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Some Characteristics of Tryptophan Uptake in *Claviceps* species

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Tryptophan serves as a precursor for the biosynthesis of alkaloids in the ergot fungus, *Claviceps purpurea* (Fries) Tulasne, and also is believed to act as an inducer of the enzymes necessary for alkaloid production. The characteristics of the transport system responsible for the accumulation of tryptophan in ergot mycelium were investigated, with the goal of clarifying the complex relationships among tryptophan uptake, size of the free intracellular pool of tryptophan, and alkaloid production. The characteristics of tryptophan uptake were studied by pulse feeding radioactively labeled tryptophan to cultures of *Claviceps* species, strain SD-58, which represented a variety of ages and nutritional states. Tryptophan accumulation in strain SD-58 is mediated by an energy-requiring system which exhibits specificity for neutral aromatic and aliphatic L-amino acids, is pH and temperature dependent, and shows saturation at high substrate concentrations. Tryptophan transport is a function of the intracellular concentration of free tryptophan, the nitrogen deficiency of the mycelium, the rate of growth, and alkaloid production, which were measured in *Claviceps* strain SD-58 growth in several culture media, some of which promoted alkaloid production and some of which did not. The results indicate that the initial velocity of tryptophan transport is not directly related to alkaloid production.

Tryptophan plays a central role in the biosynthesis of ergot alkaloids, a group of secondary metabolites which were first isolated from sclerotia of *Claviceps purpurea* (Fries) Tulasne. In addition to serving as a biogenetic precursor for a portion of the ergoline ring system of the alkaloids (35), Floss and Mothes (7), Bu'Lock and Barr (4), Robbers and Floss (23), and Vining (34) have reported that tryptophan may serve as an inducer of the enzymes required for alkaloid synthesis. Related to this is the accumulation of tryptophan in the fungal mycelium as evidenced by work in our laboratory (J. E. Robbers et al., submitted for publication) which indicates a two- to threefold temporary increase of intracellular, free tryptophan and a 20- to 25-fold increase of tryptophan synthetase activity during the transition period between growth and the alkaloid production phase. Rehacek et al. (22) also have observed that a certain minimum concentration of cell pool tryptophan is required to promote alkaloid synthesis in *C. paspali* and *C. purpurea*. Also influencing the intracellular accumulation of tryptophan is the ability of the ergot mycelium to actively take up tryptophan from the culture medium (31). In this regard, Teuscher (30) reported that out of 23 strains of *Claviceps* which he examined, only those strains which could take up, store, and metabolize large quantities of tryptophan could produce alkaloids in submerged culture. The ability to accumulate, either by way of active uptake or biosynthesis, a large intracellular pool of free tryptophan very likely is an important factor in alkaloid production.

An increasing number of kinetic and genetic studies of amino acid transport in fungi have been published in recent years. Among the filamentous fungi in which amino acid uptake has been studied in detail are *Neurospora crassa* and *Penicillium chrysogenum*. Wiley and Matchett (38, 39), Matchett et al. (18), Pall (19-21), Zalokar (40), DeBusk and DeBusk (5), Stadler (28), and Lester (16) are investigators who have turned their attention to *Neurospora*. Amino acid uptake in *P. chrysogenum* has been studied in detail by Benko et al. (1, 2).

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Wiley and Matchett (38) have investigated the specificity and kinetics of L-tryptophan uptake in germinated conidia of *N. crassa*. They found that the uptake of L-tryptophan is mediated by a distinct stereospecific system which shows strong pH and temperature dependence, unidirectionality, and saturation kinetics ($K_m = 5 \times 10^{-5}$ M) and is competitively inhibited by L-phenylalanine and L-leucine. In additional studies, these same workers (39) examined the metabolic control of tryptophan transport in *Neurospora* and concluded that the rate of transport is regulated by the size of the intracellular pool of tryptophan. Wiley (37), by using cold, osmotic shock, has isolated a tryptophan-binding protein from germinated conidia of *Neurospora* which has a dissociation constant for binding approximately equal to the K_m for tryptophan transport. Stuart and DeBusk (29) have partially characterized several glycoprotein subunits of the amino acid transport system of *Neurospora* conidia which could be extracted from the conidia with KCl.

By using the information and techniques gained from earlier work with *Neurospora* and *Penicillium*, the primary concerns of this investigation were to characterize the system, or systems, responsible for the active transport of tryptophan in *Claviceps* species (strain SD-58), to gain some insight into the metabolic control of the uptake, and to see whether there is any correlation between tryptophan uptake and alkaloid biosynthesis.

MATERIALS AND METHODS

Organism. The strain of ergot utilized for this study was *Claviceps* species strain SD-58 (11). It originally was isolated from sclerotia obtained from the host, *Pennisetum typhoideum* Rich. and is deposited in the culture collection of the Department of Medicinal Chemistry and Pharmacognosy, Purdue University.

Growth of cultures and preparation of inocula. The general procedure for cultivation of strain SD-58 consists of inoculating flasks of NL 406 liquid culture medium with a portion of the mycelium from a Czapek-Dox agar slant of the fungus and incubating the inoculated flask at 25 C and 150 rpm on a New Brunswick model VS or G-54 rotary shaking device (New Brunswick Scientific Co.). Culture medium NL 406 comprises the following (per liter of distilled water): mannitol, 50.0 g; sucrose, 50.0 g; succinic acid, 5.4 g; yeast extract (Difco), 3.0 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.0044 g; and sufficient NH_4OH to bring the pH of the solution to 5.4.

After a submerged culture, prepared as described above, had grown for 10 days, 3 ml of the mycelial suspension was used to inoculate a second shaking flask culture which, after 10 days of growth, was employed as an inoculum (3 ml of inoculum per 100 ml of culture medium) in 500-ml Erlenmeyer flasks for the cultures used in uptake studies and other experiments. Cultures prepared by the above procedure consisted chiefly of small mycelial aggregates and had a generally uniform appearance and consistency.

