The role of β-alanine in the biosynthesis of nitrate by *Aspergillus flavus*

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MARSHALL, K. C. 1965. The role of β-alanine in the biosynthesis of nitrate by *Aspergillus flavus*. Antonie van Leeuwenhoek **31**: 386–394.

D-Serine (0.05M) inhibited nitrification by *Aspergillus flavus* in media containing either peptone, aspartate, α-alanine or β-alanine as the sole nitrogen source. A similar inhibition was observed in an aspartate + peptone medium, but nitrate was formed in a β-alanine + peptone medium in the presence of the inhibitor. Exceptionally high yields of nitrate were obtained in the β-alanine + peptone medium. In replacement cultures, D-serine inhibited nitrification of aspartate but not of β-alanine. Manometric studies indicated that aspartate was decarboxylated by *A. flavus* and that the reaction was inhibited by D-serine. In view of these results, it is suggested that aspartate is a precursor and β -alanine is an intermediate in nitrification by this fungus.

INTRODUCTION

Nitrite and nitrate formation in a medium containing peptone was observed first with *Aspergillus flavus* (Schmidt, 1954) and later with *A. wentii* (Malavolta, de Camargo and Haag, 1955) and *A. oryzae* (Marshall and Alexander, 1961). More recent studies with *A. flavus* have shown that bound hydroxylamine, nitrite and nitrate are produced in media containing as the sole nitrogen source either peptone, certain individual amino acids or ammonium salts (Marshall and Alexander, 1962). The products of nitrification in all these media do not appear until the end of the active growth phase of the fungus. The term nitrification is used in the broad sense (Alexander, Marshall and Hirsch, 1961) to include the biological conversion of nitrogen in both organic and inorganic compounds from a reduced to a more oxidized state.

Marshall and Alexander (1962) have speculated on the involvement of β-alanine and β-nitropropionic acid in nitrification by *A. flavus* because of (1) higher nitrate yields in a medium containing β-alanine than in one containing α-alanine, and (2) the demonstration that cell-free extracts of the fungus catalyzed the production of nitrite from β-nitropropionic acid. Using a

replacement culture technique, Doxtader and Alexander (1964) obtained significantly higher yields of nitrate from aspartate, β-alanine and β-nitropropionate than from a variety of other nitrogen sources, including α-alanine. The above evidence suggests the following pathway in nitrification by *A. flavus*:

Aspartate → β-alanine → β-nitropropionate → nitrite → nitrate.

The isotopic labelling experiments of Birch et al. (1960) have indicated that aspartate, but not β-alanine, may be a precursor of the β-nitropropionate synthesized by a different fungus, *Penicillium atrovenetum*. Birkinshaw and Dryland (1964) have presented further evidence that the β-nitropropionate produced by this fungus is formed from aspartate or oxalacetate. They also demonstrated that fumaric acid could not be involved directly in the synthesis of β-nitropropionate, as had been proposed by Hylin and Matsumoto (1961). However, Birkinshaw and Dryland did not attempt to confirm the observation by Birch et al. (1960) that β-alanine is not a precursor of the β-nitropropionate produced by *P. atrovenetum*.

The present investigation was initiated to examine in more detail the possible role of β-alanine in nitrification by *A. flavus*. The observation that D-serine inhibited the synthesis of β-alanine from aspartate in a species of *Erwinia* (Grula and Grula, 1963) suggested that this inhibitor may be useful in the proposed investigation.

MATERIALS AND METHODS

Organism. The culture of *Aspergillus flavus* (F-16-1) used in these experiments was kindly provided by Dr. E. L. Schmidt, Department of Bacteriology, University of Minnesota, Minneapolis, U.S.A.

Media. In the initial experiment, a peptone-containing medium (Eylar and Schmidt, 1959) was used. In later investigations, the peptone was either replaced or supplemented by the addition of either α-alanine, β-alanine or neutralized aspartic acid to give a final concentration of the individual amino acid of 5×10^{-2}M. The pH was 7.0. All cultures were incubated in a static condition at 28 C. D-Serine solutions sterilized by filtration were added aseptically.

Replacement cultures. The technique employed was similar to that mentioned by Doxtader and Alexander (1964). Using actively nitrifying peptone-grown cultures, the culture medium was replaced with either aspartate, α-alanine or β-alanine in 0.02M phosphate buffer (pH 7.0). D-Serine was added to appropriate flasks. The final concentration of the amino acids, including D-serine, was 4×10^{-3}M.

Manometric technique. Mycelial mats from cultures of *A. flavus* grown on a peptone medium for 4 or 5 days were washed in 0.02M phosphate buffer (pH 5.7) and uniform portions (17 to 18 mg/flask) were suspended in the

same buffer in Warburg flasks. Neutralized aspartic acid (50 μmoles) and, in the appropriate flasks, D-serine (50 μmoles) were added from the side arm of the flasks after equilibration at 30 C. Endogenous activity of the mycelium was determined in flasks receiving no substrate. Respiration was measured by conventional manometric methods (Umbreit, Burris and Stauffer, 1957), CO_2 production being determined by the direct method and the results corrected for CO_2 absorption in the pH 5.7 buffer.

Chemical methods. Nitrite, nitrate and bound hydroxylamine were determined as described previously (Marshall and Alexander, 1962). Qualitative tests for the presence of β-alanine in culture filtrates were made using one-dimensional paper chromatography in a butanol – acetic acid – water (4 : 1 : 5) solvent.

