substrate concentrations. Furthermore, compounds of similar structure to a transport substrate, often competitively inhibited transport of that substrate. The saturation and competition, moreover, often followed Michaelis-Menten kinetics. By studying the competitive inhibition of the transport of one substrate by other potential substrates, the range of substrate specificities of a particular transport system can be determined.

The applicability of the kinetic approach is limited by the existence of more than one transport system of overlapping specificities in a given organism. In Ehrlich cells, for instance, almost all neutral amino acids studied appear to be transported by both of two transport systems present. The activities of the two systems have proved difficult to separate (19). In addition, the applicability is further limited by the possible existence of noncompetitive inhibition of transport by various compounds (20).

The kinetic and genetic approaches both have their limitations.

The limitations, however, lie in different areas. The two approaches, then, can complement each other in delimiting the activities of various transport systems. Although the second section of this thesis is involved primarily with kinetic information, the role of the mtr locus is also considered.

A number of studies of amino acid transport in Neurospora have been made where the uptake of one amino acid has been inhibited by other amino acids (21-26). In most of these cases, proper kinetic analysis of the inhibition has not been done to determine whether or not the inhibition is competitive. The uptake of phenylalanine (21) and arginine (22) into Neurospora conidia is inhibitable by a large number of amino acids, basic and neutral. Furthermore, the uptake of the aromatic D-amino acids into Neurospora mycelia is also inhibitable by a wide variety of L-amino acids (23). If these inhibitions are competitive, they imply the existence of an amino acid transport system with a very broad range of substrate specificity. Experiments discussed below show that such a transport system does exist.

Wiley and Matchett (24) have studied tryptophan uptake in germinated conidia. They have shown that L-tryptophan uptake is competitively inhibited by L-phenylalanine and L-leucine but that it is not inhibited by basic or acidic amino acids. Their results imply the existence of a transport system with limited specificity responsible for the uptake of large, neutral amino acids.

Finally, the mtr mutant, briefly mentioned above, was isolated by Lester because of its resistance to 4-methyltryptophan (26). It was subsequently determined to be deficient in the uptake of 4-methyl-

tryptophan, L-tryptophan, L-phenylalanine, and L-leucine, but has normal uptake of the basic amino acids (25, 26). It may be deficient, then, in the uptake of those amino acids with an affinity for the uptake system of Wiley and Matchett. Thus, although the studies performed are not definitive at this point, the mtr mutant may correspond to the permeaseless mutants in bacteria. The permease controlled by the mtr locus may be responsible for the transport studied by Wiley and Matchett.

There appear to be two groups of information in conflict. One group implies the existence of a transport system with broad specificity which is responsible for most of the uptake in conidia (21, 22) and older mycelia (23). Another group implies that in germinated conidia most of the uptake of neutral amino acids is catalyzed by a system of more limited specificity. This apparent conflict is resolved below.

MATERIALS AND METHODS

Strain

Wild type strain ST74A (27) was used in most transport experiments. The D-amino acid oxidaseless strain, oxD-8, was used in transport studies of the aromatic D-amino acids (28).

The mtr mutant strain has been found to be deficient in the transport of tryptophan and several other neutral amino acids (26, 27). Transport was studied in an mtr strain carrying the ylo-1 marker which was obtained from the Fungal Genetics Stock Culture Center (FGSC #1117).

Chemicals

All chemicals used were of reagent grade quality except as noted below. Table sugar was used as sucrose in the culture medium. Commercial grade Triton X-100 was obtained from Rohm and Haas.

L-alanine, β-alanine, γ-aminobutyric acid, 2-aminoethanol, L-arginine, L-asparagine, L-aspartic acid, L-histidine, L-leucine, L-lysine, L-phenylalanine, pyruvic acid, and L-tryptophan were obtained from Sigma Chemical Company. Glycine, N-acetyl-L-leucine, and L-leucinamide were obtained from Nutritional Biochemicals Corporation. L-α-amino butyric acid, D-phenylalanine, and L-ornithine, were obtained from Calbiochem.

Glycine-C14 (50 mc./mm.), L-phenylalanine-H3 (2.5c./mm.), L-leucine-4,5-H3 (2c./mm.), and L-aspartic acid-C14 (113 mc./mm.) were obtained from Schwarz Bioresearch, Inc. D-phenylalanine-1-C14 (25 mc./mm.) and L-tryptophan-3-C14 (22 mc./mm.) were obtained from New

England Nuclear Corporation. L-Asparagine-C14 (u) (102 mc./mm.) was obtained from Nuclear Chicago Corporation.

Maintenance and Growth of Neurospora Cultures

All Neurospora strains were maintained on agar slants of Horowitz complete medium (29). The liquid medium used was one-half strength Vogel's medium N salts (30) containing one-half percent sucrose. 125 ml. Erlenmeyer flasks containing 20 ml. of liquid medium were inoculated with about 10^4 conidia in aqueous suspension. The cultures were grown without shaking for forty-eight hours at 25°C. Having formed mycelial pads, the cultures were gently shaken at 25°C. on a reciprocal shaker for about twenty-four hours. The three day old cultures were then used to study amino acid uptake.

The dry weight of the mycelial pads formed after three days growth, as described above, was about 50 mg. The medium had a pH of about 5.8 with the strains used.

Uptake of Amino Acids

Stock solutions were prepared of isotopically labelled amino acids diluted in specific activity with unlabelled amino acids. Aqueous stock solutions of other compounds which were to be tested as possible inhibitors of transport were prepared.

In uptake method 1, pads prepared as described above had labelled amino acid from a stock solution added to the medium. The pads were shaken moderately on a reciprocal shaker at 25°C. for two minutes, harvested on a Buchner funnel and washed thoroughly with ice cold distilled water. The pads were then dropped into cold 5% trichloroacetic

acid (TCA). When various compounds were to be tested as inhibitors of uptake, one of two techniques was used. For some experiments, the inhibitor or inhibitors were added to the medium of the shaken cultures about thirty seconds before the addition of the labelled amino acid. For other experiments, aliquots of inhibitor and labelled amino acid were mixed and added together.

Uptake method 2 was used when a large fraction (c. 20%) of either the labelled amino acid or inhibitor was taken up when using uptake method 1. In uptake method 2, the inhibitor, if any, and 60 ml. of distilled water, previously equilibrated at 25°C., were added to the cultures. An aliquot of labelled amino acid was then added to the culture medium and the cultures shaken vigorously for one minute at 25°C. on a rotary shaker. The pads were harvested on a Buchner funnel and washed thoroughly with ice cold distilled water. These pads were then dropped into cold 5% TCA.

Extraction and Measurement of Counts Taken Up

Each mycelial pad was extracted with 5 ml. of cold TCA for at least two hours. In most cases, two identically treated pads were pooled for the extraction. The pads were spun down. One-half to one ml. of TCA extract was neutralized with an equal volume of .6M tris. This mixture was counted in a toluene base scintillation fluid using Triton X-100 as a solubilizer (23).

RESULTS

Tryptophan Uptake

Active transport of amino acids is very rapidly occurring in three day old pads of wild type strain 74A. A number of amino acids can be rapidly accumulated into the mycelium to a concentration of several milligrams per gram of cell water over their concentration in the medium. The transport of L-arginine and D-phenylalanine was shown, in exploratory experiments, to be inhibited over 95% by 10^{-2}M azide or 10^{-3}M dinitrophenol (DNP). Thus, the accumulation depends on metabolic energy. Furthermore, high concentration gradients of D-phenylalanine can be produced through transport into the mutant strain oxD-8 which cannot metabolize D-amino acids (28). These results confirm the existence of active transport of amino acids under the conditions studied.