Labeled amino acids. L-Tryptophan-β-^{14}C (54.5 or 56.5 Ci/mol specific activity) and L-arginine-*guanido*-^{14}C (45.7 Ci/mol specific activity) were obtained from Amersham/Searle, DL-5-Methyltryptophan-^3H, D-tryptophan-^3H, and L-tryptophan-^3H had been previously prepared by acid-catalyzed tritium exchange (12).

Appropriate portions of unlabeled amino acids and deionized, distilled water were added to samples of the radioactively labeled amino acids in order to obtain "feeding solutions" having the desired concentration and specific activity for individual experiments.

Determination of tryptophan uptake. The basic procedure for the assay of labeled tryptophan accumulated in *Claviceps* strain SD-58 combined features of the techniques used by Wiley and Matchett (38), Benko et al. (1), and Pall (19). Mycelial samples were freed from the culture medium by suction filtration, pressed between sheets of dry filter paper, weighed, and added to the desired volume of phosphate buffer (0.05 M, pH 5.9) for incubation with the labeled amino acid. Aseptic conditions were not maintained during the assays for amino acid transport activity.

Uptake method "A" involved incubation of the mycelium suspended in phosphate buffer with the added carbon 14- or tritium-labeled amino acid in an open 150-ml beaker equipped with a magnetic stirrer to insure vigorous aeration. Temperature was controlled by jacketing the small (150 ml) beaker with a larger beaker filled with water of the desired temperature, which proved adequate to maintain the required temperature, 25 C, during the brief duration of most experiments.

Uptake method "B" consisted of placing the mycelial sample and buffer in a 125-ml Erlenmeyer flask, adding the labeled amino acid, and incubating the mixture on a rotary shaker in a constant-temperature (25 C) culture room. This uptake assay method was utilized when long incubations were required or when only one or a few samples were taken.

In both uptake methods "A" and "B," the weight of wet pressed mycelium used was 200 mg per each 10 ml of incubation medium, corresponding to a dry weight of slightly more than 2 mg/ml. Unless otherwise noted, the pH used throughout was 5.9. After the mycelial sample was placed in the buffer solution, a 10-min period of stirring or shaking allowed resuspension and aeration of the mycelia. At this point, the labeled amino acid was added, and an electric timer was started. Samples of the stirred or shaken incubation suspension were removed by pipetting at appropriate time intervals and were filtered rapidly with suction through sintered glass funnels held in a filtering manifold. The volume of the sample taken was noted, and the time at which the last trace of liquid disappeared from the mycelial

mat was recorded as the time of sampling. The mycelial mats were quickly washed with two volumes of cold (approximately 4 C), deionized water, removed from the funnel, and placed in an 80 C forced-air oven to dry for 12 h. The dried mycelial samples were finely powdered, and between 5 and 10 mg were weighed into a scintillation vial, to which was added 1 ml of boiling, deionized water, and the mixture was placed in a boiling-water bath for 3 min. After cooling, 10 ml of Bray's (3) scintillation solution was added, and the vial was swirled to ensure thorough mixture of its contents. The samples thus prepared were counted in a Beckman LS 100 liquid scintillation system. After counting for 2 min, or for a time sufficient to limit the counting error to 2%, internal carbon 14 or tritium standards were added to the counting vials, and the vials were counted again to determine the counting efficiency. The counting efficiency, along with the specific activity of the amino acid feeding solution, enabled the amount of labeled amino acid in the mycelium to be calculated. By using these conditions, a counting efficiency of approximately 60% was obtained for carbon 14, which correlates with the results of Benko et al. (1).

The standard incubation medium consisted of potassium phosphate buffer (pH 5.9, 0.05 M) consisting of KH_2PO_4 and K_2HPO_4 or potassium-sodium phosphate buffer (pH 5.9, 0.05 M) consisting of KH_2PO_4 and Na_2HPO_4.

Determination of free intracellular tryptophan. Tryptophan was assayed in the mycelium, or in the aqueous culture medium, which had been extracted to remove alkaloids by the Spiess and Chambers (27) method. For determination of tryptophan in the mycelium, freeze-dried mycelial samples were finely powdered in a mortar and pestle, and weighed portions (usually 100 mg) were extracted with 5 ml of deionized water in a small test tube suspended in a boiling-water bath for 30 min. The mycelial powder was separated out by centrifugation, and the supernatant fluid was assayed by the colorimetric method based on the reaction of tryptophan with *p*-dimethylaminobenzaldehyde.

Determination of alkaloid production and mycelial dry weight. Dry-weight determinations were carried out on samples by using a tared piece of Whatman no. 1 filter paper to collect the mycelium, drying the paper plus mycelium for 24 h in a forced-air oven at 50 C and recording the net weight after drying.

Quantitation of alkaloids in the culture filtrate was accomplished by adding 2 ml of the van Urk (33) reagent, as modified by Smith (26), to 1 ml of alkaloid containing sample, allowing the characteristic blue-purple color to develop for 30 min and measuring the absorbancy at 580 nm in a Beckman model B spectrophotometer. The quantity of alkaloids in μg present in the 1-ml sample was calculated (on the basis of a standard curve obtained by using elymoclavine) by multiplying the absorbancy at 580 nm by 38.7 and by any dilution factor which may have been incurred in sample preparation. In order to obtain the sample for alkaloid determination, 2 ml of the culture filtrate obtained from harvesting by filtration was made alkaline by adding 0.2 ml of 10% NH_4OH, and the alkaloids were extracted into 2 ml of chloroform. A portion (usually 1.0 or 0.1 ml) of the chloroform extract was evaporated to dryness at room temperature. Succinic acid (2%, 1 ml) was added to the dried residue, and the resulting solution was used for alkaloid quantitation. This extraction procedure was essential in cultures which contained added tryptophan.

