Tryptophan Transport in *Neurospora crassa*

II. Metabolic Control

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The rate of tryptophan transport in *Neurospora* is regulated by the intracellular pool of tryptophan. When cells were shifted from growth in minimal medium to tryptophan-containing medium for 10 min, there was a 50% reduction in the rate of tryptophan transport. Intracellular tryptophan pools derived from indole were equally effective in reducing the rate of transport as externally supplied tryptophan. The regulatory influence of tryptophan on the transport system appears to be a property of all the amino acids transported by the tryptophan transport site or sites. Lysine and glutamic acid are not transported by the tryptophan transport site or sites and are ineffective in the regulation of tryptophan uptake. Continued protein synthesis is required for the maintenance of a functional tryptophan transport system. The half-life of the transport system, estimated by inhibiting protein synthesis with cycloheximide, was about 15 min. Turnover of the system occurred at 30 C but not at 4 C, suggesting that the breakdown of the system is enzymatically mediated. It was inferred that the rate of tryptophan transport in *Neurospora* is modulated through the maintenance of a delicate balance between the synthesis and breakdown of some component of the transport system.

Previous investigations (10) have shown that the unidirectional transport of tryptophan across the cell membrane of *Neurospora crassa* is mediated by a distinct transport system which is similar in many respects to enzyme-catalyzed reactions. The similarities include structural and stereochemical specificity, typical enzyme saturation kinetics, and a decrease in transport activity at low temperatures. The tryptophan transport site or sites appear to be specific for a family of L-amino acids, including leucine, phenylalanine, and methionine.

When tryptophan is supplied to a growing culture, transport across the cell membrane occurs until the intracellular concentration of tryptophan exceeds the external concentration several-fold. Thus, depending on the external supply of tryptophan, large intracellular pools of tryptophan may be formed. In view of this observation and the crucial role of intracellular tryptophan in determining the entry of metabolites into the multi-branched aromatic pathway (1, 4, 7), it was of interest to determine whether the tryptophan transport system can be metabolically regulated. Specifically, the experiments described in this report were carried out in an attempt to (i) obtain evidence that the rate of tryptophan accumulation in *Neurospora* is under the influence of some pattern of metabolic control and (ii) determine the identity and mode of action of factors which regulate the number or activity (or number and activity) of the tryptophan transport sites.

**MATERIALS AND METHODS**

The strains of *N. crassa* used in these experiments were: wild-type strain 74A and td-201, auxotrophic for tryptophan. The techniques used for maintenance and growth of cultures and the measurement of rates of amino acid transport and accumulation have been described (10). Tryptophan pools (2 to 80 μmoles per g of cells) were determined by the tryptophanase assay (2).

To prepare concentrated extracts for the assay of kynureninase and tryptophan synthetase activity, mycelia were lyophilized, powdered, and extracted for 30 min with 0.05 m potassium phosphate buffer, pH 7.8, at 4 C (7). Kynureninase activity was determined by measuring anthranilic acid production in a Turner fluorometer (6). Tryptophan synthetase activity was assayed by measuring the disappearance of indole (11).

**RESULTS**

Effect of intracellular pool tryptophan on the rate of tryptophan uptake. The rate of tryptophan transport in *Neurospora* is markedly affected by the conditions of growth. When conidia were germinated and grown for 8 hr in minimal medium, washed, and incubated with 1.0 × 10⁻⁴ M
L-tryptophan-3-$^{14}$C, the rate of transport was 3.94 μmoles per min per g of cells (dry weight). Cells grown in medium containing 3 mM tryptophan, on the other hand, transported tryptophan at a rate of 0.86 μmole per min per g of cells. The reduction in the rate of transport in the tryptophan-grown cells occurred without any significant alteration in the rate of growth or protein synthesis. The intracellular tryptophan pool in the tryptophan-grown cells, however, was 20 μmoles per g of cells (dry weight), compared to only 1.8 μmoles in cells grown in minimal medium. From these observations, it was postulated that the reduction in the rate of transport was related to the higher concentration of intracellular tryptophan in the tryptophan-grown cells. To test this point, transport was measured in cells which had been preloaded with tryptophan.

For this experiment, cells were grown for 9 hr in minimal medium, abruptly shifted to minimal medium containing 3 mM L-tryptophan [cell concentration, 1 mlg of cells (dry weight) per ml], and incubated for 15 min at 30°C. This 15-min incubation insured preloading of the intracellular tryptophan pool. Control cells received the same treatment without tryptophan. The preloading process was terminated by pouring the culture onto frozen, crushed minimal medium. Rapid cooling of the cells did not detectably alter the concentration of tryptophan in the intracellular pool. The mycelia were then washed twice in cold mineral salts medium and tryptophan uptake was measured at 30°C. The observed rates are given by the slopes of the curves presented in Fig. 1. As shown, the rate of transport by preloaded cells was reduced by a factor of 5 (i.e., from 3.9 to 0.76 μmoles per min per g of cells).