The uptake of 10^{-5}M L-tryptophan was shown, in preliminary experiments, to be largely inhibitable by L-arginine. In typical three day old pads of 74A, inhibition values of 70 to 85 percent were obtained with 10^{-4}M L-arginine from different experiments. As shown in figure 1, higher amounts of arginine than this give no greater inhibition of tryptophan uptake. Similarly, 10^{-4}M of the basic amino acids L-lysine, L-canavanine, and L-ornithine inhibited tryptophan uptake by 70 to 85 percent and higher concentrations gave no increase in inhibition. These results are in contrast to the normal response to competitive inhibitors where increasing the inhibitor concentration gives increasing inhibition, asymptotically approaching complete inhibition. Two possible explanations for the above results suggest themselves: (1) Tryptophan may be

Fig. 1. L-TRYPTOPHAN UPTAKE IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF L-ARGININE. Three day old mycelial pads of 74-A had various amounts of L-arginine added to the medium. They were shaken with 10^{-5}M tryptophan according to uptake method 1. In various experiments of this type, 10^{-5}M L-arginine appeared to give maximal inhibition of tryptophan uptake. Concentrations as high as 4×10^{-3}M L-arginine gave little or no increase in inhibition.

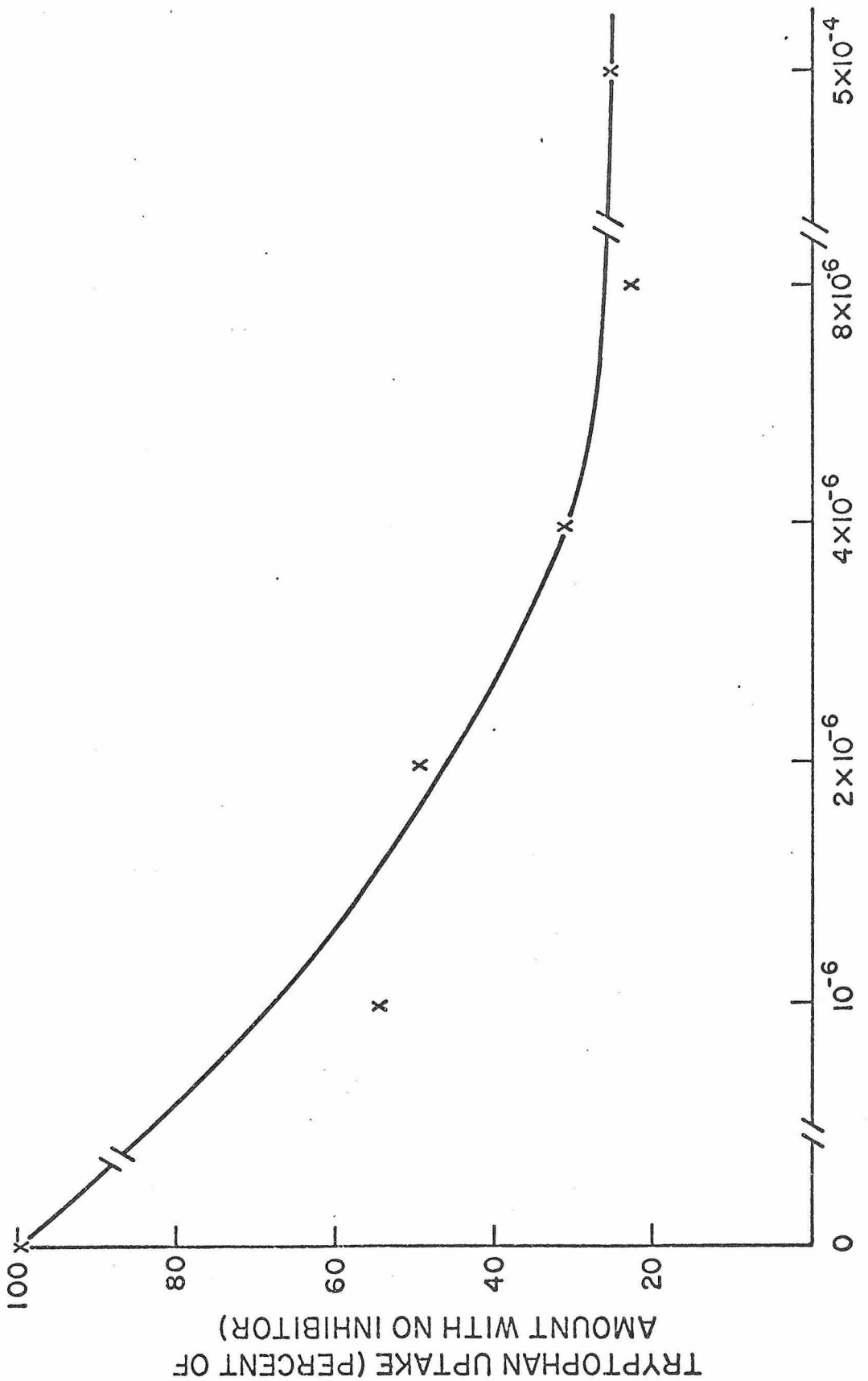

TRYPTOPHAN UPTAKE (PERCENT OF AMOUNT WITH NO INHIBITOR)
100
80
60
40
20
0
10^{-6}
2×10^{-6}
4×10^{-6}
8×10^{-6}
5×10^{-4}
ARGININE CONCENTRATION (M)

Figure 1

taken up by two transport systems, one competitively inhibited by arginine and the other not inhibited. Once the transport system which is sensitive to arginine inhibition is completely inhibited, no further inhibition can occur. (2) Alternatively, a single tryptophan transport system might be, in some way, only partially inhibited by arginine. This could occur, for example, through a common step in tryptophan and arginine transport as suggested by Koch (5). The inhibition, in this case, would be of some noncompetitive type. It will be shown below that the first of these explanations is consistent with the data. The second is not. Two distinct transport systems appear to be active under the conditions studied.

If two transport systems are active here, then they can be expected to differ, in general, in their affinities for various amino acids. That is, the Michaelis constants (K_m) and inhibition constants (K_i) for a particular amino acid will probably be different for the two systems. Conversely, the demonstration of two different constants for a given amino acid is evidence for the existence of two transport systems. The following method was used to determine such constants for the proposed two transport systems:

The uptake of a particular amino acid was observed at a given concentration, both with no arginine present and with sufficient arginine to give maximum inhibition. If two transport systems are present, the uptake of the amino acid being observed in the presence of arginine should be due only to the arginine insensitive transport system. The activity of the arginine sensitive transport system should be equal to the uptake in the absence of arginine minus the uptake in the presence

of arginine. In most cases 10^{-3}M, 2 x 10^{-3}M, or 4 x 10^{-3}M L-arginine was used to achieve maximum inhibition.

For example, assume that at 10^{-4}M L-tryptophan, 30 millimicromoles of tryptophan per minute per mycelial pad were taken up, and at 10^{-4}M L-tryptophan plus 10^{-3} L-arginine, 8 millimicromoles of tryptophan per minute per pad were taken up. The activity of the arginine insensitive transport system at 10^{-4}M L-tryptophan is 8 millimicromoles per minute per pad. The activity of the arginine sensitive uptake system at 10^{-4}M L-tryptophan is, then, 30 minus 8 or 22 millimicromoles per minute per pad. By determining these activities at various concentrations of L-tryptophan and analyzing the values on a Lineweaver-Burk plot, the K_m's can be determined for the two transport systems. Similar experiments can be performed for amino acids other than tryptophan which may also be transported by the proposed two transport systems.