Claviceps strain SD-58 was grown in culture medium NL 406 to achieve "normal" growth and alkaloid production. When 1.0 g of KH_2PO_4 per liter was added to culture medium NL 406, cultures which produced no alkaloids were obtained. Alkaloid production was, in part, restored by the addition of 4 mM DL-tryptophan to the NL 406 high-phosphate cultures.

RESULTS

Linearity of tryptophan uptake. Plotting the quantity of accumulated L-tryptophan-β-^{14}C as a function of incubation or sampling time in *Claviceps* species, strain SD-58, results in uptake curves such as those illustrated in Fig. 1. The values were obtained by incubation with 10^{-4} M L-tryptophan-β-^{14}C (0.5 Ci/mol). In each case, the resulting curve is linear during the early stages of the incubation with labeled tryptophan. The initial velocity of tryptophan uptake, a property of cultures which could be reproducibly measured, is represented by the slope of the best straight line determined by three points on the uptake curve, usually representing samples taken during the first 5 min of the incubation period. Initial velocity is expressed as the number of micromoles of labeled tryptophan accumulated per gram (dry weight) of mycelium during 1 min of incubation time (μmoles per gram per minute). As will be noted in a later section, Fig. 1 shows that there are large differences among initial uptake velocities determined in cultures of different ages.

Distribution of tryptophan uptake. Radioactive label begins to accumulate in the fungal mycelium immediately after the addition of L-tryptophan-β-^{14}C (0.5 Ci/mol) to the incubation suspension. Extraction of the individual mycelial samples with boiling water and subsequent counting of both the aqueous extract and the extracted mycelium indicated that most of the C-14 taken up is in a free state and readily extractable from the mycelium. Figure 2 shows the results of one study of the distribution of radioactive label in a 36-h-old culture strain SD-58. The curve labeled "insoluble tryptophan" may represent labeled tryptophan which has been incorporated into protein. Similar results were obtained in an additional experi-

ment in which samples of mycelium were extracted with cold 5% trichloroacetic acid. In either case, the total cellular uptake of tryptophan is equal to the sum of the curves labeled "extractable" and "insoluble" in Fig. 2.

That the radioactivity accumulated by the fungus actually represents tryptophan, and not products of tryptophan metabolism or degradation, was demonstrated by thin-layer chromatography of the aqueous extracts by using the following solvent systems: *n*-butanol-glacial acetic acid-water (65:13:22) and methyl acetate-isopropanol-25% ammonia (45:35:20). Radiochromatographic analysis of samples taken at 1.3, 2.1, 15.0, 29.2, and 42.8 min indicated that all of the radioactivity in the extracts resided in a single spot, which was not distinguishable from authentic L-tryptophan in two chromatographic solvent systems.

The early samples in Fig. 2 indicate that the initial accumulation of tryptophan follows a linear pattern. A rapid depletion of the labeled tryptophan in the extracellular medium, due to the rapid uptake by the mycelium, may account for the departure from linearity which occurs after 4 or 5 min. The initial uptake velocity determined in this experiment is 1.5 μmol per g per min.

These results also indicate transport against a concentration gradient. A calculation, based on the concentration of labeled tryptophan in the intracellular water (ca. 88% of the wet weight of the mycelium), indicates that the intracellular concentration after the incubation had proceeded for 42.8 min and was about 100 times greater than that in the extracellular medium.

Effects of metabolic inhibition on tryptophan transport. Figure 3 illustrates the pronounced inhibitory effect of 2,4-dinitrophenol on tryptophan transport in a mycelial suspension prepared from a 48-h-old *Claviceps* culture. The mycelium was preincubated in either potassium phosphate buffer (normal uptake curve) or in a 10^{-3} M solution of 2,4-dinitrophenol in potassium phosphate buffer. Under these conditions, the 2,4-dinitrophenol produced an initial velocity of tryptophan uptake which was less than 10% of the uninhibited value. The small amount of tryptophan which accumulated in the mycelium in the presence of the inhibitor may be attributed to adsorption, intercellular trapping by small mycelial aggregates, or some component of tryptophan transport which is not energy dependent. Similar studies, summarized in Table 1, indicate that tryptophan uptake is also inhibited in the presence of 10^{-3} M sodium azide and in the absence of oxygen (achieved by bubbling nitrogen through the incubation suspension).

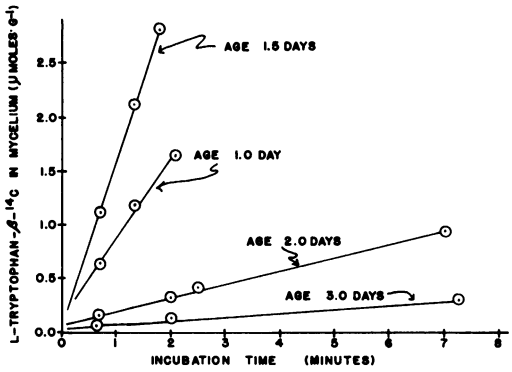

FIG. 1. *Linearity of* L-*tryptophan uptake in Claviceps species, strain SD-58.*

Unidirectionality of tryptophan transport. The results of an attempt to wash radioactively labeled tryptophan from 36-h-old mycelium of *Claviceps* are shown in Fig. 4. Mycelium was preloaded with tryptophan by incubation in a 10^{-4} M solution of L-tryptophan-β-^{14}C for 15 min. When preloaded mycelium (containing more than 11 μmole/g of labeled tryptophan) was resuspended into phosphate buffer for 15 min, less than 10% of the label was lost. Incubation in a 10^{-3} M solution of 2,4-dinitrophenol, however, caused more than 50% of the accumulated label to be lost in fewer than 10 min. It has been observed that labeled tryptophan is not lost from preloaded mycelium after replacement into deionized water or into phosphate buffer containing 5×10^{-3} M unlabeled DL-tryptophan. These data indicate that tryptophan accumulation in this *Claviceps* strain does not involve a simple equilibration across the cell membrane. In addition, if some form of adsorption to the extracellular surface were important in the accumulation of label in 36-h-old cultures, replacement into the unlabeled tryptophan should have washed away a significant portion of the label by isotope dilution.