During the 15-min preloading process, the intracellular tryptophan pool increased from 2 to 40 μmoles of tryptophan per g of cells.

The relationship between the size of the intracellular pool and the reduction in rate of transport was of interest. Accordingly, samples of cells, treated as described above, were removed at various intervals during the preloading process. In Fig. 2, the ratio of postshift to preshift rates of transport in these cells are plotted as functions of the duration of the preloading process. The reduction in the transport rate is apparent after 5 min of preloading. The initial rate of uptake was reduced to about one-half the original rate after 10 min and reached a minimum after 20 min. Control cells transported tryptophan at essentially the preshift rate. After the shift to preloading medium, the pool size increased from 2 to 40 μmoles per g of cells after 5 min. The pool size after 20 min was 42 μmoles per g of cells. These results suggested that the decrease in the rate of tryptophan uptake following the shift to preloading medium was related to the accumulation of tryptophan in the intracellular pool. An implication of these results is that pool tryptophan or some metabolite derived from the pool controls the rate of tryptophan entry.

Effect of endogenously formed tryptophan pools on the rate of tryptophan uptake. Previous investigations have shown that indole and tryptophan are transported across the cell membrane of N. crassa by independent transport systems (10). Further, free indole is not accumulated by the cell but enters the cell and is immediately converted to tryptophan (8). Thus, if intracellular pool tryptophan or a metabolite of tryptophan is responsible for the observed reduction in the rate of transport, pools formed from indole should provide a stringent test of this contention. Figure 3 shows the result of such a test. The experiment was conducted in the manner described in Fig. 2, except that preloading involved a shift to minimal medium containing indole. Indole does not inhibit the rate of growth of germinated conidia at the concentration used. Control cells in this experiment showed a slight increase in the rate of transport. The ratio of rates increased from an initial value of 1.0 at zero-time to 1.34 at 2 hr after the shift. Preloaded cells, on the other hand, showed a reduction in the rate of tryptophan uptake.
Fig. 2. Relationship between the duration of preloading with tryptophan and the rate of tryptophan uptake. Cells were shifted from growth in minimal medium to fresh minimal medium or minimal medium plus 3 mM L-tryptophan. Samples were removed at intervals from the culture medium and washed twice at 4°C; the rate of uptake was measured at 30°C. The uptake medium contained: minimal medium (pH 5.8), 1 × 10⁻⁴ M L-tryptophan-3⁠⁻¹⁴C, and 478 μg/ml of cells (dry weight).

Transport to approximately one-half the preshift rate after 20 min and to a minimal value after 60 min. During incubation in indole, the intracellular tryptophan pool increased from 1.8 to 6.0 μmoles of tryptophan per g of cells after 15 min, and to 10 μmoles per g of cells after 60 min.

It appears from these data that intracellular tryptophan pools derived from exogenously supplied indole are at least as effective in reducing the rate of tryptophan transport as pools derived from externally supplied tryptophan. It seems clear, therefore, that the reduction in the rate of tryptophan transport after a shift to tryptophan medium is related primarily to the accumulation of tryptophan in the intracellular pool and is independent of the concentration of tryptophan in the external medium.

Response of the tryptophan transport site or sites to pools of other amino acids. The criterion of competitive inhibition of amino acid uptake has been used to recognize distinct transport groups or families of amino acids in Neurospora (10). The members of a family compete with each other for transport but not with members of other groups. On this basis three groups are currently recognized. Diamino acids, such as arginine and lysine, comprise one group. Dicarboxylic acids, such as glutamic acid and aspartic acid, constitute a second group. The third, and apparently most heterogeneous, group consists of neutral amino acids such as tryptophan, phenylalanine, methionine, and leucine. An important consideration is whether the group concept applies to inhibitions exerted by amino acids in intracellular pools. This point was tested by the experiment detailed in Table 1. In this experiment, cells were preloaded, as described above, with representative members of each transport group. Lysine and glutamic acid, both members of distinct groups unrelated to tryptophan, are without effect on tryptophan uptake when present in the intracellular pool. In contrast, leucine, a member of the tryptophan group, is almost as effective as tryptophan itself as an inhibitor of tryptophan uptake.

The concentration of lysine, leucine, and glutamic acid in the intracellular pools was not determined. However, transport studies indicated that the rates of transport of these amino acids were adequate to produce large intracellular pools.