Similarly, the inhibition constants or K_i's for various amino acids can be found. The uptake of L-tryptophan can be measured in the presence of an inhibitor, such as leucine, both in the presence and absence of arginine. In this way, the effects of other inhibitors on the two proposed transport systems can be determined. For example, using leucine as a potential inhibitor, assume that 20 millimicromoles of tryptophan per minute per pad are taken up from a medium containing 10^{-4}M L-tryptophan plus 10^{-5}M L-leucine. Assume further that 8 millimicromoles of tryptophan per minute per pad are taken up from a medium containing 10^{-4}M L-tryptophan plus 10^{-5}M L-leucine and 10^{-3}M L-arginine. Then the activity of the arginine insensitive transport system in the presence of the leucine inhibitor is 8 millimicromoles of tryptophan per minute per pad

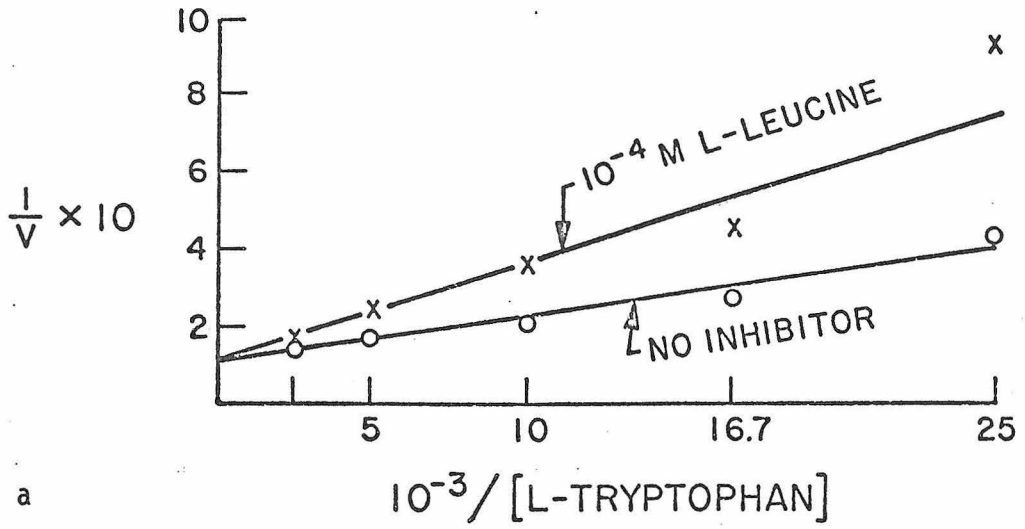
and the activity of the arginine sensitive transport system is 12 millimicromoles of tryptophan per minute per pad. Further consideration of these kinds of calculations are given in the appendix. The activities of the two systems at various concentrations of tryptophan can be found in the presence or absence of an inhibitor. Analyzing these values on a Lineweaver-Burk plot should yield K_i's for the two uptake systems.

Experiments of the type described above with no inhibitor present were performed to determine the Michaelis constants for L-tryptophan uptake of the two presumed uptake systems. Two typical experiments are shown in figure 2. The K_m for the arginine insensitive transport system is 6×10^{-5}M. The K_m for the arginine sensitive system is 4-5 $\times 10^{-5}$M. Due to the inaccuracies inherent in these determinations, the values obtained are not considered to be significantly different from each other.

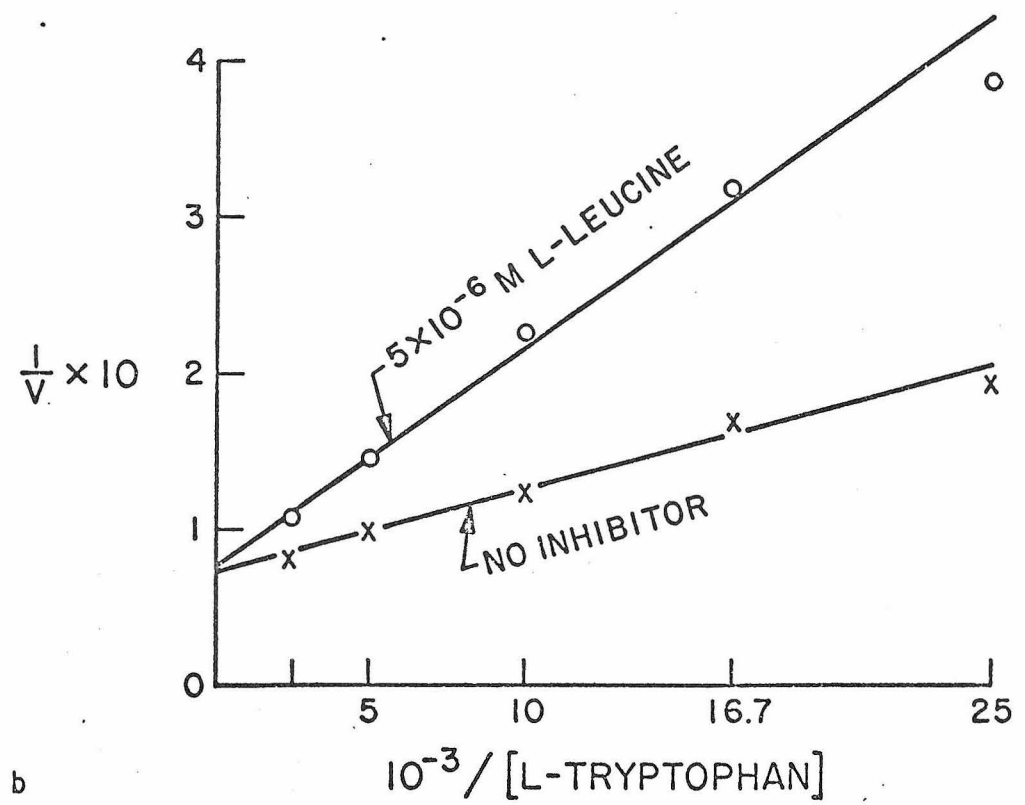
As is shown in figure 2, L-leucine is a competitive inhibitor of L-tryptophan uptake by both transport systems. The arginine sensitive transport system, however, requires much less leucine to be effectively inhibited than does the arginine insensitive system. Typical K_i values for L-leucine found for the two uptake systems are 3×10^{-6}M and 1.1×10^{-4}M respectively. Moreover, as shown in figure 3, L-leucine itself shows an arginine sensitive and arginine insensitive uptake as does tryptophan. Typical K_m determinations for these are 4×10^{-6}M and 1.2×10^{-4}M respectively. These are not considered to be significantly different from the K_i values for leucine given above but are certainly significantly different from each other.

The values determined for leucine show that the binding sites of the arginine sensitive and arginine insensitive transport systems differ

Fig. 2. L-TRYPTOPHAN UPTAKE IN THE PRESENCE OF L-LEUCINE. The inhibition of the arginine insensitive and the arginine sensitive components of L-tryptophan uptake by L-leucine was determined as described in the text. The inhibition of the arginine insensitive uptake of L-tryptophan by 10^{-4}M L-leucine, measured in the presence of 4 x 10^{-3}M L-arginine, is shown in figure 2a. It was measured by using uptake method 1. Figure 2b shows the inhibition of the arginine sensitive component of L-tryptophan uptake by L-leucine. The arginine insensitive component was measured using 2 x 10^{-3}M L-arginine. All values plotted in figure 2b were determined using uptake method 2. As is noted in the text, the arginine sensitive component of L-tryptophan uptake is substantially inhibited by much lower concentrations of L-leucine than is the arginine insensitive component. Furthermore, for both components, the inhibition appears to be competitive. For both plots, v is expressed as millimicromoles of L-tryptophan taken up per minute per pad.


