Metabolism of Glutamic Acid in *Aspergillus ochraceus* During the Biosynthesis of Ochratoxin A

C. W. BACON,* J. D. ROBBINS, AND D. BURDICK

Richard B. Russell Research Center, Athens, Georgia 30604

The uptake and utilization of glutamic acid in the biosynthesis of ochratoxin A by *Aspergillus ochraceus* were studied. Uniformly labeled L-[^{14}C]glutamic acid was incorporated into both the phenylalanine and isocoumarin moieties of ochratoxin A. Penicillic acid was also labeled. During the early stages of development, the amino acid was used mainly for the synthesis of ribonucleic acid and protein. A portion of glutamic acid was oxidized and was recovered as metabolic $^{14}CO_2$. The initial uptake velocity of glutamic acid decreased with age and was pH and temperature dependent. No relationship was found between the initial uptake velocities and ochratoxin A biosynthesis.

Aspergillus ochraceus Wilhelm produces ochratoxin A, a dihydroisocoumarin derivative linked through its 7-carboxy group to L-β-phenylalanine (23). This mycotoxin has a mean lethal dose (oral) of 100 to 200 μg per day-old cockerels and 20 mg/kg for rats (21). Many strains belonging to the *A. ochraceus* group are capable of producing this toxin and concomitantly another, penicillic acid (5, 21). Such a strain of *A. ochraceus*, isolated from poultry litter, produced both toxins on poultry feed under varying conditions of moisture and temperature (2).

Some initial studies on the biosynthesis of ochratoxin A have been done. Searcy et al. (19) reported that the isocoumarin portion of the mycotoxin was derived via acetate condensation and that the phenylalanine moiety was synthesized via the shikimic acid pathway. The work of Maebayashi et al. (15) confirmed this route for biosynthesis. Furthermore, activity was incorporated into the carboxy carbon of the isocoumarin moiety by using either [^{14}C]formate or methionine-S-^{14}CH$_3$ (15, 22).

Several investigators (6, 7, 14, 20) have studied the production of ochratoxin A in chemically defined media. The medium of Ferreira (6) is used for submerged culture production of the mycotoxin and that of Lai et al. (14) for surface culture.

According to Ferreira (6, 7), glutamic acid (or proline) exerts an inducer-like effect in ochratoxin A biosynthesis in submerged culture. Proline was considered to be converted to glutamic acid via pyrroline-5-carboxylic acid as reported in many microorganisms (18). How-

ever, other amino acids are also known to be converted to glutamic acid (18) but they do not effect the production of the mycotoxin (7). That glutamic acid influences toxin production is based on data which show that if the amino acid is not available during the early stages of fungal development (0 to 24 h), the maximum level of toxin is not produced, and that no toxin is produced if the amino acid is added 72 h later (6). The accumulation of the toxin started after 3 days and was completed within 24 h. In this respect, ochratoxin A differs from some mycotoxins in that it is produced during the logarithmic phase of growth in liquid media. Ferreira (6) further demonstrated that analogues and antagonists of glutamic acid and proline inhibited production of the toxin. The inhibition was reversed by increasing the concentrations of glutamic acid or proline and lactic acid. Recently, Steele et al. (20) studied ochratoxin A production in Ferreira's medium and stated that glutamic acid was removed from the medium during the first 3 days.

The above investigations suggest that glutamic acid or its metabolic product might be used as a convenient tool to determine some of the regulatory parameters involved in the production of ochratoxin A. This paper reports the uptake and utilization of glutamic acid by *A. ochraceus* in submerged culture during the production of ochratoxin A.

MATERIALS AND METHODS

Organism. The organism used in this investigation was *A. ochraceus* 107. Cultures were maintained at 4 C on Czapek agar and subcultures used for inocula-

tions were prepared as described previously (2).

Culture conditions. The culture medium (FM) was that of Ferreira (6) as modified by Steele et al. (20). Triple-baffled shake flasks (125 ml) containing 50 ml of medium were inoculated with 1 ml of a spore suspension (5 × 10⁹ spores/ml) and incubated at 26 C on a rotary shaker at 250 rpm (2.54-cm circular orbit).