Effect of tryptophan starvation on transport activity. Experiments were performed to test the effects of tryptophan deprivation on the rate of
tryptophan transport, protein synthesis, and intracellular pool utilization in *Neurospora* mutant strain td-201, auxotrophic for tryptophan. Conidia were germinated and grown in minimal medium supplemented with 150 μg per ml of tryptophan for 15 hr. The mycelia were harvested, resuspended in minimal medium supplemented with 50 μg per ml of L-tryptophan, and incubated for an additional hour. This incubation does not cause a reduction in the rate of tryptophan transport in this mutant strain; no reduction occurs until the intracellular pool of tryptophan exceeds 60 to 80 μmoles per g of cells. The reason for this quantitative difference in the response of the mutant and wild-type strains to tryptophan is not clear, but presumably reflects some alteration in the regulation of tryptophan uptake in the mutant strain. Immediately after the 1-hr incubation in tryptophan medium, the germinated conidia of td-201 were harvested, washed twice at 4°C, and resuspended in minimal medium without tryptophan [40 mg of cells (dry weight) per ml]. The culture was then divided into two flasks. To one flask, 1 × 10^{-4} M uniformly labeled 14C-L-lysine was added to measure protein synthesis. To the second, 1 × 10^{-4} M unlabeled lysine was added to measure pool utilization and, subsequently, transport activity. Both flasks were incubated at 30°C with vigorous aeration.

The results of this experiment are shown in Fig. 4. The intracellular pool of tryptophan decreased from an initial value of 40 μmoles of tryptophan per g of cells to a minimal value of 0.5 μ mole after 300 min. The size of the pool was reduced to approximately one-half the original value after 45 to 50 min of incubation. Protein synthesis (as measured by the incorporation of radioactivity from uniformly labeled 14C-L-lysine into trichloroacetic acid-precipitable material) continued at a constant rate for approximately 60 min. After 60 min, the incorporation of lysine into protein diminished and finally ceased as the intracellular pool of tryptophan reached exhaustion. The rate of transport remained at a constant value (5.5 μ moles per min per g of cells) for approximately 60 min and decreased to practically zero after 130 min of incubation. Transport activity remained at the reduced level for at least 300 min. Thus, the cessation of protein synthesis was followed by a depression in the rate of uptake.

Figure 5 shows that these effects are completely reversed by the addition of high concentrations of tryptophan to the growth medium. In this experiment, cells were starved for tryptophan in the manner described for the experiment in Fig. 4. Initially, the rate of tryptophan transport was 5.5 μ moles per min per g of cells. This rate decreased to about 1.0 μ mole per min per g of cells after starvation for 60 min, and to approximately 0.1 after 120 min. Incubation in tryptophan-free medium was continued for 150 min, after which the culture was divided into four flasks. Flask 1 received 10^{-4} M uniformly labeled 14C-L-lysine, 7.4 × 10^{-4} M L-tryptophan, and cycloheximide at a final concentration of 2 μg per ml. Cycloheximide at this concentration completely inhibits protein synthesis. Flask 2 received the same additions except that lysine was nonlabeled. Flasks 3

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**Table 1. Effect of other amino acid pools on the rate of tryptophan uptake**

<table>
<thead>
<tr>
<th>Amino acid pool*</th>
<th>Ratio of postshift rate to preshift rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Lysine</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>1.08</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Each amino acid was present in the preloading medium at a final concentration of 3 mm. The cells were preloaded at 30°C for 30 min. Control cells were incubated in minimal medium. After preloading, the mycelia were chilled to 0 to 3°C, washed twice, and the rate of uptake measured. The uptake medium contained 10^{-4} M L-tryptophan-3-14C (specific activity, 6.6 μc/μmole) and 450 μg/ml of cells (dry weight).

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**Fig. 4. Effect of tryptophan deprivation in a tryptophan auxotroph on pool utilization, transport, and protein synthesis. Cells with a preestablished pool of tryptophan were transferred to medium without tryptophan; 10^{-4} M uniformly labeled 14C-L-lysine (specific radioactivity, 2 μc/μmole) was added to measure protein synthesis. Intracellular tryptophan was measured by the tryptophanase assay. The transport medium contained 10^{-4} M L-tryptophan-3-14C (specific radioactivity, 4 μc/μmole) and 350 μg/ml of cells (dry weight).**
and 4 received the same additions as 1 and 2, respectively, except that cycloheximide was omitted. All flasks were incubated at 30 C. Samples were removed for the measurement of protein synthesis (flasks 1 and 3) and tryptophan transport activity (flasks 2 and 4). Tryptophan transport activity increased from approximately 0.10 to 1.8 μmoles per min per g of cells after 60 min under recovery conditions and reached a maximal value of 7 after 90 min. Cells treated with cycloheximide, on the other hand, showed only a slight incorporation of 14C-lysine and no increase in tryptophan transport activity.