10
8
6
4
2
1/V × 10
10^-4 M L-LEUCINE
NO INHIBITOR
5
10
16.7
25
10^-3 / [L-TRYPTOPHAN]

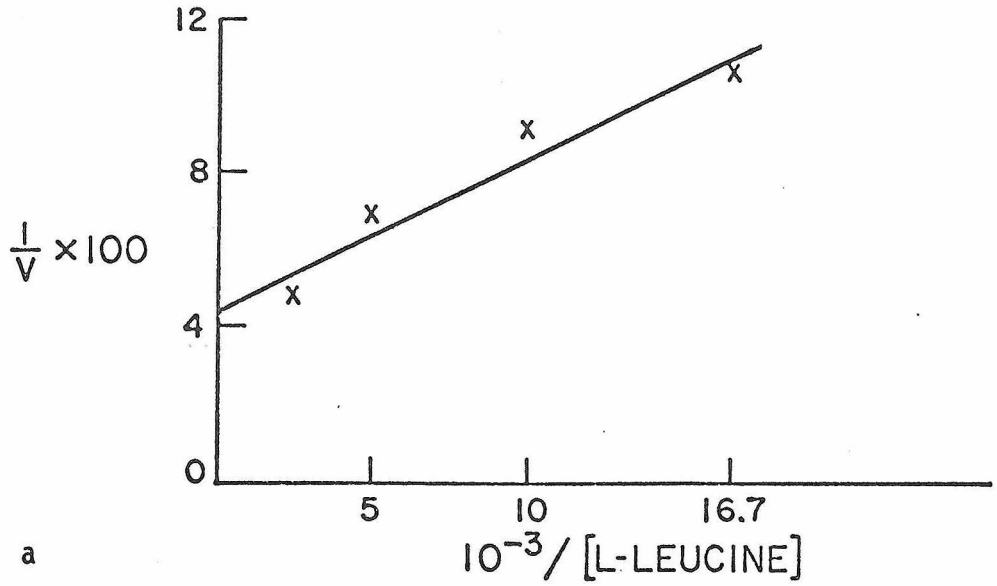
a


4
3
2
1
0
1/V × 10
5×10^-6 M L-LEUCINE
NO INHIBITOR
5
10
16.7
25
10^-3 / [L-TRYPTOPHAN]

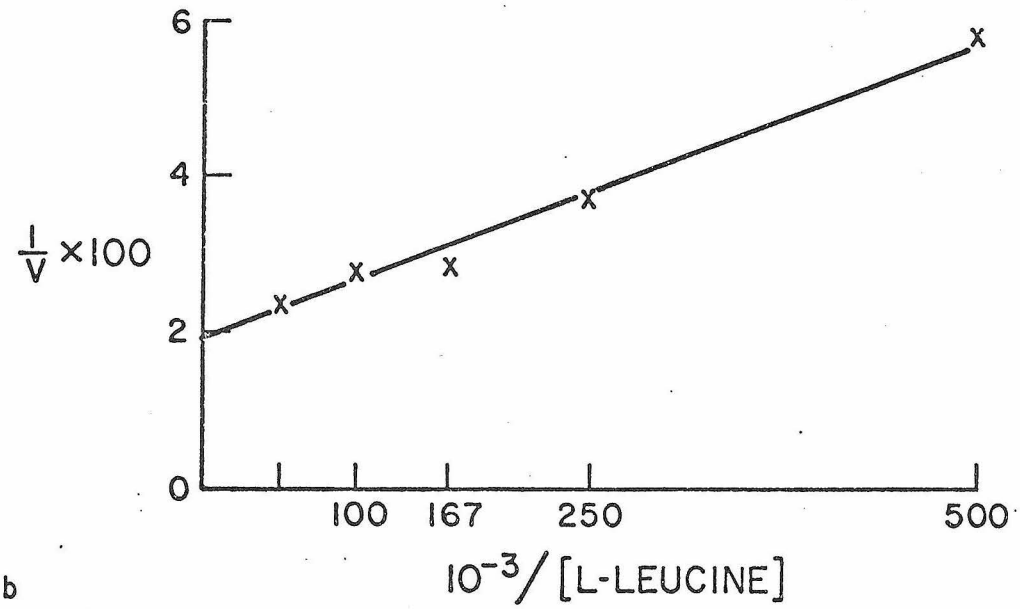
b

Figure 2

Fig. 3. L-LEUCINE UPTAKE BY THE ARGININE INSENSITIVE AND ARGININE SENSITIVE TRANSPORT SYSTEMS (SYSTEM I AND SYSTEM II). The uptake of isotopically labelled L-leucine into three day old pads of strain 74A, shown in figure 3a, was measured in the presence of 4×10^{-3}M L-arginine by uptake method 1. It denotes the activity of the arginine sensitive transport system for the transport of L-leucine. The arginine sensitive leucine uptake into three and a half day old pads of strain 74A, plotted in figure 3b, was calculated as described in the text from values obtained using uptake method 2. Where L-arginine was used in the latter experiment, its concentration in the medium was 2×10^{-3}M. For both graphs, v is expressed as millimicromoles of L-leucine taken up per minute per pad.


12
8
4
0
$\frac{1}{V} \times 100$
5
10
16.7
10^{-3}/ [L-LEUCINE]

a


6
4
2
0
$\frac{1}{V} \times 100$
100
167
250
500
10^{-3}/ [L-LEUCINE]

b

Figure 3

from each other in their affinity for L-leucine. This finding supports the proposition that two separate and distinct transport systems are active here.

L-phenylalanine behaves very similarly to L-leucine. Early experiments showed that the arginine sensitive transport system is effectively inhibited by much lower concentrations of L-phenylalanine than is required to inhibit effectively the arginine insensitive system. In addition, L-phenylalanine itself demonstrates an arginine sensitive and arginine insensitive uptake, the Michaelis constants for which are 2×10^{-6} and 5×10^{-5}.

The arginine sensitive and arginine insensitive transport systems do show different binding properties and, therefore, do appear to be separate and distinct transport systems. Henceforth, the arginine insensitive amino acid transport system will be called amino acid transport system I. The arginine sensitive system will be called amino acid transport system II.

The binding constants obtained above are compared in table I with the binding constants obtained by Wiley and Matchett (24). There is a striking similarity between the values listed for system I and those reported by Wiley and Matchett. Furthermore, both system I and the transport studied by those authors are not inhibited by lysine. System II differs from system I both in the binding constant values obtained and in its sensitivity to inhibition by lysine and other basic amino acids.

Amino acid transport system I, the arginine insensitive system, would appear to be identical to the transport system previously studied

TABLE I AFFINITY CONSTANTS

Amino acid	Arginine sensitive transport system (transport system II)		Arginine insensitive transport system (transport system I)		Wiley and Matchett (24)
	K_m	K_i	K_m	K_i	K_m or K_i
L-tryptophan	$4\text{-}5\times10^{-5}$M		6×10^{-5}M		5×10^{-5}M
L-leucine	4×10^{-6}M	3×10^{-6}M	1.2×10^{-4}M	1.1×10^{-4}M	1.1×10^{-4}M
L-phenylalanine	2×10^{-6}M	1.5×10^{-6}M	5×10^{-5}M		4×10^{-5}M

All values reported here were obtained from measurements of amino acid transport into three day old pads of strain 74A. The K_m values were determined by measuring the uptake of isotopically labelled L-tryptophan, L-leucine, or L-phenylalanine in the presence and absence of 4×10^{-3}M L-arginine. All K_i values were determined by measuring the uptake of isotopically labelled L-tryptophan in the presence and absence of L-leucine or L-phenylalanine. K_m and K_i values for L-leucine and L-phenylalanine for transport system II were obtained using uptake method 2. All other values were obtained using uptake method 1. The affinity constants were calculated as described in the text.

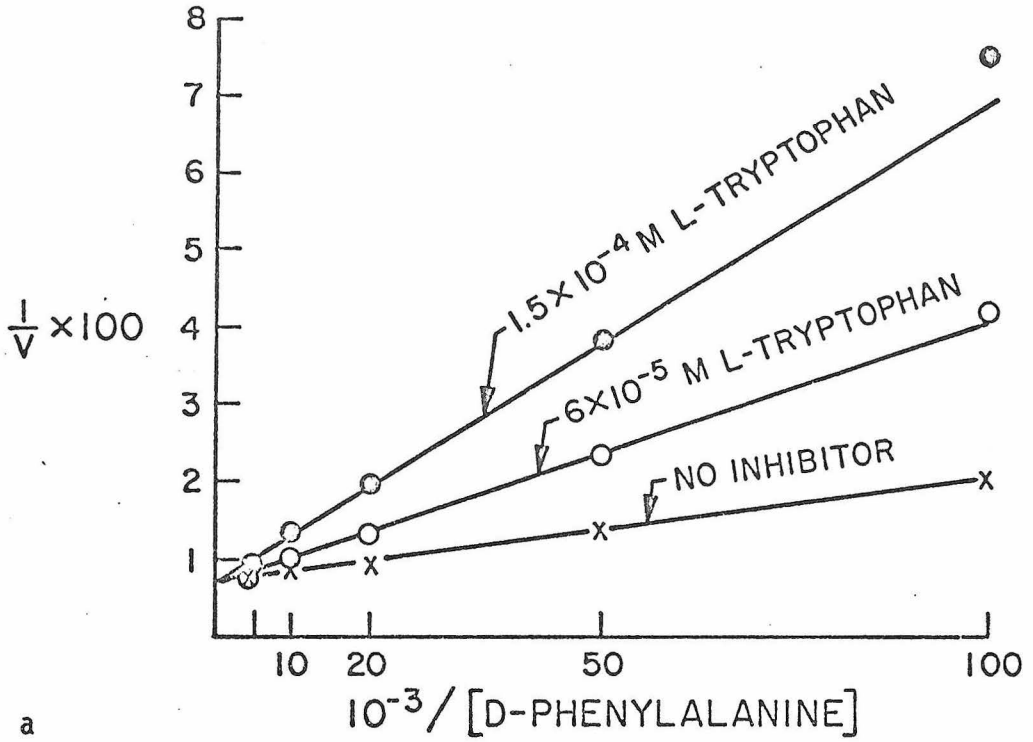
by Wiley and Matchett. Amino acid transport system II, however, is different from any previously described. Its sensitivity to inhibition by basic amino acids suggests that it may be the transport system with affinity for a wide range of amino acids proposed in the introduction. Experiments described below show that this is, indeed, the case.