Further evidence to support an active transport process. The pH dependence of tryptophan transport velocity is illustrated in Figure 5, and the effect of temperature is shown in Fig. 6. Although detailed studies to determine the exact pH and temperature optima were not conducted, the results shown in these two figures illustrate the marked variation in uptake velocity with changes in these conditions. The pH of the uptake medium was adjusted by using appropriate ratios of McIlvane's (17) 0.2 M disodium phosphate and 0.1

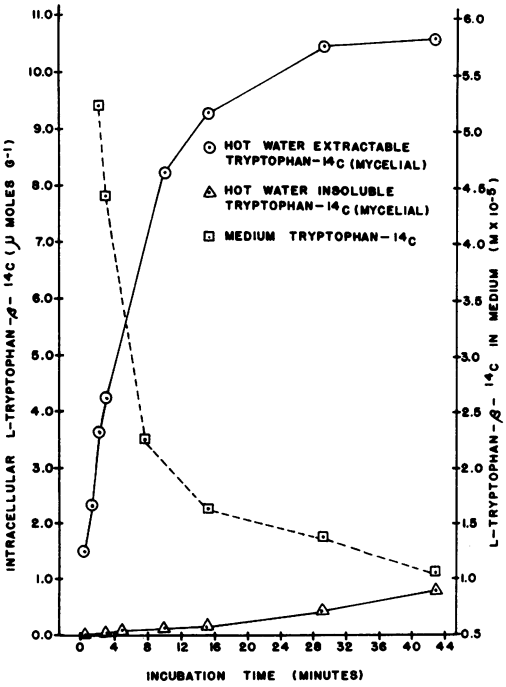

FIG. 2. *Distribution of labeled tryptophan accumulated by a 36-h-old culture of Claviceps species, strain SD-58.*

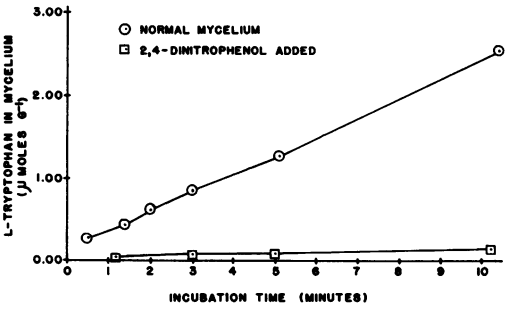

FIG. 3. *Effect of treatment with 2,4-dinitrophenol on uptake of L-tryptophan in Claviceps species, strain SD-58.*

M citric acid buffer solution at an overall concentration of 0.05 M. Increases in temperature produced a steady increase in uptake velocity between 10 and 30 C and the Q_{10} calculated between 20 and 30 C was approximately 2.6.

The effect of varying tryptophan concentration on the rate of uptake of the amino acid was studied over a wide range of concentrations (10^{-6} M to 3×10^{-3} M), and the results were expressed as a Lineweaver-Burk plot. Saturation of the transport system was observed at high tryptophan concentrations, and a K_m of

TABLE 1. *Inhibition of tryptophan uptake*

Inhibitor and concn (M)	Normal value for initial velocity of L-tryptophan uptake (μmol per g per min)[a]	Value of uptake with inhibitor (μmol per g per min)	Inhibition (%)
2,4-Dinitrophenol (10^{-3})	0.97	0.025	97
Sodium azide (10^{-3})	0.97	0.037	96
Nitrogen, bubbled through suspension	0.97	0.20	79

[a] 36-hr. culture.

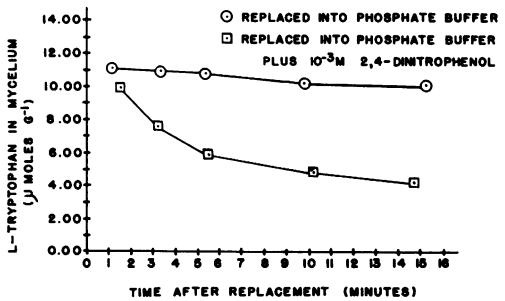

FIG. 4. *Loss of L-tryptophan-β-^{14}C from mycelium in Claviceps species, strain SD-58.*

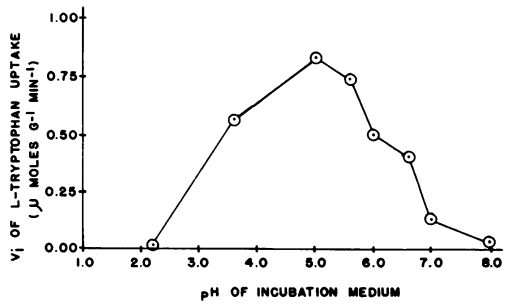

FIG. 5. *Effect of pH on initial velocity of tryptophan uptake in Claviceps species, strain SD-58.*

1.54×10^{-4} M and maximum reaction velocity (V_{max}) of 3.0 μmol per g per min were calculated. A similar experiment with another young culture indicated that the uptake process had a K_m of 1.7×10^{-4} M and a V_{max} of 3.0 μmol per g per min. The K_m values obtained for tryptophan uptake in this study are very close to those obtained for phenylalanine uptake in *Aspergillus nidulans* (25) and *N. crassa* (5), which are 2×10^{-4} and 10^{-4} M, respectively. Since the K_m values in this experiment and