Measurement of L-glutamic acid uptake and initial velocity. Uniformly labeled L-[¹⁴C]glutamic acid with a specific activity of 200 mCi/mmol (Internation Chemical and Nuclear Corp., Irvine, Calif.) was diluted with unlabeled L-glutamic acid (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 0.064 M. Each flask contained 0.01 μCi/ml. The uptake of the amino acid was measured under the following standard conditions. After incubation, flasks were removed from the shaker and their contents were filtered through membrane filters (0.45-μm pore size). Portions (1.0 ml) of the filtrate were used for determining mycotoxin and sucrose concentration and for measuring ¹⁴C activity. The mycelium was washed with three 5-ml portions of a salt solution, pH 6.2, at 4 C. This wash solution (FM salts) was the same as the nutrient solution except glutamic acid and sucrose were omitted. Washing removed no significant radioactivity from the mycelium. The washed mycelium was dried in a 70 C oven for 12 h and weighed. The dried mycelium was ground into a fine powder with a mortar and pestle. A weighed amount was placed in a scintillation vial, 2 ml of 80% ethyl alcohol was added, and the vial was placed in an 80 C water bath for 30 min. After cooling, 15 ml of Bray scintillation fluid (4) was added, and the solution was counted as described below. This procedure provided a measure of total incorporation of labeled glutamic acid. To determine the incorporation of specific carbons into ochratoxin A, cells were incubated for 3 days in FM and one of the following specifically labeled glutamic acid (0.01 μCi/ml) was added: DL-[5-¹⁴C], 11.4 mCi/mmol; DL-[3, 4-¹⁴C], 14 mCi/mmol; and DL-[1-¹⁴C], 3.6 mCi/mmol. These specifically labeled compounds were purchased from Schwarz/Mann, Orangeburg, N.Y.

The method used to determine the initial uptake velocity of glutamic acid as a function of mycelial age was modified from that of Benko et al. (3). Cells were cultured for designated times in FM with either glutamic acid or 7.43 g of KNO₃ per liter (an equimolar concentration of nitrogen). When KNO₃ was used as a nitrogen source, it allowed growth but did not induce toxin formation. After a given time, the mycelium was washed by filtration as described earlier and pressed between dry filter paper. Usually, 300 mg of wet, pressed mycelium was resuspended in 25 ml of FM in 125-ml culture flasks, labeled glutamic acid was rapidly added (0.01 μCi/ml), and the flask was incubated as before for times up to 1 h. Three-milliliter samples were removed from the reaction mixture at each interval (10, 20, 40, and 60 min), rapidly filtered onto a membrane filter, and washed with 10 ml of cold (4 C) FM salts. The mycelium was then dried in a 70 C oven for 12 h and finely powdered, and total uptake was determined as described earlier.

Initial uptake velocities were expressed as nanomoles of glutamic acid per milligram (dry weight) of mycelium per hour.

Collection and measurement of ¹⁴CO₂. The production of ¹⁴CO₂ from L-[U-¹⁴C]glutamic acid was measured in a fermentation gas train. Each fermentation vessel (1,000 ml) contained 250 ml of the culture medium and 0.01 μCi of [U-¹⁴C]glutamic acid per ml. The fermentor received air that passed serially through concentrated H₂SO₄, distilled water, 20% KOH, Ascarite, and distilled water. The air entered the fermentor through a glass tube located below the surface of the medium. Air was delivered under a pressure of 2 lb/in². The fermentor was incubated at 26 C on a rotary shaker (250 rpm, 2.54-cm circular orbit). The atmosphere above the suspension was driven under pressure through a tube of Drierite to a series of three receiving tubes. Each tube contained 50 ml of a solution of ethanolamine in ethylene glycol monomethyl ether (11) to trap respired CO₂. Each tube was renewed after 24 h. During the course of these experiments we found that the first tube was adequate to trap all the C¹⁴O₂ for 24 h, thus in subsequent experiments the contents of the remaining two tubes were not collected for analysis. Two-milliliter samples of the trapping solution from tube 1 were placed into 15 ml of a scintillation fluid (11) and counted.

Fractionation of mycelium. The incorporation of ¹⁴C activity into the various classes of substances was measured by a modification of the procedure of Bacon et al. (1). Briefly, a weighed amount of washed mycelium (usually 10 to 20 mg) described above was extracted serially with 5 ml of an 80% ethyl alcohol-2% formic acid solution for 30 min, and chloroform-methyl alcohol (1:1) for 15 min. These two fractions were combined and evaporated to dryness under a stream of nitrogen. This fraction was extracted three times with 5 ml of diethyl ether, the extracts were combined, and the volume was reduced to 1 ml (ether-soluble fraction). The residue after ether extraction was taken up with 80% ethyl alcohol (alcohol-soluble fraction). The initial mycelial residue was then extracted with 5% cold (4 C) trichloroacetic acid for 15 min (cold trichloroacetic acid fraction). The remaining mycelium was then fractionated into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (1). Protein was determined by hydrolyzing the residue remaining after DNA extraction with 5 ml of 6 N HCl in an autoclave at 121 C for 8 h. The HCl was removed by evaporation of the fraction under vacuum at 30 C. The residue was dissolved in 5.0 ml of distilled water.