D-Phenylalanine Uptake

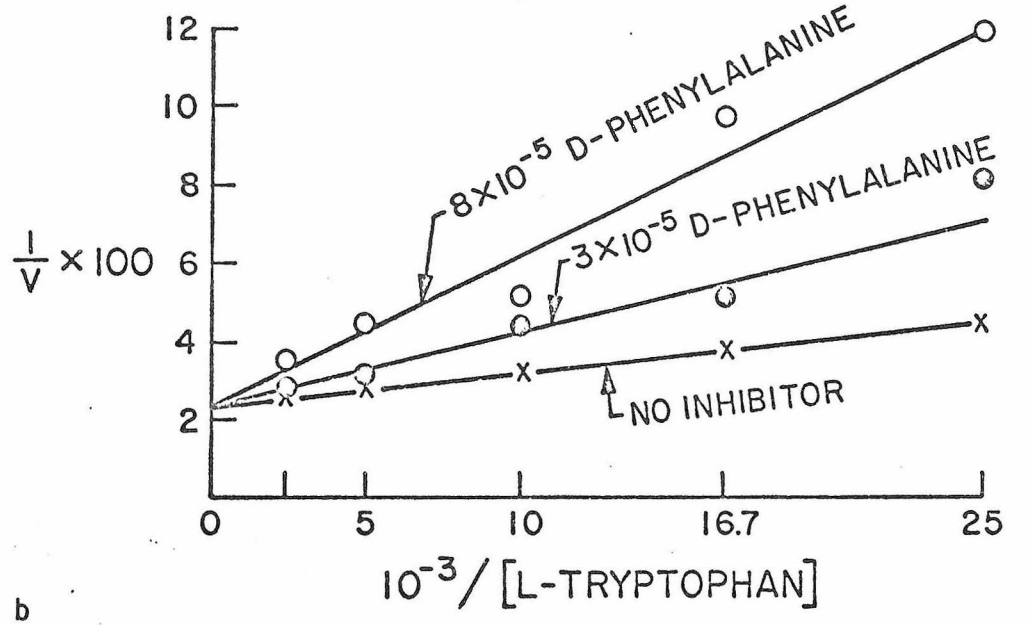
D-phenylalanine uptake was previously found to be inhibited by a very large variety of L-amino acids (23). It was of interest to determine if it was transported by either of the transport systems delineated above. Preliminary experiments showed that L-tryptophan uptake was inhibited by D-phenylalanine but that the inhibition did not exhibit strictly competitive kinetics when analyzed on a Lineweaver-Burk plot. This was not surprising, however, in light of the above findings that tryptophan is taken up by two transport systems. If D-phenylalanine is a competitive inhibitor for one but not for the other systems, then its effect on total tryptophan uptake would not appear to be competitive.

Additional work showed that D-phenylalanine is a competitive inhibitor for L-tryptophan uptake by transport system II but shows little or no affinity for transport system I. Figure 4b shows the inhibition of the arginine sensitive L-tryptophan uptake by D-phenylalanine. The K_i measured here for D-phenylalanine is 2-3 x 10^{-5}M. Similarly, as shown in figure 4a, the uptake of D-phenylalanine is competitively inhibited by L-tryptophan. This uptake is completely inhibitable by arginine and shows a K_m of 2-3 x 10^{-5}. The K_i for L-tryptophan on the uptake of D-phenylalanine is 4-5 x 10^{-5}.

Fig. 4. UPTAKE OF D-PHENYLALANINE BY TRANSPORT SYSTEM II. Figure 4a shows the total uptake of isotopically labelled D-phenylalanine and its inhibition by two concentrations of L-tryptophan. The tryptophan inhibition appears to be competitive. Figure 4b shows the arginine sensitive component of L-tryptophan transport (by transport system II) and its inhibition by two concentrations of D-phenylalanine. The inhibition appears again to be competitive. Both sets of values were determined using uptake method 1 as described in the text. The uptake was measured into three day old pads of strain 74A. v is expressed as millimicromoles of isotopically labelled amino acid (D-phenylalanine and L-tryptophan respectively) taken up per minute per mycelial pad.


8
7
6
5
4
3
2
1
1/V x100
1.5X10-4 M L-TRYPTOPHAN
6X10-5 M L-TRYPTOPHAN
NO INHIBITOR
10
20
50
100
10-3/ [D-PHENYLALANINE]

a


12
10
8
6
4
2
1/V x100
8X10-5 D-PHENYLALANINE
3X10-5 D-PHENYLALANINE
NO INHIBITOR
0
5
10
16.7
25
10-3/ [L-TRYPTOPHAN]

b

Figure 4

Thus, the K_i for D-phenylalanine on L-tryptophan uptake by system II is equal to the K_m for D-phenylalanine. Correspondingly, the K_i for L-tryptophan on D-phenylalanine uptake is equal to the K_m for L-tryptophan uptake by system II ($4\text{-}5 \times 10^{-5}$M). These equalities and the competitive nature of the inhibitions clearly show that D-phenylalanine is transported by amino acid transport system II.

As noted above, D-phenylalanine uptake has been shown previously to be inhibited by a wide variety of L-amino acids (23). Since D-phenylalanine is taken up by transport system II, system II may have an affinity for this same wide range of L-amino acids.

Substrate Specificity of Amino Acid Transport System II

A number of amino acids have been investigated to determine whether they are taken up by transport system II. The transport of all the amino acids listed in table II is inhibited by arginine. Furthermore, the transport of L-leucine or L-tryptophan by transport system II is inhibited by each of these amino acids. The Michaelis constants listed were based on the assumption that the arginine sensitive uptake of the individual amino acids was due to transport system II. The K_i's were measured against the uptake of either L-tryptophan, L-leucine, or L-phenylalanine by transport system II. In all cases, the inhibition appeared to be competitive when analyzed on a Lineweaver-Burk plot.

Thus, a very wide range of L-amino acids demonstrate an affinity for transport system II. They include D- as well as L-amino acids, small neutral as well as large neutral amino acids, acidic as well as basic amino acids.

TABLE II AMINO ACIDS TRANSPORTED BY TRANSPORT SYSTEM II

Amino acid	K_m	K_i
L-tryptophan	$4\text{-}5 \times 10^{-5}$M	
L-leucine	4×10^{-6}M	3×10^{-6}M
L-phenylalanine	2×10^{-6}M	1.5×10^{-6}M
D-phenylalanine	$2\text{-}3 \times 10^{-5}$M	$2\text{-}3 \times 10^{-5}$M
L-asparagine	8×10^{-6}M	10^{-5}M
glycine	5×10^{-6}M	8×10^{-6}M
L-arginine		2×10^{-7}M
L-aspartic acid	1.2×10^{-3}M	

All K_m's and all K_i's were determined on mycelial pads of strain 74A. Three day old pads were used for studies on all amino acids except arginine, where four day old pads were used. The inhibition constants, or K_i's were measured against the arginine sensitive uptake of L-tryptophan, L-phenylalanine, or L-leucine. The Michaelis constants, or K_m's were determined for the arginine sensitive uptake of each amino acid. All results were analyzed on Lineweaver-Burk plots.

