Nitrification of Aspartate by *Aspergillus flavus*

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Heterotrophic conversion of L-aspartic acid to nitrification products by *Aspergillus flavus* was studied in a replacement incubation system. Numerous amino acids supported nitrification; aspartate and glutamate were about equivalent as the best sources of nitrate. Addition of sodium bicarbonate to the incubation system substantially enhanced nitrate formation for all nitrifiable amino acids except aspartic acid, but the basis for the bicarbonate effect is obscure. The yield of nitrate from L-aspartate was not approached by forms of aspartic acid resulting from substitution on the beta carbon, the amino nitrogen, or the gamma carboxyl group or by aspartate presented as the D-configuration. There was no relationship between nitrate formation and the occurrence of such possible intermediates as nitrite, bound hydroxylamine, ammonia, aspergic acid, and beta-nitropropionic acid. Uniformly labeled 14C-L-aspartate that was nitrified in replacement incubation led to no accumulation of label in possible nitrification products in the culture filtrate. Label was found in components of the mycelium after acid hydrolysis, with heaviest accumulation in what appeared to be glucosamine and an unidentified compound, possibly acetylglucosamine. Detectable label was redistributed into serine, glycine, and threonine.

Formation of nitrate in the fungus *Aspergillus flavus* is now well established. This product, usually associated with the growth of autotrophic nitrifying bacteria, was first observed as a product in the culture filtrate of *A. flavus* grown heterotrophically on peptone medium (12). Of the various amino acids subsequently shown to support nitrate formation by *A. flavus* in chemically defined culture media, aspartic acid is of particular interest. This amino acid and asparagine were among the best substrates for nitrification when supplied as the sole source of carbon and nitrogen to growing or replacement cultures (6; G. E. Becker and E. L. Schmidt, Bacteriol. Proc., p. 9, 1964; K. G. Doxtader and M. Alexander, Bacteriol. Proc., p. 9, 1964). Marshall (10) emphasized the possibility of \( \beta \)-alanine as an intermediate in fungal nitrification and considered aspartic acid to be its precursor. Studies with *Penicillium atrovenetrum* (3, 13, 14) point to aspartic acid as the most attractive precursor of \( \beta \)-nitropropionic acid (BNP) since the BNP carbon skeleton is derived intact from aspartate. Such data are pertinent as BNP was reported in culture filtrates of *A. flavus* as early as 1951 (4) and may be an intermediate in nitrate formation by this fungus (2, 7).

A number of studies have been designed to explore the nitrification of given nitrogen sources by *A. flavus*. In summarizing this work, Doxtader and Alexander (6) point out the conflicting nature of much of the data and state that it is still premature to propose generalizations on the pathway or pathways of fungal nitrification. The purpose of this report is to give further consideration to aspartic acid as a specific substrate for nitrate production by *A. flavus*. Replacement cultures in which the nitrogen carrier of interest was presented in sterile buffer to washed, actively nitrifying mycelia were used throughout, in preference to growing cultures and in lieu of active cell-free extracts that are not yet available.

**MATERIALS AND METHODS**

The soil isolate *A. flavus* F-16-1 used throughout was described previously (8). The culture was maintained in sterile soil and was recovered on Sabouraud maltose agar; spore suspensions obtained on slant transfers of the same medium were used to grow inoculum. Inocula and mycelia used for replacement were grown in the following media: solution A, grams per liter: peptone, 16.0; glucose, 8.0; KH2PO4, 2.0; adjusted to pH 7.0 with 5 N NaOH; solution B, grams per liter: MgSO4·7H2O, 50.0; MnSO4·H2O, 1.0; FeSO4·7H2O, 1.0. The two solutions were autoclaved for 20 min at 121 C; after cooling, 10 ml of solution B was added aseptically to 1 liter of solution.
A and distributed in 100-ml quantities into 250-ml Erlenmeyer flasks. Inocula for replacement cultures were prepared by introducing 2 ml of a dense spore suspension into the regular medium and incubating on a rotary shaker at 28 C for 2 days. A 5-ml amount of a thick suspension of small pellets (<2 mm) was used per flask to initiate replacement cultures. After 5 days of incubation in shaking flasks at 28 C, the medium was removed, and the small mycelial pellets were washed in the flask with several replacements of demineralized water until the washings were negative for nitrate and nitrite.