It is conceivable that the absence of lysine incorporation into protein in the cycloheximide-treated cells is a reflection of the inability of the cell to take up lysine from the external medium and that protein synthesis occurs, but at the expense of endogenously synthesized lysine. Although this question was not unequivocally resolved, experiments described later in the text show that cycloheximide completely inhibits the synthesis of the enzyme kynureninase in Neurospora.

These results suggest that net protein synthesis is an obligatory requirement for the synthesis of a functional tryptophan transport system. Since tryptophan uptake is a highly accumulative process in Neurospora and apparently dependent on a coupled exergonic reaction, it seems unlikely that protein synthesis is required simply to provide a thermodynamic driving force for the transport system. Further, if this were the case, maximal uptake would be expected to occur coincident with the resumption of a constant rate of protein synthesis. Figure 5 shows, however, that there was about a 30-min lag following the resumption of a constant rate of protein synthesis before the maximal rate of uptake was attained.

It is possible that depletion of energy reserves required for the transport process is responsible for the reduction in the rate of uptake. However, glycogen synthesis and sucrose utilization are not inhibited by tryptophan deprivation or by treatment with cycloheximide. The simplest interpretation of these results is that protein synthesis is required for the continuous regeneration of some labile component of the transport system.

Metabolic turnover of tryptophan transport activity. If the transport system, or some component of it, is unstable and requires protein synthesis for its continuous regeneration, then specific inhibition of protein synthesis should result in the decay of transport activity. To test this hypothesis, cells of Neurospora strain 74A were treated with cycloheximide and transport activity was measured at various intervals after the cessation of protein synthesis. In this experiment, conidia were germinated and grown in medium containing 150 μg per ml of tryptophan. Although transport activity in these cells was reduced relative to cells grown in minimal medium, the kinetics and specificity of the remaining activity were the same as those shown for the transport system in strain td-201 (10). The fact that 74A was grown under the same conditions as td-201 (Fig. 5) and that the kinetic and specificity characteristics of the two systems were identical suggests that the labile component demonstrated in this experiment is the same as that observed with td-201 in Fig. 5.

To initiate the experiment, washed mycelia were resuspended (10 mg per ml, dry weight) in minimal medium containing 2 μg (per ml) of cycloheximide. Control and cycloheximide-treated cells were incubated at 30 and 4 C. Samples were removed from the growth flasks at appropriate intervals, and metabolic activity was terminated by pouring the cells over frozen,
crushed minimal medium. The mycelia were then washed twice in cold (4°C) minimal medium, and the rate of tryptophan uptake was measured at 30°C.

The results of this experiment are shown in Fig. 6. Tryptophan transport activity at 30°C decreased from 0.89 to a minimal value of 0.1 μmole per min per g of cells after 30 min of exposure to cycloheximide. The rate of transport in control cells incubated at 4°C remained essentially constant for 40 min. It is conceivable that the reduction in tryptophan transport activity during cycloheximide treatment may have resulted from an increase in the intracellular concentration of amino acids which may feed back and inhibit the transport system. This possibility, however, does not seem likely, since the size of the tryptophan pool remained relatively constant during the 40-min incubation period (3 to 4 μmoles per g of cells, dry weight). Since the drain from the tryptophan pool into protein was not present in these cells, the tryptophan pool size would be expected to increase. The observation that the pool size did not increase probably reflects the fact that tryptophan is an effective feedback inhibitor of anthranilate synthetase (3), the first enzyme specific for tryptophan biosynthesis. For the same reasons, it seems unlikely that the pool sizes of the other amino acids of the tryptophan family would be significantly increased.

An interpretation of these results is that the tryptophan transport system in Neurospora turns over at an extremely high rate (half-life of about 15 min) and that continued protein synthesis is required for the maintenance of a functional transport system. The fact that transport was not diminished at 4°C suggests that the decay of the transport component is temperature-sensitive, which in turn suggests that the turnover process may be enzymatically mediated. The inference is that the rapidly turning-over component is protein in nature. This is not an unlikely speculation, in view of the recent isolation of protein components from the cell membrane of Escherichia coli with binding characteristics which are similar in many respects to amino acid transport systems observed in vivo (9).