Fig. 5. INHIBITION OF TRANSPORT SYSTEM II BY L-ARGININE. The inhibition of the arginine sensitive uptake of L-phenylalanine into four day old pads of 74A by 2 x 10^{-6}M L-arginine is shown in figure 5. The inhibition appears to be competitive. v is expressed as millimicromoles of L-phenylalanine taken up per minute per pad.

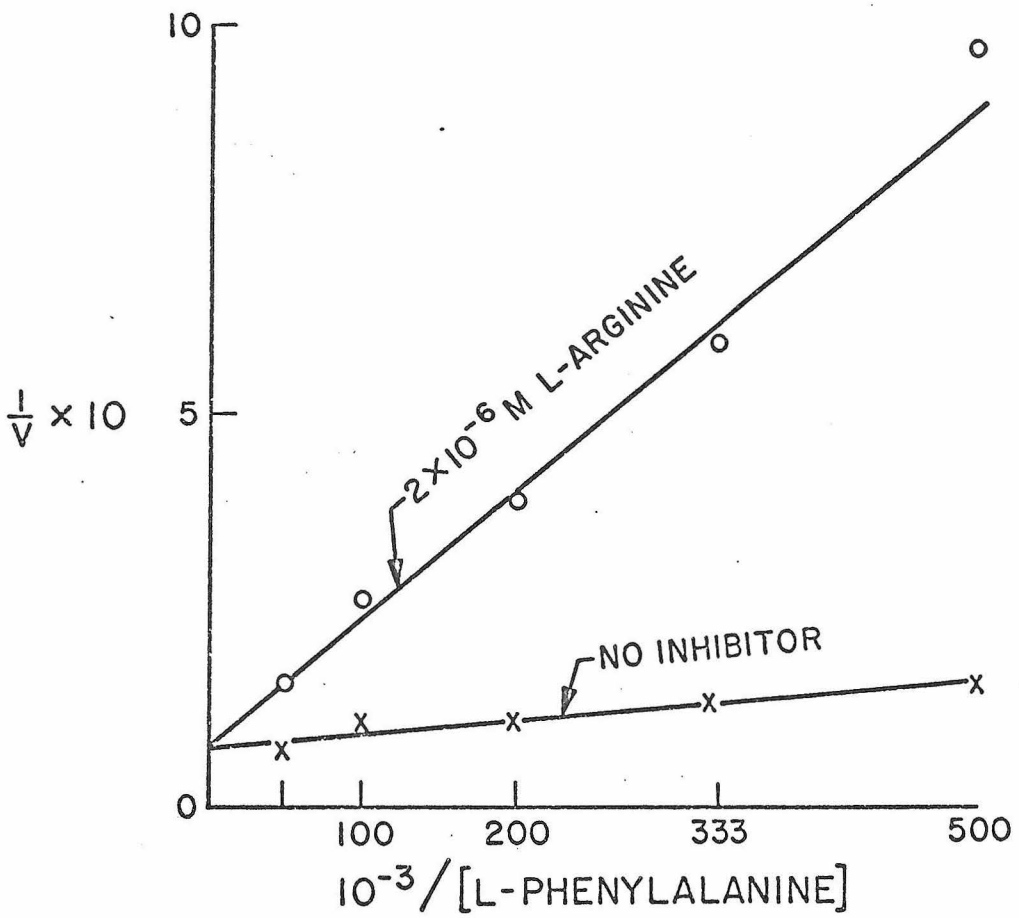

10
5
0
$\frac{1}{V} \times 10$
2×10^{-6} M L-ARGININE
NO INHIBITOR
100
200
333
500
10^{-3} / [L-PHENYLALANINE]

Figure 5

Two amino acids require special comment. Arginine has an extremely low K_i, about 2 x 10^{-7}M. The low K_i and, therefore, high affinity, together with the very high transport activity of three day old pads for arginine, made an accurate determination of K_i difficult. Four day old pads, which have lowered transport activity for arginine as well as for other amino acids, were used for this determination. The inhibition of L-phenylalanine uptake by arginine is shown in figure 5. The very high K_m and, therefore, low affinity of aspartic acid for system II, presents another problem. Small contaminating amounts of other amino acids with higher affinities in either the labelled or unlabelled aspartic acid could produce considerable uptake or cause inhibition of the uptake of other amino acids which was not due to the aspartic acid itself. The uptake of 3 x 10^{-4}M aspartic acid into three day old pads was inhibited about 75% by 10^{-3}M or 4 x 10^{-3}M L-arginine. Even when over 50% of the aspartic acid was taken up into mycelial pads in the absence of arginine, the uptake into similar pads in the presence of arginine was about 75% less. Thus, the arginine inhibitable uptake is the uptake of the aspartic acid itself rather than of some minor impurity in the labelled aspartic acid. It is assumed that the arginine sensitive transport was due to the activity of amino acid transport system II. It is not clear whether the high K_m for transport of aspartic acid apparently demonstrated by transport system II is due to a low affinity for the negatively charged compound or due to a relatively high affinity for the small amount of uncharged aspartic acid present at the pH (5.8) of the medium. Studies at other pH's will be necessary to resolve this problem.

Further experiments were performed to determine if transport system II is inhibitable by compounds other than those tested above. The uptake of 10^{-6}M L-phenylalanine was observed in the presence or absence of several potential inhibitors. Since the K_m of L-phenylalanine for transport system II is much lower than that for transport system I, the uptake of 10^{-6}M L-phenylalanine into three day old pads of 74A is primarily due to the activity of transport system II. The inhibition of this uptake by 10^{-4}M of various compounds is shown in table III.

All α-amino acids tried were effective in inhibiting uptake. This further confirms the wide range of substrates which apparently have a good affinity for transport system II. However, none of the nonamino acids tried were effective inhibitors. Inhibition of less than 20% is not considered significantly different from zero percent. Thus, in order to retain affinity, the amino group of an amino acid cannot be replaced by a hydrogen, hydroxyl group or keto group, nor can it be acetylated. Similarly, the carboxyl group cannot be replaced by a hydrogen or amide, nor can it be involved in a peptide bond. However, β-alanine and probably γ-amino-n-butyric acid (GABA) do inhibit uptake. Therefore, β-amino acids and probably also γ-amino acids have some affinity for transport system II.

Control of Transport System II

Transport system II is responsible for about 75% of the tryptophan uptake in three day old pads. Only the remaining 25% of the uptake is due to transport system I. However, according to Wiley and Matchett (24), most, if not all, of the uptake of L-tryptophan in germinated

TABLE III

INHIBITION OF UPTAKE OF 10^{-6}M L-PHENYLALANINE BY VARIOUS COMPOUNDS

Compound	Percent inhibition of uptake
None	(0)
L-alanine	94
β-alanine	80.3
Lactic acid	0
Pyruvic acid	0
Propionic acid	0
α-amino-n-butyric acid	93.6
γ-amino-n-butyric acid	25.0
L-histidine	91.8
Histamine	12.3
L-leucine	91.9
N-acetyl-L-leucine	2.3
Leucinamide	11.2
L-leucyl-glycine	6.0
L-serine	91.5
2-aminoethanol	0

All potential inhibitors were added to a concentration in the medium of 10^{-4}M. Three day old pads of 74A were used to measure the uptake of 10^{-6}M L-phenylalanine by uptake method 2.