A one-milliliter portion of each fraction was tested for radioactivity. Water-immiscible fractions were counted in a scintillation fluid of the following composition: 2,5-diphenyloxazole, 4 g; 1,4-bis-[2]-(5-phenyloxazolyl)benzene, 100 mg; and toluene, 1 liter. Water-miscible fractions were counted in the scintillation fluid of Bray (4).

Biochemical analysis of fractions. Fractions were reduced to 2 ml under a stream of nitrogen at 40 C before analysis. Amino acids were analyzed according

to a two-dimensional procedure (10) on Eastman chromagram cellulose sheets without a fluorescent indicator. Amino acids were identified by co-chromatography and visualized with a ninhydrin-collidine spray reagent to determine locations. The reagent consisted of 0.1 g of ninhydrin, 100 ml of 95% ethyl alcohol, 4 ml of collidine, and 30 ml of acetic acid. Sprayed sheets were heated at 65 C for 15 min in a convection oven and the spots were marked with a pencil. Each identified area was scraped from the thin-layer plate, the cellulose was placed in a vial and extracted with 15 ml of Bray scintillation fluid by shaking on a wrist action shaker for 30 min, and the solution was counted.

Both ochratoxin A and penicillic acid were analyzed from culture filtrates as described previously (2). Ochratoxin A was purified by preparative thin-layer chromatography and hydrolyzed to separate the phenylalanine and iscoumarin moieties according to Van der Merwe et al. (23).

Measurement of radioactivity. Radioactivity of the samples was measured in a liquid scintillation system (Nuclear-Chicago Corp., Des Plaines, Ill., model Mark II). All counts were corrected for both background and counting efficiency due to quenching by using the appropriate external standards. Counting efficiencies of 60 to 84% were obtained with these procedures.

RESULTS

Time course of glutamic acid uptake. The data in Fig. 1 show the course of changes in total incorporation, dry weight, ochratoxin A yield, and respired $^{14}CO_2$ in the fermentation vessel. Glutamic acid was rapidly taken up by cells and, of the total amount taken up, 57% accumulated during the first day of incubation. Within 5 days, 5% of the labeled carbon was evolved as $^{14}CO_2$.

Initial uptake velocities. The initial uptake velocity of glutamic acid decreased with age of culture (Fig. 2). The decrease in specific activ-
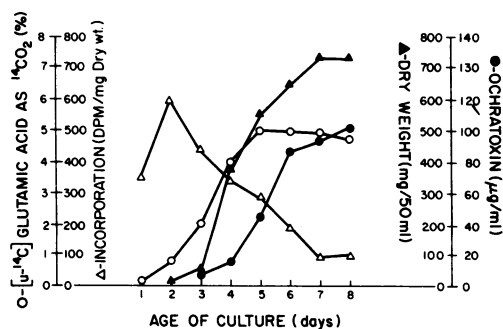
FIG. 1. *Incorporations and $^{14}CO_2$ evolution from L-[U-^{14}C]glutamic acid (0.01 µCi/ml), growth, and ochratoxin A synthesis in A. ochraceus 107 in a fermentation gas train.*
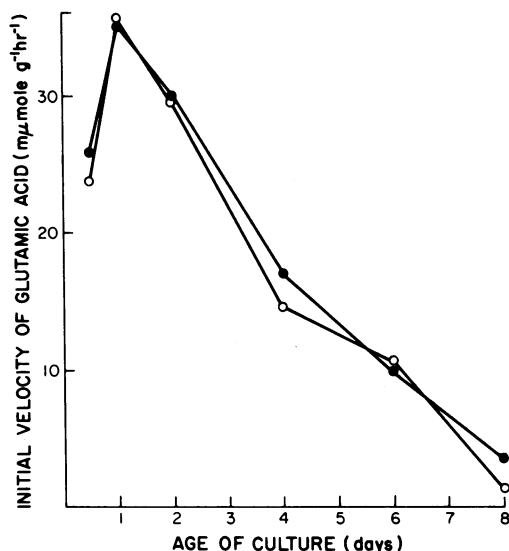
FIG. 2. *Initial uptake velocity by cells cultured in the presence or absence of glutamic acid. Symbols: O, uptake by cells preincubated in medium with glutamic acid; ●, uptake by cells preincubated in medium with KNO_3.*

ity was greatest between 2- and 4-day-old cells. The uptake velocities did not differ markedly in cells preincubated in the presence or absence of glutamic acid.