The turnover of the tryptophan transport activity in Neurospora does not appear to be a generalized property of tryptophan biosynthetic or degradative enzymes. Figure 7 shows that kynureninase activity in germinated conidia remains constant after prolonged treatment with cycloheximide at 30°C. In this experiment, germinated conidia of wild-type strain 74A were induced for kynureninase activity by incubation in the presence of 150 μg per ml of l-tryptophan for 3 hr previous to exposure to cycloheximide. Kynureninase specific activity (μmoles of anthranilic acid formed per hour per milligram of cells) was 0.4 before treatment with cycloheximide was initiated. The specific activity was unchanged after incubation for 50 min. Cells incubated in the absence of cycloheximide showed an increase in specific activity reflecting continued inductive synthesis of the enzyme. Similar results were observed for tryptophan synthetase activity. Thus, if the labile transport component is a protein, its degradation is clearly not the result of generalized protein turnover.

**DISCUSSION**

The results described in this report show that the rate of tryptophan transport in Neurospora is regulated by the intracellular pool of tryptophan or some product of tryptophan metabolism. When germinated conidia were shifted from minimal medium to medium containing high concentrations (3 mM) of tryptophan, 5 to 20 min was required to fill the intracellular pool from its preshift level. During this period of
tryptophan accumulation, the rate of tryptophan transport was reduced by a factor of approximately 5. There appears to be no significant efflux of intracellular tryptophan in *Neurospora* (10). The fact that the shifting of cells to indole-containing medium also results in a reduction in the rate of tryptophan transport suggests that this reduction is a function of the intracellular pool of tryptophan and only secondarily related to the high external concentration of tryptophan. The regulatory influence of tryptophan pools appears to be only partially specific; pools of leucine (an amino acid transported by the tryptophan uptake site or sites) are as effective as tryptophan in regulating the rate of influx of tryptophan. Similarly, large tryptophan pools inhibit the rate of entry of leucine. In contrast to the inhibitory effects of leucine pools on the rate of tryptophan uptake, preloading cells with lysine or glutamic acid has no effect on the rate of tryptophan uptake. These data suggest that pools formed from amino acids of the tryptophan transport family are capable of regulating the rate of entry of any amino acid of that transport family; hence, each amino acid is able to inhibit the transport system.

There appears to be a quantitative difference in the external concentration of tryptophan required to reduce the rate of tryptophan transport in the mutant strain td-201 and wild-type 74A. Conidia of td-201, germinated and grown in media containing 10 to 150 μg per ml of tryptophan, transport tryptophan at essentially the same rate. When cells of wild-type strain 74A, on the other hand, are grown in 10 to 150 μg per ml of tryptophan, the transport rates obtained are three- to sevenfold less than those observed in cells grown in minimal medium (Fig. 4 and 6). Considerable variations in the final intracellular tryptophan pool sizes have been observed by G. Lester (personal communication) for various strains of *Neurospora* grown in comparable external tryptophan concentrations. With one exception, all mutant strains tested appeared to form much larger pools than wild-type strain 74A. Whether these differences in final pool size represent alterations in the transport system or in the utilization of tryptophan pools in the mutants is not known.

The rapid reduction in the initial rates of tryptophan transport following a brief period of preloading with tryptophan poses a basic question. What is the nature of the metabolic factors which permit the cells to make such a fine and immediate adjustment? If we assume that the decrease in the rate of transport represents a decrease in the number of functional transport sites or some component of the sites, two explanations may be a priori invoked to explain the results. (i) Pool tryptophan or some metabolite derived from the pool feeds back and inhibits the activity of the transport site. (ii) Tryptophan or a metabolite represses the synthesis of the transport site (or sites) or some component which turns over at an extremely rapid rate. These two possibilities are not mutually exclusive, and it is conceivable that both are active in the regulation of tryptophan uptake during growth. It is clear from the results described above that continuous net protein synthesis is required for the maintenance of a functional transport system. Further, the data show that some component of the tryptophan transport system turns over at a relatively rapid rate and that this turnover may be enzymatically mediated.

The turnover of amino acid transport activity does not appear to be unique to *Neurospora*. Elías and Rosenberg (5) have shown that puromycin inhibits the uptake of α-aminoisobutyric acid in rat kidney cortex. It was suggested that puromycin inhibits the synthesis of a rapidly turning-over peptide (or peptides) which catalyzes amino acid transport.

The results presented in this report imply that the rate of tryptophan uptake is modulated by the maintenance of a delicate balance between the biosynthesis and degradation of some component of the transport site or sites, perhaps a
polypeptide. Thus, as the pool size increases, tryptophan or some metabolite feeds back and represses the synthesis of the rapidly turning-over transport component.

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LITERATURE CITED