Replacement solutions added to the washed pellets were composed of 50 ml of the nitrification substrate at pH 6.5 and an usual concentration of 0.1 M, in a 1 M phosphate buffer (pH 6.5, 2 M in experiments with bicarbonate). Solution and pH adjustments of substrate substances were accomplished in minimal volumes with either 20% NaOH or 42.5% H3PO4. Heat-labile substances were sterilized by filtration; others were autoclaved. Mycelial pellets were shaken in the replacement solutions, usually for 5 days, and the filtered solution was collected for analysis. All replacements were carried out in duplicate.

In experiments with uniformly labeled ^14C\text{-L-}

aspartic acid, cold L-aspartate was added to obtain a 0.1 M concentration (1.0 µCi/ml), and both filtrate and mycelium were examined after the replacement period. The mycelium was freeze-dried, ground, and hydrolyzed by autoclaving 0.5 g in 5 ml of 6 M HCl for 18 hr at 121 C in a sealed glass tube. After the tube was opened, excess acid was removed by repeated evaporation under vacuum. Chromatograms were prepared by spotting 100 µl of culture filtrate or hydrolysate on Whatman 3 MM filter sheets [9 by 11 inch (ca. 23 by 28 cm)]. The chromatograms were run in two dimensions by using the ascending technique and the solvent systems described by Steward et al. (16). Amino acids were detected with 0.2% ninhydrin in 1-butanol. Radioautograms were prepared from Kodak Royal Blue X-ray film; incubation periods up to a month were used.

All replacement filtrates were examined for the presence of BNP. Filtrates were cooled in an ice bath, acidified with 42.5% H3PO4 and extracted several times with equal volumes of ethyl ether. The ether extracts were combined and evaporated; the residue was taken up in a small amount of chloroform and spotted in duplicate on Whatman no. 1 paper. Sheets were chromatographed with an ascending solvent system of phenol-water-formic acid (75:25:1). After the developed chromatogram was dried, the duplicates were separated and one was placed in an ammonia-saturated chamber for 0.5 hr. Both strips were placed in an oven at 90 C for 1 min and then sprayed with a-naphthylamine-sulfanilic acid solution. The presence of BNP was indicated by the appearance of a cherry red spot near the solvent front on the paper treated with ammonia and the absence of a reaction on the duplicate control.

Quantitative determinations of nitrate were carried out by the phenol-disulfonic acid method described by Jackson (9). Nitrate determinations were made by the Griess-Ilosvay test with sulfanilic acid and a-naphthylamine. Ammonium nitrogen was determined by nesslerization, and bound hydroxylamine was estimated as described by Novak and Wilson (11). Spot tests for nitrate were made by diphenylamine-sulfuric acid reagent. Aspergillus acid was shown by fractionation and co-chromatography to be the only ferric chloride-reactive substance present in the replacement culture filtrates in significant amounts. Subsequent estimations were made colorimetrically (Klett Colorimeter, blue filter) with acidified ferric chloride reagent (2.0 g of ferric chloride dissolved in 10 ml of concentrated HCl and diluted to 140 ml with water). Authentic aspergillus acid was used as a standard.

RESULTS

Preliminary screening of 16 individual amino acids added in replacement solution to actively nitrifying, peptone-grown A. flavus mycelia confirmed previous experience that L-aspartate and L-glutamate were the best sources of nitrate nitrogen, approached only by β-alanine. Although nitrate yields were good, conversion of aspartate was nevertheless a slow process in replacement culture. A 5- to 6-day period was required for maximum production (Fig. 1). Since time response to L-glutamate was very similar to that of aspartate, the routine 5-day incubation period for the replacement systems was adopted. Comparisons of nitrate yields from aspartate and glutamate at various concentrations are shown in Table 1. Both amino acids followed much the same pattern, with

![Graph](image-url)

**FIG. 1.** Production of nitrate by *A. flavus* in a 6-day period in replacement culture with L-aspartate as substrate (75 µmoles/ml).
nearly equivalent amounts of nitrate being produced at each amino acid concentration. Throughout this work, the replacement mycelium consisted of small (<2 mm) pellets since these were observed to be more active in nitrification than larger pellets. Although well washed, these pellets always demonstrated substantial endogenous nitrification as indicated in Table 1 for the zero amino acid levels.