These data also show that 12-h germinating conidia had high uptake velocities. Such spores had reached the swollen stage of spore development. In the FM medium spore germination (emergence of germ tubes) was 75 to 85% synchronized, which is sufficiently high to suggest that these data represent uptake by germinating spores. Since these cells were suspended in a nitrogen-sufficient medium, another experiment was performed under nitrogen starvation. Spores were incubated in FM medium without a nitrogen source and, since nitrogen is essential for germination, no outgrowth was achieved. However, such spores showed no significant deviation in initial uptake velocities from spores incubated for comparable periods in FM medium with nitrogen.

Utilization of glutamic acid. The appearance of label in all fractions of mycelium showed that glutamic acid was assimilated (Table 1) and suggests that the effect on ochratoxin A biosynthesis is indirect as suggested by Ferreira (7). The percentage of incorporation into RNA decreased with age, whereas the ethanol-soluble fraction increased with age. A high percentage of glutamic acid taken up in 1- and 2-day-old cells was incorporated into

TABLE 1. *Distribution of ^{14}C from L-[U-^{14}C]glutamic acid in cellular fractions*

Age of culture (days)	Fraction (% radioactivity)					
	Ethanol	Pro-tein	RNA	DNA	Lipid	5% Cold trichloroacetic acid
1	20	10	62	3	2	3
2	28	14	47	3	4	3
3	41	16	34	7	5	3
4	43	22	20	8	3	7
5	49	16	18	10	4	8
6	52	20	20	9	4	2
7	58	12	17	6	6	0
8	59	12	19	3	4	1

RNA whereas a smaller percentage was incorporated into protein.

That glutamic acid served as a precursor for both ochratoxin A and penicillic acid is shown in Table 2. The organism was cultured in the presence of the labeled compound (0.1 μCi/ml) for 6 days. There was 0.519% incorporation into ochratoxin A and 1.685% into penicillic acid. Acid hydrolysis of ochratoxin A revealed that of total activity incorporated, 41.9% and 53.4% were in the phenylalanine and isocoumarin moieties, respectively. Higher amounts (25%) of the amino acid were incorporated into ochratoxin A when it was added to 3-day-old cells that had been incubated with nonlabeled glutamic acid (Table 3). Using this technique, specifically labeled glutamic acid revealed that carbons 3 and 4 were significantly used for the synthesis of the mycotoxin (Table 3). Thin-layer chromatography showed that glutamic acid was also utilized for the synthesis of other amino acids. Lysine (183 nCi/mM), isoleucine (80 nCi/mM), proline (80 nCi/nM), and γ-aminobutyric acid (160 nCi/mM) were identified as the radioactive amino acids in the ethanol-soluble fraction during the 8-day incubation. The percentage of radioactivity in this fraction increased markedly after the third day, the period when ochratoxin A began to accumulate (Fig. 1).

The system responsible for the uptake of glutamic acid was temperature and pH dependent (Fig. 3); the optima for uptake were pH 6 and 28 C.

DISCUSSION

Glutamic acid is utilized for the synthesis of both ochratoxin A and penicillic acid. Twenty-five percent of the labeled amino acid was incorporated into ochratoxin A when it was added to 3-day-old cells that had been incubated with nonlabeled glutamic acid initially. Significant amounts of the amino acid were used for the synthesis of RNA during the early stages of development and since RNA has a high turnover rate the label in this pool decreased. Glutamic acid is incorporated into protein, lipids, and amino acids. Thus, it is not only used for mycotoxin biosynthesis. Glutamic acid serves as a source for Krebs cycle acids, amino acids, and RNA precursors (16). Any regulatory role assigned to this amino acid in mycotoxin synthesis should consider these many catabolic fates.