Fig. 6. RELATIVE ACTIVITIES OF TRANSPORT SYSTEM I AND TRANSPORT SYSTEM II IN NEUROSPORA CULTURES OF VARIOUS AGES. Mycelial pads of various ages of wild type strain 74A were prepared as described in the text. The uptake of 10^{-5}M L-tryptophan into these pads was measured in the presence and absence of 10^{-3}M L-arginine. The inhibition of tryptophan uptake by arginine is plotted. The inhibition of tryptophan uptake by arginine reported by Stadler (25) is plotted in the position of the "S". The inhibition of tryptophan uptake by lysine reported by Wiley and Matchett (24) is plotted in the position of the "W". Although the growth conditions used by those investigators differ from the procedures used in this thesis, their results appear to agree well with those reported here. The transport activity not inhibited by basic amino acids is assumed to be due to transport system I. The basic amino acid inhibitable transport is assumed to be due to transport system II. These assumptions have been verified, as reported above, in three day old pads of 74A. The first assumption has been verified by Wiley and Matchett in germinated conidia.

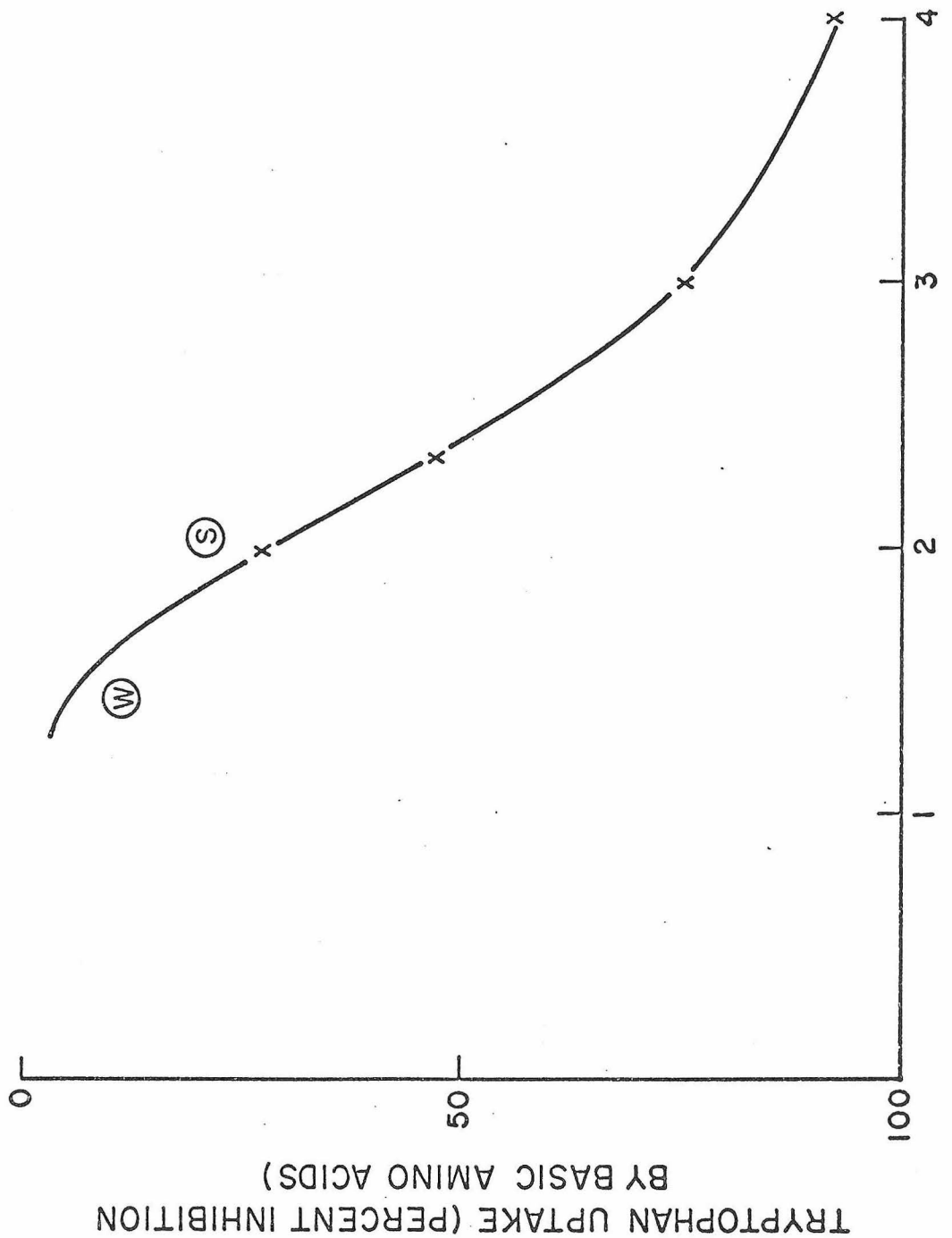

TRYPTOPHAN UPTAKE (PERCENT INHIBITION
BY BASIC AMINO ACIDS)
0
50
100
1
2
3
4
AGE (days)
W
S

Figure 6

conidia is due to amino acid transport system I. Therefore, the relative activities of the two transport systems must be in some way dependent on the physiological state of the cultures.

The relative amounts of tryptophan uptake by the two transport systems in cultures of different ages were determined by measuring the fraction of the uptake of L-tryptophan that was inhibited by arginine. The cultures of various ages were prepared as were the three day old cultures except that two day old cultures were not shaken before uptake while the four day old cultures were shaken for two days preceding uptake.

As shown in figure 6, the relative activity of transport system II increases strikingly in older cultures. The uptake of most neutral amino acids in young cultures is primarily due to transport system I, but in older cultures, system II predominates.

Older cultures under these conditions have ceased growth due to the depletion of the carbon source, sucrose, from the medium. Two to three times heavier cultures can be obtained by simply using more sucrose in the initial medium. It would appear possible, then, that the limitation of growth by carbon starvation in some way induces amino acid transport system II. It is not clear whether growth limitation due to other causes might also be effective in inducing this transport system.

Deficiency of the mtr Mutant Strain

The mtr strain, as noted above, is deficient in the transport of L-tryptophan, L-leucine, and several other neutral amino acids in germinated conidia (26) and young mycelia (25). Exploratory experiments

showed that in three day old cultures, however, the transport of tryptophan and leucine approached that in wild type cultures. Subsequent experiments were performed to determine which of the two transport systems was deficient in the mutant strain.

In three day old pads of mtr, over 99% of the uptake of 10^{-4}M L-tryptophan and over 98% of the uptake of 4 x 10^{-4}M L-leucine are inhibited by 4 x 10^{-3}M L-arginine. This compares with about a 75% inhibition found for wild type 74A. Moreover, even in two day old cultures where wild type shows only 30% inhibition, over 99% of the uptake of L-tryptophan is inhibited by arginine in the mtr mutant. Thus, transport system I, the arginine insensitive system, is specifically missing in the mtr mutant strain but transport system II is present in normal amounts. The mtr mutant, then, behaves as a permeaseless mutant in that it is deficient in the activity of a specific transport system.

DISCUSSION AND CONCLUSIONS

Considerable progress has been made toward the understanding of two amino acid transport systems in Neurospora. Amino acid transport system I is equivalent to that previously studied by Wiley and Matchett (24). Those authors have determined that transport system I will transport a wide range of neutral L-α amino acids.

Transport system I is missing in cultures of the mtr mutant, although transport system II is present at normal levels. The specificity of the defect in mtr mutant, together with the fact that only one locus has been implicated in this transport deficiency (25), makes it quite likely that the mtr locus is the structural gene for the specific transport protein, or permease, of transport system I.

Amino acid transport system II has high activity in old, carbon-starved, cultures but, unlike system I, has little or no activity in young, rapidly growing, cultures. It has a very broad range of substrates, transporting amino acids with hydrophobic and hydrophilic side chains, both D- and L-amino acids, and basic as well as neutral amino acids. It demonstrates an affinity for β- as well as α-amino acids.

Previous work showing that a very broad range of amino acids inhibits the transport of L-phenylalanine (21) and L-arginine (22) into ungerminated conidia suggests that transport system II is present in conidia as well as in old mycelial cultures.