In the course of preliminary experiments with certain nitrogen sources, it was noted that bicarbonate had a stimulatory effect on nitrate production. Because of this, the individual amino acids were studied in replacement culture with and without the addition of sodium bicarbonate. A summary of the results for nitrate and possibly related nitrification products is given in Table 2. Apart from the sulfur amino acids, cysteine and methionine and the aromatic amino acids L-histidine, DL-phenylalanine, and

| Amino acid | Nitrate | Nitrite | Bound hydroxylamine | Ammonia | Aspergilliacid | pH |
|------------|---------|---------|---------------------|---------|---------------|----|
| L-Alanine  | 6.8     | 0.03    | 0.15                | 35.0    | 1.4           | 7.4|
| + HCO₃⁻    | 10.0    | 0.05    | 0.05                | 35.5    | 1.2           | 7.9|
| β-Alanine  | 10.8    | 0.04    | 0.08                | 38.5    | 0.8           | 7.3|
| + HCO₃⁻    | 16.5    | 0.13    | 0.03                | 39.5    | 1.0           | 7.8|
| L-Arginine | 6.5     | 0.02    | 0.09                | 50.0    | 1.5           | 8.2|
| + HCO₃⁻    | 12.5    | 0.03    | 0.05                | 52.0    | 1.7           | 7.8|
| L-Aspartate | 14.5   | 0.19   | 0.04                | 35.0    | 0.7           | 7.9|
| + HCO₃⁻    | 11.3    | 0.12    | 0.03                | 24.0    | 0.1           | 8.5|
| L-Cystine  | <1.0    | 0.01    | 0.01                | 3.0     | 0.9           | 6.6|
| + HCO₃⁻    | <1.0    | 0.01    | 0.03                | 6.0     | 1.6           | 7.4|
| L-Glutamate| 15.0    | 0.10    | 0.04                | 22.0    | 1.2           | 7.5|
| + HCO₃⁻    | 18.0    | 0.15    | 0.04                | 38.0    | 1.1           | 8.0|
| L-Glycine  | 8.5     | 0.04    | 0.09                | 42.0    | 0.6           | 7.3|
| + HCO₃⁻    | 9.8     | 0.08    | 0.07                | 38.0    | 0.9           | 8.4|
| L-Leucine  | 4.3     | 0.01    | 0.03                | 22.0    | 2.5           | 7.1|
| + HCO₃⁻    | 8.0     | 0.03    | 0.04                | 36.5    | 1.8           | 7.4|
| L-Lysine   | 5.8     | 0.03    | 0.07                | 20.5    | 0.2           | 7.0|
| + HCO₃⁻    | 9.0     | 0.10    | 0.04                | 35.5    | 1.0           | 7.5|
| L-Methionine| <1.0   | 0.15    | 0.01                | 7.6     | <0.1          | 7.0|
| + HCO₃⁻    | <1.0    | 0.31    | 0.01                | 7.3     | <0.1          | 8.0|
| DL-Proline | 3.1     | 0.02    | 0.15                | 8.1     | 2.9           | 7.0|
| + HCO₃⁻    | 6.5     | 0.02    | 0.07                | 8.2     | 1.5           | 7.6|
| DL-Serine  | 2.9     | 0.01    | 0.03                | 10.0    | 1.5           | 7.5|
| + HCO₃⁻    | 9.0     | 0.07    | 0.03                | 9.0     | 1.1           | 8.0|
| DL-Valine  | 2.8     | 0.01    | 0.20                | 8.4     | 3.5           | 7.2|
| + HCO₃⁻    | 11.0    | 0.03    | 0.07                | 9.0     | 1.8           | 7.3|
| Endogenous control | 4.3 | 0.01 | 0.14 | <1.0 | 0.3 | 6.8 |
| + HCO₃⁻    | 6.5     | 0.06    | 0.06                | <1.0    | 0.1           | 7.7|