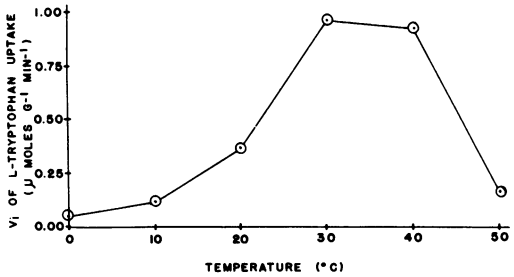

FIG. 6. *Initial velocity of tryptophan uptake as a function of temperature in Claviceps species, strain SD-58.*

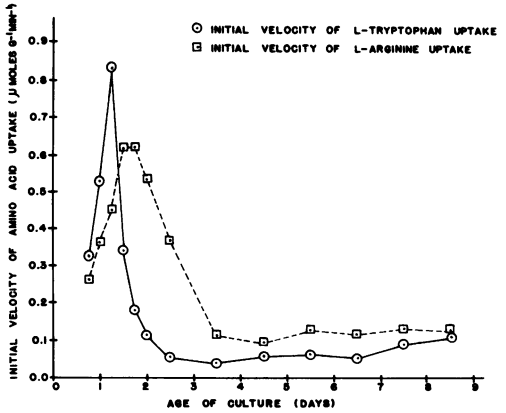

FIG. 7. *Initial velocities of* L-*tryptophan and* L-*arginine uptake as a function of culture age in Claviceps species, strain SD-58.*

those reported by other authors are properties of suspensions of fungal mycelia and may be greatly influenced by the conditions under which they are measured, the direct comparison of these values in studies using widely different techniques for measurement of amino acid uptake is difficult. The data for concentration, pH, and temperature dependence strongly suggest that tryptophan uptake in *Claviceps* strain SD-58 is a permease-mediated process.

Culture age and initial velocity of tryptophan uptake. It was observed early in this investigation that tryptophan uptake velocity fluctuated greatly with the age of the culture examined, as is indicated in Fig. 1. The solid line in Fig. 7 represents initial velocity of tryptophan transport measured periodically during a culture period of 8.5 days. A striking feature of the results is the sharp peak in uptake velocity at a culture age of approximately 1.5 days, which is followed immediately by a decline to a much lower level. This lower rate of transport is maintained, with only a slight fluctuation, throughout the remainder of the culture period. This pronounced peak and decline in tryptophan transport was observed in every group of SD-58 cultures grown in culture medium NL 406.

The extent to which this peak and decline in the rate of amino acid transport is specific for tryptophan was examined by studying the uptake of L-tryptophan-β-^{14}C and L-arginine-*guanido*-^{14}C in the same series of cultures. At each sampling time, part of the mycelium from a single culture was utilized for a measurement of the initial velocity of tryptophan uptake, and a second portion was used for the determination of arginine uptake. As is seen in Fig. 7, the initial velocity of arginine uptake follows a similar pattern of peak and decline, but the peak in arginine transport does not coincide with the peak in tryptophan transport. The decrease in arginine transport velocity begins 12 to 15 h later than the corresponding decrease in tryptophan transport. Arginine was chosen as a second amino acid for study, because competition studies indicated that arginine and tryptophan enter the mycelium by different transport systems. A second difference in the pattern of uptake for the two amino acids is that the initial velocity of arginine transport never reaches as low a level as that for tryptophan.

Gottlieb et al. (9) and Jones (14) have reported decreases in amino acid uptake with increasing age in fungi, but not such pronounced decreases as those observed in *Claviceps* in these experiments. Recently, Whitaker and Morton (36) have described the considerable influence of the age of mycelium on the extent of leucine accumulation in *Penicillium griseofulvum*. The greatest capacity to accumulate leucine was attained at a culture age of 33 h in cultures of *P. griseofulvum*, and older cultures showed a marked decline in transport ability, which the authors attributed to the cessation of growth and the beginning of mycelial fragmentation and autolysis.

The possible significance of the pattern of variation of tryptophan transport with age will be discussed in a later section.

Specificity studies in 36-h-old cultures. Competition studies, carried out by adding a 10^{-3} M concentration of a selected nonlabeled amino acid simultaneously with 10^{-4} M L-tryptophan-β-^{14}C, produced results such as those shown in Table 2. Neutral aromatic and aliphatic amino acids show significant inhibition of tryptophan uptake. The basic amino acids L-lysine and L-arginine are rather poor inhibi-

TABLE 2. *Effect of selected amino acids on tryptophan uptake*

Inhibitor	Concn (mM)	Inhibition of the initial rate of uptake (%)
L-Phenylalanine	1	86
L-Tryptophan	1	85
L-Alanine	1	71
L-Leucine	1	67
L-Methionine	1	62
DL-5-methyl-tryptophan	2	62
L-Histidine	2	36
L-Arginine	2	12
L-Lysine	2	5
D-Tryptophan	2	4
L-Aspartic acid	2	1

tors, as is aspartic acid. A very slight degree of inhibition, or none at all, is shown by D-tryptophan, which indicates the stereospecificity of the process involved. The fact that DL-5-methyl-tryptophan gives significant inhibition of L-tryptophan transport indicates that this tryptophan analogue may, to some extent, utilize the same mode of entry into the cell as L-tryptophan.