The early evolution of $^{14}CO_2$ from glutamic acid suggested that it is metabolized via a decarboxylation reaction. Glutamate decarboxylase has been reported in numerous bacterial cells, plants, and fungi (9). The finding of γ-aminobutyric acid in the ethanol-soluble fraction is suggestive of this reaction. Direct evidence for the participation of these pathways was not obtained in this study. It was established, however, that in addition to other cellular fractions the amino acid serves as a precursor for both moieties of ochratoxin A, since the isocoumarin skeleton is derived via the acetate-malonate pathway and the phenylalanine moiety via the shikimic acid pathway (19, 15). Since all fractions were labeled, the effect of glutamic acid on ochratoxin A biosynthesis would appear to be indirect as indicated by Ferreira (7). These data indicate that carbons 3 and 4 are mainly used in the synthesis of ochratoxin A. Presumably it is these fragments

TABLE 2. *Radioactivity in ochratoxin A and penicillic acid from L-[U-^{14}C]glutamic acid*

Mycotoxin	Radioactivity (dpm)	Sp act (nCi/mmol)
Ochratoxin A	58,240	47.7
Isocoumarin	31,200	24.7
Phenylalanine	24,400	17.6
Penicillic acid	173,680	164.6

TABLE 3. *Incorporation of [^{14}C]glutamic acid into ochratoxin A*

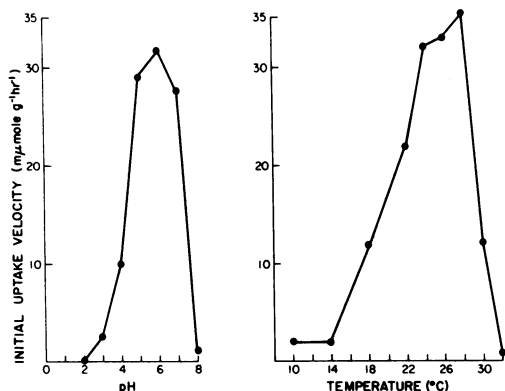| ^{14}C-labeled glutamic acid	Ochratoxin A (nCi/mmol)	Radioactivity (%)
L-[U-^{14}C]	90.3	25.02
DL-[1-^{14}C]	0.0	0.00
DL-[3,4-^{14}C]	82.7	20.70
DL-[5-^{14}C]	16.1	0.24

FIG. 3. *Influence of pH and temperature on uptake of [U-¹⁴C]glutamic acid by A. ochraceus.*

of glutamic acid which exert a direct effect on the synthesis of ochratoxin A.

Glutamic acid was taken up by germinating spores. At the time glutamic acid was disappearing from the medium, the spores were entering the swelling stage. Perhaps during this stage the amino acid is first utilized for RNA synthesis. These spores were not fractionated to determine the distribution of label. The initial rates of uptake of glutamic acid decreased with age. Such decreases in amino acid uptake with age have been reported from other fungi (8, 12, 13). We found no relationship between initial uptake velocities and ochratoxin A synthesis. While 6- and 8-day-old cells had low uptake velocities, they produced maximum levels of ochratoxin A. Robertson et al. (17) concluded that the initial velocity of tryptophan uptake was not directly related to alkaloid production by *Claviceps*. Earlier studies indicated that the decrease in uptake with age was due to either the decreasing activity in the protein-synthesizing system or to decrease in permeability of cells (8).

The uptake of glutamic acid is pH and temperature dependent, two criteria which offer support for an active transport system (17). Bacon et al. (2) reported that, with *A. ochraceus* 107, high temperatures (above 22 C) and moisture favored the production of ochratoxin A over that of penicillic acid on poultry feed. Because the uptake was similar with cells preincubated with either glutamic acid or inorganic nitrogen, the uptake system appears to be constitutive as has been reported for other fungal systems (12).

Although there may be no relationship between initial uptake velocities, there is one between the initial uptake velocity and the induction of ochratoxin A biosynthesis by glutamic acid. That is, the amino acid must be

added to young cells with high initial uptake velocities to achieve maximum synthesis.

An examination of the level of glutamic acid in the ethanol-soluble fraction revealed that the total activity in the ethanol-soluble fraction increases with time (Table 1). This suggests that during this time a pool of ochratoxin A precursors and structurally related compounds were accumulating in the cells and that the synthesis of ochratoxin A followed the increase. This pool did not decrease as the level of the mycotoxin increased; however, the amount of toxin produced is actually quite small and may not have an effect on the size of the pool. The identity of the major components of this fraction should indicate how the catabolism of glutamic acid indirectly effects ochratoxin A biosynthesis. We are investigating that relationship.

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