* Five days of replacement incubation in buffer with washed A. flavus mycelium. Sodium carbonate and amino acid concentrations: 100 μmoles/ml.
DL-tryptophan (not reported in Table 2), the remaining amino acids supported some nitrification. Again, aspartate and glutamate were clearly the best sources for nitrate nitrogen. Addition of bicarbonate increased the nitrate derived from all nitrifiable amino acids except L-aspartate and was without effect on those that failed to support nitrate formation. No correlation was observed between nitrate and the formation of ammonia, bound hydroxylamine, or aspersergic acid. The decrease in nitrate nitrogen that occurred with L-aspartate supplemented with sodium bicarbonate was probably due to the excessively high pH that developed. In all subsequent trials at lower levels of sodium bicarbonate and more favorable pH circumstances, the bicarbonate had essentially no effect on the nitrate formed from aspartic acid.

The effect of bicarbonate concentration on the conversion of 100 μmoles of L-glutamate per ml was studied (Table 3). Increasing concentrations of sodium bicarbonate were reflected in increased nitrate yields up to the maximum of 80 to 100 μmoles per ml, but none of the other forms of nitrogen measured could be correlated with the nitrate changes.

The general equivalence of aspartate and glutamate as sources for nitrate and the indications that glutamate conversion to nitrate was enhanced by bicarbonate although aspartate was not suggested that glutamate may have been converted to aspartate. Such a conversion could take place if aspartate was resynthesized from glutamate and bicarbonate through a glyoxylate pathway. Certain intermediates of the tricarboxylic acid cycle were tested in replacement culture with ammonium phosphate in the presence and absence of sodium bicarbonate. The data in Table 4 show general response to bicarbonate for all acids except oxalacetic and are not suggestive of a glyoxylate cycle, as might be indicated if α-ketoglutaric acid were selectively stimulated by bicarbonate.

A further test of the specificity of aspartate was carried out with various forms of aspartic acid supplied in replacement. None of the substrates yielded as much nitrate as the L-aspartate (Table 5). The nitrification that was observed for L-asparagine and carbamyl-DL-aspartate was probably due to hydrolysis to L-aspartate. Substitution on the beta carbon, the amino nitrogen, in the gamma carboxyl group or use of the D form of aspartate resulted in poor nitrification relative to L-aspartate.

Further indication that aspartate may be a specific substrate for nitrification by A. flavus was sought in intermediates that might appear in the replacement filtrates. Nitrite, bound hydroxylamine, and aspersergic acid were not correlated with nitrate. Repeated attempts made to detect BNP in actively nitrifying aspartate replacement systems were negative. If the replacement medium consisted of a buffered 5% glucose solution rather than aspartate, BNP could be found readily in ether extracts of acidified culture filtrates.

An observation that DL-methionine interfered somewhat with the utilization of aspartate suggested that some intermediate of interest might accumulate in a methionine-aspartate replacement system. At a high concentration of

| Table 3. Effect of sodium bicarbonate on nitrification by A. flavus* |
|-----------------------------|-----------------|---------------------|---------------------|---------------------|-----------------|
| Sodium bicarbonate | Nitrification product (μmoles/ml) | | | | |
| | Nitrat | Nitrite | Bound hydroxylamine | Aspersergic acid | pH | |
| 0 | 6.8 | 0.04 | 0.17 | 2.7 | 7.8 | |
| 20 | 7.0 | 0.05 | 0.08 | 2.8 | 7.9 | |
| 40 | 9.0 | 0.06 | 0.09 | 2.2 | 7.8 | |
| 60 | 10.5 | 0.07 | 0.06 | 1.8 | 7.8 | |
| 80 | 11.3 | 0.07 | 0.05 | 1.8 | 7.9 | |
| 100 | 11.5 | 0.08 | 0.04 | 1.7 | 8.0 | |
| 150 | 8.8 | 0.07 | 0.04 | 1.0 | 8.5 | |
| 200 | 9.0 | 0.06 | 0.04 | 0.3 | 9.1 | |