Tritiated D-tryptophan and DL-methyl-tryptophan were added to 36-h-old mycelium in an attempt to investigate further the specificity of tryptophan uptake. Table 3 shows the results of these uptake experiments. The 5-methyl analogue of tryptophan was transported into the mycelium, but with an initial velocity approximately 25% of that for L-tryptophan under identical uptake conditions. The fact that the 5-methyl analogue is taken up is of some interest to us, because it is also able to induce clavine alkaloid production in strain SD-58 (7, 23). D-Tryptophan appears to be taken up with a very low initial velocity. This again indicates that the amino acid transport system responsible for tryptophan uptake is very stereospecific and agrees well with the competition data listed in Table 2. Stereospecificity of the L-tryptophan transport system, as evidenced by lack of competition of D-tryptophan with L-tryptophan, was reported in *N. crassa* by Wiley and Matchett (38). Teuscher (31) found that D-tryptophan was taken up and utilized by *Claviceps* strain SD-58 over a period of several days and at a rate which was somewhat slower than that for L-tryptophan. Our studies with unlabeled D-tryptophan indicate that during the course of a 48-h-incubation period, large amounts of D-tryptophan may be taken up by strain SD-58, even though the initial velocity of transport for the D isomer is very low. It is not known whether the D-tryptophan accumulates in the cells as the D isomer; it has been shown, however, that *Claviceps* strain SD-58 can convert D-tryptophan into the L isomer (8).

Metabolic regulation of tryptophan transport. The tryptophan uptake activity described thus far is that which develops "naturally" during the growth of *Claviceps* strain SD-58 in culture medium NL 406. We observed that various growth conditions as well as other factors can affect tryptophan uptake.

That ammonium ion concentration was important in the regulation of tryptophan transport was indicated by the observation that initial uptake velocities were consistently lower when measured with an ammonium-potassium phosphate buffer (0.05 M, pH 5.9) as the incubation medium than when a potassium-sodium phosphate buffer (0.05 M, pH 5.9) was used. Apparently, the ammonium ions were causing partial inhibition of tryptophan transport, the initial velocity of tryptophan uptake being 30 to 40% lower in the buffer containing ammonium ions.

The effect of nitrogen starvation on L-tryptophan uptake was investigated by replacing mycelia from 33-h-old cultures into nitrogen-sufficient and nitrogen-deficient incubation media. Part of the mycelia were replaced into NL 406-YE culture medium, which contained no yeast extract but contained ammonium ions as a nitrogen source. The remainder of the mycelia were replaced into medium NL 406-N, which contained no nitrogen source. Samples of each of the two sets of cultures were taken over a 14-h period, and the initial velocity of tryptophan uptake was determined in each sample. Figure 8 illustrates the results of this experiment, and it can be seen that after 9 h of incubation in the replacement media, the initial velocity of tryptophan uptake in the nitrogen-deficient medium was approximately 12

TABLE 3. *Comparison of the initial uptake rates for tritiated L-tryptophan, D-tryptophan, and DL-5-methyltryptophan*

Amino acid	Initial velocity of uptake (μmol per g per min)	
	Trial A	Trial B
L-Tryptophan-^{3}H	0.82	0.87
D-Tryptophan-^{3}H	0.032	0.046
DL-5-Methyltryptophan-^{3}H	0.23	0.19

times greater than that in the nitrogen-sufficient medium. When the last samples were measured at 14-h, the difference was greater than 20-fold.

Grenson et al. (10), Benko et al. (1, 2), and Pall (20) have published considerable evidence in support of a general amino acid permease which is regulated by the ammonium ion concentration. In particular, Benko et al. (1, 2) found the same kind of increase in the initial uptake velocity of amino acids upon nitrogen starvation as was noted in this investigation. In addition, Hackette et al. (13) have studied and characterized a specific ammonium transport system in *P. chrysogenum* which does not develop coincidentally with the general amino acid permease during nitrogen starvation. In order to determine whether a general amino acid permease is responsible for the increase in tryptophan uptake in nitrogen-starved cultures of *Claviceps* strain SD-58, a comparison was made of the concentration dependence and specificity of tryptophan uptake in mycelia from 36-h-old SD-58 cultures incubated in nitrogen-sufficient and nitrogen-deficient media for 12 h. The K_m and V_{max} values obtained with the nitrogen-sufficient mycelium were 2.2×10^{-4} M and 0.5 μmol per g per min, respectively. The K_m value was similar to that obtained in normal 36-h-old cultures, but the V_{max} was only one-third to one-sixth as high as in normal cultures. The K_m value for the nitrogen-starved mycelium was 8.1×10^{-5} M, which is approximately one-half of the average value for normally growing 36-h-old cultures. The nitrogen-starved culture showed a V_{max} for tryptophan uptake of 7.4 μmol per g per min, which is between two and four times as high as that in normal cultures of this age. Nitrogen starvation apparently increases the affinity of the transport system for tryptophan and increases the rate of tryptophan transport.

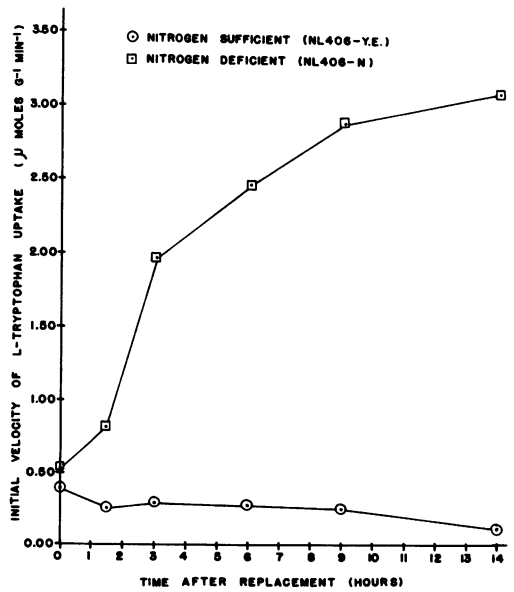

FIG. 8. *Development of L-tryptophan transport activity during nitrogen starvation.*

TABLE 4. *Effect of selected amino acids on initial velocity of tryptophan transport in nitrogen-sufficient and nitrogen-deficient cultures*

Inhibitor (1 mM)	Inhibition of the initial rate of tryptophan transport (%)	
	(N-sufficient)	(N-deficient)
L-Phenylalanine	85	90
L-Tryptophan	82	86
L-Leucine	60	76
L-Arginine	12	29
L-Aspartic acid	5	15
D-Tryptophan	3	19

An examination of the specificity of tryptophan uptake was undertaken by using competition data obtained by adding a 10-fold excess of selected unlabeled amino acids. The results are shown in Table 4. There is a decreased specificity for tryptophan in the nitrogen-starved cultures as evidenced by the inhibitory effects produced by L-arginine, L-aspartic acid, and D-tryptophan.