The Michaelis constants for system II of most neutral and basic amino acids investigated are quite low, the majority being between 10^{-5} and 10^{-7}M. The low K_m's should make this system quite efficient in

removing traces of amino acids from the medium. In carbon-starved cultures, then, transport system II may serve as a scavenger, taking up any remaining exogenous amino acids.

The technique used here to separate the activities of two transport systems studied may well be applicable in other systems. Any substrate which will competitively inhibit one, but not the other, of two simultaneously active transport systems can be utilized to separate their activities.

There are two previous reports of amino acid transport systems in fungi which are similar to transport system II in having a very broad range of substrates. Surdin et al (31) has reported that most amino acids in yeast are taken up by a single transport system. Other authors (32) have disputed Surdin's conclusion and have reported a number of relatively specific transport systems in yeast (32-34). Perhaps an amino acid transport system similar to transport system II described above, which is active in yeast under some physiological conditions but not others, would aid in integrating the interpretations of the two groups.

Recently, a general amino acid transport system with affinity for a broad range of amino acids has been reported in nitrogen-starved cultures of Penicillium chrysogenum (35). The dependence of the activity of this system on starvation conditions may be similar to that reported above for transport system II in Neurospora.

A number of questions about amino acid transport in Neurospora remain unresolved. There are almost certainly other transport systems than the two delimited above. Growth antagonisms of various amino

acid requiring auxotrophs of Neurospora probably occur through competition for uptake of the different exogenous amino acids added to the medium. Such antagonisms provide clues as to what transport systems remain to be studied. Arginine requiring mutants are inhibited by lysine (36) and lysine requiring mutants are inhibited by arginine (37). These growth antagonisms imply the existence of a specific transport system for basic amino acids. Histidine requiring mutants are inhibited by any of a large number of neutral amino acids plus either arginine or lysine (38). Thus, in young cultures, histidine is probably transported by two amino acid transport systems. These two are most likely transport system I and the transport system for basic amino acids postulated above. Some studies on these two transport systems are reported in a recent abstract (39).

Only a portion of the transport of L-aspartic acid, reported in this thesis, is inhibited by L-arginine. Since aspartic acid has no affinity for transport system I (24), the residual uptake must be due to some as yet unexplored transport system. Thus, at least four amino acid transport systems are implicated in Neurospora. No evidence for additional transport systems has appeared in results obtained by the author. However, most experiments were performed at relatively low amino acid concentrations; consequently, systems having high Michaelis constants would probably not have been detected. In addition, a number of amino acids have not been studied. Furthermore, some transport systems may only be present under physiological conditions other than those studied. Nevertheless, it seems likely that the four systems outlined above are responsible for most of the amino acid transport

activity of Neurospora.

Even more attention will be required to obtain an understanding of the integration of transport with the other activities of the mycelia or conidia. Clearly, the synthesis or activity of system II is under some kind of physiological control. The control of various transport systems is demonstrated through changes in several mutant strains (18, 37, 40, 41). Certainly, a number of useful physiological and genetic studies of the control of amino acid transport can be pursued on such mutants.

Finally, the most critical studies on transport must center on the transport proteins themselves and their integration into the membrane structures and energy metabolism of the cell. Relevant studies are further along in other systems (7-11) than in Neurospora. However, experiments on transport proteins such as those reported by Woodward and Munkres (42, 43) on other enzymes in Neurospora may yield information concerning the role of membrane structure in transport.

APPENDIX: RESOLUTION OF THE TWO PROPOSED AMINO ACID TRANSPORT SYSTEMS

The finding that tryptophan transport can be resolved into an arginine inhibitable and an arginine uninhibitable transport component has led to the proposal, described in the results section, that two separate and distinct transport systems may be capable of transporting L-tryptophan in Neurospora crassa. Alternatively, part of the tryptophan transport, due to a single transport system, may in some non-competitive way be inhibited by arginine.

If two transport systems are active in tryptophan transport, they will probably differ, in general, in their affinities for a potential competitive inhibitor. Alternatively, if only one transport system is present, the arginine sensitive and arginine insensitive components of tryptophan transport should demonstrate the same affinity for a potential competitive inhibitor. It is shown in the results section that the arginine sensitive and arginine insensitive components of tryptophan transport differ in their affinity for L-leucine, thus supporting the conclusion that two transport systems are active. The analysis leading to this finding is considered in detail below.

Table IV shows the uptake of isotopically labelled L-tryptophan into three and a half day old pads of 74A in the presence of 4×10^{-3}M L-arginine. The uptake with no leucine present is shown in column "a". The uptake in the presence of 10^{-4}M L-leucine is shown in column "b". The uptake of tryptophan was measured using uptake method 1 and is expressed in millimicromoles of L-tryptophan taken up per minute per mycelial pad. The data are plotted on a Lineweaver-Burk plot in figure

TABLE IV INHIBITION OF THE ARGININE INSENSITIVE COMPONENT OF L-TRYPTOPHAN UPTAKE BY L-LEUCINE

Concentration of L-tryptophan in the medium	Uptake in the presence of 4×10^{-3}M L-arginine	Uptake in the presence of 4×10^{-3}M L-arginine and 10^{-4}M L-leucine
4×10^{-5}M	2.30	1.06
6×10^{-5}M	3.67	2.22
10^{-4}M	4.30	2.76
2×10^{-4}M	5.88	4.17
4×10^{-4}M	7.22	6.12

The uptake of isotopically labelled L-tryptophan into three day old pads of 74A was measured using uptake method 1. The uptake is expressed in millimicromoles of L-tryptophan taken up per minute per mycelial pad.

2a. The results show leucine to be a competitive inhibitor and yield an inhibition constant (K_i) for L-leucine on the arginine insensitive component of L-tryptophan transport of about 10^{-4}M.

The determination of a similar inhibition constant for L-leucine on the arginine sensitive component of L-tryptophan uptake is more difficult. The L-tryptophan uptake in the absence of arginine should be due to the sum of the activities of the arginine sensitive and arginine insensitive components. Therefore, the activity of the arginine sensitive component, in the presence of some particular concentration of L-tryptophan, will equal the uptake of tryptophan in the absence of arginine minus the uptake of tryptophan in the presence of a saturating amount of arginine. These differences are calculated in table V both where leucine was present during the uptake and where leucine was absent. The values obtained in column "c" and column "f" are plotted in figure 2b. They show that leucine is a competitive inhibitor of the arginine sensitive component of tryptophan uptake. The inhibition constant (K_i) for L-leucine is 3×10^{-6}M. This is quite different from the K_i determined above for the arginine insensitive component (10^{-4}M). Therefore, the binding of the two components for leucine are different. It can therefore be concluded that there are two different transport systems with two different active sites which transport tryptophan under the conditions studied.

TABLE V

INHIBITION OF THE ARGININE SENSITIVE COMPONENTS OF L-TRYPTOPHAN UPTAKE BY L-LEUCINE

Concentration of L-tryptophan in the medium	a. Uptake with no inhibitor present	b. Uptake in the presence of $2x10^{-3}M$ L-arginine*	c. Activity of the arginine sensitive uptake component (a-b)	d. Uptake in the presence of $5x10^{-6}M$ L-leucine	e. Uptake in the presence of $2x10^{-3}M$ L-arginine and $5x10^{-6}M$ L-leucine*	f. Activity of the arginine sensitive uptake component in the presence of $5x10^{-3}M$ L-leucine (d-e)
$4x10^{-5}M$	6.28	.65	5.63	3.74	.87	2.87
$6x10^{-5}M$	7.28	1.42	5.86	4.50	1.14	3.36
$10^{-4}M$	9.78	1.28	8.50	6.02	1.65	4.37
$2x10^{-4}M$	11.72	1.63	10.09	8.74	1.41	7.33
$4x10^{-4}M$	15.00	2.83	12.17	11.28	2.11	9.11

The uptake of isotopically labelled L-tryptophan into three and one-half day old pads of 74A was measured using uptake method 2. The uptake is expressed in millimicromoles of L-tryptophan taken up per minute per mycelial pad.

*The considerable scatter in the values obtained for uptake in the presence of arginine is due, at least in part, to the low number of counts measured in these cases.

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