* Five days of replacement incubation with 100 μmoles of L-glutamic acid per ml.

| Table 4. Effect of sodium bicarbonate on nitrate formation by A. flavus after 5 days replacement incubation with tricarboxylic acid cycle intermediates and ammonium nitrogen |
|-----------------------------|-----------------|---------------------|---------------------|---------------------|
| Substratea in ammonia-buffer | Nitrat formed (μmoles/ml) | Nitrite formed (μmoles/ml) | Final pH | |
| α-Ketoglutarate | 2.5 | 0.01 | 7.7 | |
| + HCO₃⁻ | 2.8 | 0.02 | 7.8 | |
| Isocitrate | 2.4 | 0.01 | 7.4 | |
| + HCO₃⁻ | 4.0 | 0.02 | 7.8 | |
| Oxalacetate | 5.5 | 0.03 | 7.7 | |
| + HCO₃⁻ | 3.8 | 0.01 | 8.2 | |
| Fumarate | 3.1 | 0.02 | 7.5 | |
| + HCO₃⁻ | 4.1 | 0.03 | 7.8 | |
| Succinate | 3.6 | 0.02 | 7.7 | |
| + HCO₃⁻ | 3.9 | 0.02 | 7.8 | |
| Ammonia (control) | 1.6 | 0.01 | 6.8 | |
| + HCO₃⁻ | 2.4 | 0.01 | 7.4 | |
| L-Glutamate | 8.5 | 0.04 | 7.8 | |
| L-Aspartate | 6.5 | 0.03 | 7.7 | |

a Carbon compounds and ammonium phosphate, 80 μmoles/ml; sodium bicarbonate, 100 μmoles/ml. Phosphate buffer, 1% (pH 6.5); bicarbonate, 2%.
methionine (50 μM/ml), the concentration of aspartate was reduced from an initial level of 100 μM/ml to 35 μM/ml during a 5-day replacement period. The final concentration of aspartate was 18 μM/ml in the absence of methionine. When methionine was added to 100 μM of aspartate per ml, including 1 μCi of uniformly labeled 14C-L-aspartate per ml, no BNP or unusual amounts of bound hydroxylamine or aspergillic acid accumulated in the culture filtrate. However, the acid hydrolysate of the mycelium gave evidence of selective incorporation of the label (Fig. 2). Comparison with a chromatogram of known compounds indicated that the majority of the activity was located in the glucosamine position and in an unknown position. The unknown corresponded rather closely to the position of known acetylglucosamine but did not give a consistent color reaction with acetyl acetone p-dimethylaminobenzaldehyde. Substantial activity was also found in serine, glycine, and threonine. The Lₘ-methionine had little bearing on the relative distribution of radioactivity in mycelial hydrolysates for similar patterns from labeled asparate were observed with or without methionine.

**DISCUSSION**

Nitrate formation by washed mycelium of *A. flavus* in replacement, as in growing culture, is a slow process that allows ample time for interconversions of nitrifiable substrates so that most nonaromatic and non-sulfur-containing amino acids support some nitrification in replacement systems. L-Aspartic and glutamic acids were clearly superior sources of nitrate. Any specificity with respect to these two amino acids must be tempered by the recognition that their metabolic versatility permits many possibilities for the transfer of nitrogen to the organic intermediates that eventually yield nitrate.

That very specific organic nitrogen intermediates are involved and not the inorganic sequence of NH₃ → NO₂⁻ → NO₃⁻ as reported by Aleem et al. (1) is evident. The evidence against the inorganic sequence as summarized by Doxtader and Alexander (7) is strong and in full agreement with this study and another (Van Gool and Schmidt, unpublished data), in which attempts to reproduce the cell-free nitrifying activity reported by Aleem et al. were unsuccessful. The general situation in the present experiments was that all nitrifiable amino acids were deaminated substantially during replacement, but there was no relationship between ammonia and nitrate. Both asparagine and β-methylaspartate (Table 5), for example, were deaminated readily but the nitrate yield from this ammonia was very low relative to aspartate even in the presence of closely analogous carbon structures.