The changes in specificity and concentration dependence could be explained by the presence of a single amino acid permease whose properties change with the age and nutritional state of the mycelium, or they might be due to changes in the relative concentrations or activities, or both, of two or more transport systems. Our data do not confirm either of these possibilities in *Claviceps* strain SD-58, but recent studies in other species of fungi suggest that the latter possibility may be the correct one. If, for example, a situation similar to that described by Pall (19, 21) in *N. crassa*, Benko et al. (2) in *P. chrysogenum*, and Grenson et al. (10) in *Saccharomyces cerevisiae* exists in strain SD-58, the task of accumulating tryptophan from the growth medium may be shared by as many as three distinct permeases, including a general amino acid permease, a permease which is specific for neutral aromatic and aliphatic L-amino acids, and a specific tryptophan permease. These permeases would proba-

bly exist in different relative amounts and with different degrees of activity, depending upon the culture age and the type and quantity of amino acids and other nutrients produced in the mycelium and supplied in the culture medium. Regardless of how many permeases are present in strain SD-58, a significant portion of the tryptophan uptake activity is mediated by a transport system whose amount or activity, or both, is regulated by the ammonium ion concentration in the incubation medium.

The size of the intracellular pool of free tryptophan appears to be an important factor regulating the initial uptake velocity for L-tryptophan under certain culture conditions. Table 5 illustrates the results obtained in a study of 36-h-old cultures of strain SD-58 grown in, or resuspended into, four different culture media. The initial velocity of L-tryptophan transport in these cultures seems to be inversely proportional to the concentration of tryptophan in the intracellular pool. The largest intracellular tryptophan pool (17.0 μmol per g) and the lowest value for the initial velocity of tryptophan transport (0.30 μmol per g per min) were obtained in a culture which was grown in medium NL 406 without yeast extract supplemented with 4 mM DL-tryptophan. The smallest intracellular tryptophan pool (3.32 μmol per g) and the highest initial velocity of tryptophan transport (0.85 μmol per g per min) were measured in a culture which was grown in medium NL 406 without yeast extract for 28 h and replaced into medium NL 406 having no nitrogen source for 8 h. The figures in Table 5 probably reflect the nitrogen sufficiency or nitrogen deficiency of the mycelia employed in the assays as well as the intracellular concentration of tryptophan.

TABLE 5. *Initial velocity of tryptophan uptake and size of the intracellular tryptophan pool*

Growth media	Extractable free tryptophan (μmol/g)	Initial velocity of L-tryptophan uptake (μmol per g per min)
NL406–yeast extract (YE)	4.82	0.67
NL406-YE plus 4 mM DL-tryptophan	17.00	0.30
NL406-YE plus 4 mM DL-tryptophan, suspended into NL406-YE	7.55	0.46
NL406-YE plus 4 mM D-d into NL406-N	3.32	0.85

Clavine alkaloid production and tryptophan uptake. A major objective of this investigation was to examine the relationship between alkaloid production and active transport of tryptophan in cultures of *Claviceps*. By growing *Claviceps* strain SD-58 in three different culture media, it was possible to obtain the three patterns of growth and alkaloid production shown in Fig. 9 through 11.

Culture dry weight, initial velocity of tryptophan transport, free intracellular tryptophan, and alkaloid production were plotted as functions of culture age in *Claviceps* strain SD-58 grown in culture medium NL 406 (Fig. 9). Growth in culture medium NL 406 enabled this strain of the ergot fungus to produce substantial quantitites of alkaloids, approximately 400 μg/ml after 9 days of growth. The initial velocity of L-tryptophan uptake dropped from a relatively high value at 1.5 days to a minimum value at 3 days. Tryptophan uptake remained at a low level during the remainder of the culture period, including the time during which alkaloid production was occurring. Growth, as measured by dry weight, proceeded rapidly for the first 3 days, but no further increase in dry weight was noted between 3 days and 7 days. The level of free tryptophan in the mycelium more than doubled between day 2 and day 5 and reached a maximum value of about 4.5 μmol per g.

The course of the growth of strain SD-58 in NL 406 culture medium supplemented with 1.0 g of KH_2PO_4 per liter is shown in Fig. 10. The additional phosphate resulted in the complete loss of alkaloid production and was accompanied by an initial velocity of tryptophan transport which remained high throughout the duration of the culture period. After the age 2.5 days, there was a slow decline in the level of intracellular tryptophan from about 4.0 to about 2.5 μmol/g, which was accompanied by an increase in the initial velocity of tryptophan uptake. The dry weight of the culture continued to increase steadily until the age of 7 days, at which time it was approximately 2.5 times as large as the dry weight obtained in plain NL 406 medium.

The addition of 4 mM DL-tryptophan to NL 406, high-phosphate cultures overcame the inhibitory effect of phosphate on alkaloid production, as may be seen in Fig. 11. The total alkaloids produced at age 9 days amounts to 200 μg per ml of culture filtrate or 10 μg per mg of dry mycelium (as compared with about 40

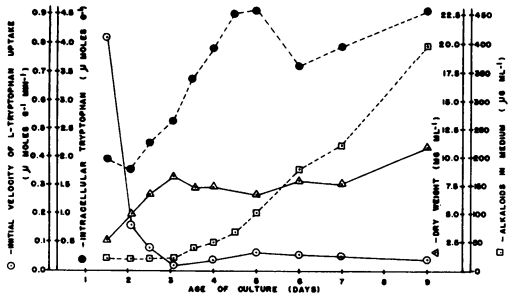

FIG. 9. *Growth, L-tryptophan uptake, free intracellular tryptophan pool, and alkaloid production as functions of age in Claviceps species, strain SD-58, grown in NL 406 culture medium.*

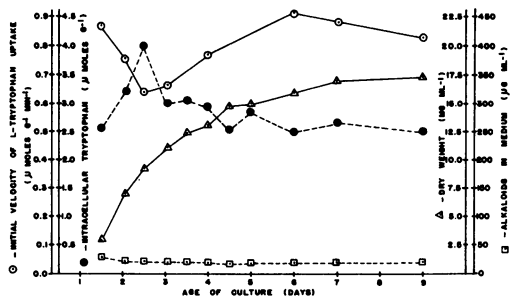

FIG. 10. *Growth, L-tryptophan uptake, free intracellular tryptophan pool, and alkaloid production as functions of age in Claviceps species, strain SD-58, grown in NL 406 high-phosphate culture medium.*

μg/mg in the NL 406 cultures of SD-58). Due to the presence of DL-tryptophan in the medium, there was an intracellular tryptophan pool of more than 28 μmol/g in the 2-day-old cultures. By the 9th day of growth, this value had decreased to less than 12 μmol/g. The decrease in the level of intracellular tryptophan and the continued steady growth rate were accompanied by a sharp increase in initial velocity of tryptophan transport between the age of 5 and 7 days.

DISCUSSION

The detailed examination of tryptophan transport in cultures of *Claviceps* species, strain SD-58, indicates that tryptophan uptake is mediated by an energy-requiring, unidirectional transport system which is specific for neutral aromatic and aliphatic amino acids. That the transport process involves the participation of a permease is also suggested by its preference for L-tryptophan over D-tryptophan, its pronounced dependence on temperature and pH, and its saturation at high L-tryptophan concentrations.

The uptake of tryptophan in cultures of strain SD-58 is a complex process which is markedly influenced by the intracellular concentration of free tryptophan, the nitrogen sufficiency or nitrogen deficiency of the mycelium, the age of the culture, and the rate of growth at the time uptake velocity is measured. Typically, the period from 36 to 48 h, during which the very sharp decrease in initial velocity of tryptophan uptake occurs in cultures grown in NL 406 medium, is characterized by an increase in culture dry weight and, apparently, no significant change in the size of the intracellular tryptophan pool. Slowing of the growth rate and increase in size of the intracellular tryptophan pool are noted only after the lowest

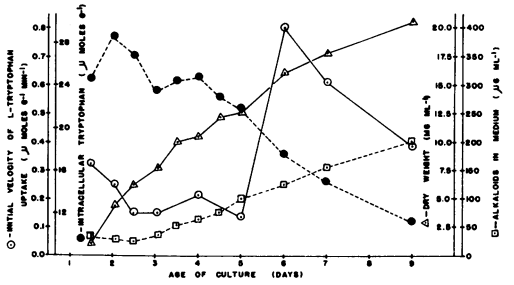

FIG. 11. *Growth, L-tryptophan uptake, free intracellular tryptophan pool, and alkaloid production as functions of age in Claviceps species, strain SD-58, grown in NL 406 high-phosphate culture medium with added D, L-tryptophan.*

level of tryptophan transport has been reached, at an age of approximately 3 days. In fact, the decline in arginine transport (Fig. 7), which begins 12 to 15 h later than the decline in tryptophan uptake, seems to coincide more closely with the decline in growth rate as measured by culture dry weight.

Another observation (Fig. 7 and 9) was the absence of any large increase, or even any significant change, in tryptophan uptake velocity during the period between 4.5 and 7.5 days, when alkaloid production was proceeding at a high rate. Other studies have shown, however, that protein synthesis occurs during the alkaloid production phase (4, 6, 15, 24) even though mycelial dry weight does not increase significantly. The only other published studies of tryptophan uptake in the ergot fungus are those of Teuscher (30–32). He observed that strains of *Claviceps* which produce alkaloids in saprophytic culture possess the ability to accumulate and metabolize large quantities of tryptophan from the culture medium. Teuscher's work consists of investigations of the uptake of tryptophan in 5- and

7-day-old cultures of *Claviceps* strain SD-58, as well as in numerous other ergot strains. Teuscher's experiments involved the incubation of mycelia suspended in phosphate buffer with large amounts (usually 2 mM) of unlabeled tryptophan for 3-day periods. Degradation of tryptophan via the indole-acetic acid and kynurenine pathways was demonstrated in his studies (32), and apparently much of the tryptophan was incorporated into alkaloids. In summary, Teuscher's data measured transport and metabolism of tryptophan in cultures which had already begun to produce alkaloids.

The utilization of carbon 14-labeled amino acids in more recent studies of amino acid uptake has made possible the detection of the accumulation of very small quantities of the amino acids. Therefore, it is hoped that the present study represents the measurement of tryptophan uptake at more nearly normal concentrations. In all three of the culture media utilized for the cultivation of *Claviceps* strain SD-58 in these experiments, the fungus is capable of accumulating L-tryptophan with a high initial velocity during some portion of the culture period, although the pattern of development and decline for this high initial velocity is different in each case. There seems to be little or no direct correlation between the initial velocity of tryptophan uptake and the production of alkaloid under the conditions examined. The more significant condition for alkaloid production, however, may be the build-up of a large intracellular pool of free tryptophan, whether by active uptake from the culture medium or by biosynthesis.

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