Not only was there no relationship between ammonia and nitrate, there was no relationship between nitrate and nitrite, bound hydroxylamine, or aspergillic acid. BNP was never detected in nitrifying replacement cultures although it may have been present at low transitory levels as an immediate precursor to nitrate and may have accumulated if a suitable inhibitor had been found. The most consistent effect on nitrate was evidenced by the addition of sodium bicarbonate to the replacement substrate. It is

### Table 5. Effects of certain structural changes and configuration on use of aspartic acid for nitrification by *A. flavus* after 5 days of replacement incubation

| Nitrogen source | Nitrification product (μmoles/ml) | pH |
|-----------------|----------------------------------|----|
|                 | Nitrate  | Nitrite | Ammonia |
| L-Asparagine    | 3.5      | 0.03    | 19.0    | 8.1 |
| DL-β-Methylaspartate | 0.6    | 0.02    | 22.5    | 8.2 |
| Carbamyl-DL-aspartate | 1.4  | 0.02    | 12.0    | 7.4 |
| D-Aspartate     | 0.9      | 0.02    | 12.0    | 7.7 |
| L-Aspartate     | 6.5      | 0.05    | 15.0    | 7.8 |
| Endogenous      | 0.9      | <0.01   | 10.5    | 6.6 |

*a* DL-Forms added at 100 μmoles/ml; others, at 50.

**Fig. 2. Two-dimensional autoradiogram of mycelium hydrolysate after replacement incubation of *A. flavus* with substrate of uniformly labeled 14C-L-aspartate diluted with L-aspartate carrier to final concentration of 1 μCi/ml and 0.1 M. Solvents: left to right, phenol; water; bottom to top, 1-butanol: propionic acid: water; x-origin, L-aspartate, 2-glucosamine, 3-serine, glycine, 4-threonine, 5-unknown.
interesting that nitrate (but not other nitrogen products) was stimulated by every nitrifiable amino acid with the exception of aspartic acid (Table 2). No explanation for this effect is available, but the work of Cantino (5) with the fungus *Blastocladiella emersonii* has made it clear that exogenous bicarbonate may induce morphogenic changes resulting from profound shifts in the synthesis of tricarboxylic acid cycle enzymes. One possibility for the conversion of glutamate to aspartate in the presence of high concentrations of bicarbonate was not borne out by nitrification data (Table 4). It is perhaps more likely that aspartate, glutamate plus bicarbonate, and possibly β-alanine plus bicarbonate lead more effectively than other amino acids to the synthesis of a common carrier of the nitrogen that ultimately undergoes oxidation. β-Nitroacrylic acid might be this carrier, leading to BNP from glutamate and aspartate as recently proposed for *P. atroveneretrum* (13). Although BNP has been shown to yield nitrate when added to *A. flavus* systems and to be a product under certain growth conditions, it has not been established that it is indeed formed by non-growing, nitrifying replacement cultures. The highly conjectural status of the mechanism of heterotrophic nitrification is likely to persist until means can be found to increase the rate of nitrate formation by whole cells or to obtain active cell-free preparations.

The aspartic acid fed to replacement cells was used much more selectively than may have been expected of such a versatile carbon source. Radioautograms of filtrates after exposure to uniformly labeled 14C-L-aspartate during 5 days of active replacement nitrification showed the presence of the label only in residual aspartic acid. Hydrolysates of the fungal mycelium, on the other hand, showed distribution of the label, with a considerable portion in glucosamine (Fig. 2). This situation could be accounted for in terms of deamination of aspartate to provide oxalacetate for the generation of glucose, since oxalacetate has been found to be an important starting material for glucosoneogenesis in mammalian tissue (15). No oxalacetate was detected, however, in our system. If it is involved in the formation of glucosamine and a nitrogen carrier, its turnover must be rapid and efficient.

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