RESULTS

When added to a peptone-containing medium, increasing concentrations of D-serine resulted in an increase in the degree of inhibition of nitrification by *A. flavus* (Table 1). In fact, a linear relationship was observed between the

TABLE 1

Inhibition by D-serine of nitrification by *Aspergillus flavus* in a peptone-containing medium.

D-Serine concentration	Products of nitrification[1]		
	Bound NH_2OH μgN/ml	Nitrite μgN/ml	Nitrate μgN/ml
After 4 days incubation			
0	0.81	1.00	22.6
5×10^{-4}M	0.86	1.02	20.4
5×10^{-3}M	1.11	0.72	18.8
5×10^{-2}M	0.17	0.04	5.0
After 7 days incubation			
0	0.67	1.67	38.8
5×10^{-4}M	0.67	0.52	28.8
5×10^{-3}M	1.08	0.63	17.6
5×10^{-2}M	0.30	0.15	5.2

[1]) All values are the average of duplicate flasks.

percentage inhibition of nitrate production after 7 days incubation and the logarithm of the D-serine concentration (Fig. 1). A bright yellow colour in the culture medium containing D-serine was noted after 4 days incubation. This colour changed to a deep orange after 7 days incubation, at which time the culture filtrate was found to have an U.V. absorption peak in the region

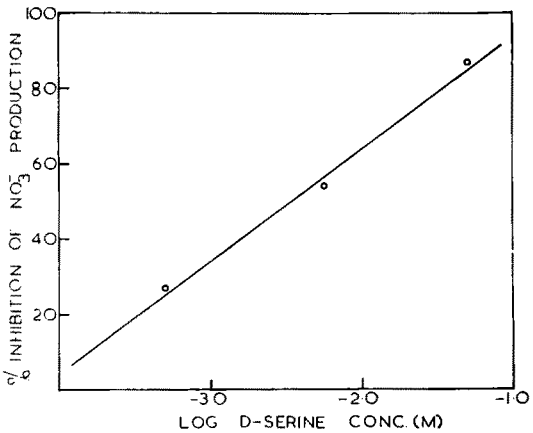

Fig. 1. Relationship between the concentration of D-serine in a peptone medium and the nitrate production by *Aspergillus flavus* after 7 days incubation at 28 C.

of 330 to 340 mμ. No absorption peak was found at this range in the control culture filtrates.

The effect of D-serine on nitrification was examined in media containing either aspartate, α-alanine or β-alanine as the sole nitrogen source. The results in Table 2 show that the formation of both nitrite and nitrate in these three media was inhibited completely by D-serine. Higher yields of nitrate were obtained in the medium containing α-alanine than in one containing β-alanine, a result that differs from earlier observations (Marshall and Alexander, 1962) where higher yields were obtained with β-alanine. When peptone was present in the medium in addition to either aspartate or β-alanine a strikingly different pattern of inhibition by D-serine was observed. Nitrification was inhibited in the aspartate + peptone medium, but was not inhibited in the β-alanine + peptone medium (Table 2). In addition, growth of *A. flavus* in the β-alanine + peptone medium was characterized by exceptionally high yields of nitrate, even in the presence of D-serine. The inclusion of aspartate and peptone in the same medium, by contrast, did not increase nitrate yields over those obtained when these nitrogen sources were employed separately. The yellow pigment mentioned earlier was observed in all flasks containing D-serine, including those in which nitrification occurred.

Qualitative paper chromatographic analyses of culture filtrates revealed that β-alanine had disappeared prior to the onset of nitrification in a β-alanine medium, while β-alanine was still present at this stage in a β-alanine + peptone medium (Table 3). This result suggests that the lack of inhibition of nitrification by D-serine in the β-alanine + peptone medium resulted from

TABLE 2

Inhibition by D-serine of nitrification by *Aspergillus flavus* in media containing amino acids in the presence or absence of peptone[1)]

Nitrogen source[2)]	Final pH	Nitrite μgN/ml	Nitrate μgN/ml	Percentage inhibition[3)]
Aspartate		2.45	35.6	
Aspartate + D-serine		0	0	100
α-Alanine		2.16	46.5	
α-Alanine + D-serine		0	0	100
β-Alanine		1.44	34.1	
β-Alanine + D-serine		0	0	100
Aspartate + peptone	7.3	1.96	38.3	
Aspartate + peptone + D-serine	8.2	0.05	5.9	84.6
β-Alanine + peptone	7.3	1.51	155.0	
β-Alanine + peptone + D-serine	8.2	0.23	165.0	-6.5

[1)] Cultures incubated for 6 days. All values are the average of duplicate flasks.
[2)] The concentration of all amino acids, including D-serine, was 0.05M.
[3)] Based on the nitrate-N in the control treatment in each instance.

TABLE 3

The relationship between the onset of nitrification and the disappearance of β-alanine in two different media[1)]

Incubation time (days)	Mycelium wt. mg/flask	Nitrite μgN/ml	Nitrate μgN/ml	β-alanine
β-Alanine medium				
0	0	0	0	+++
3	25	0	0	++
4	43	0	0	-
5	49	0.20	9.5	-
6	50	0.23	16.3	-
7	46	0.31	19.8	-
β-Alanine + peptone medium				
0	0	0	0	+++
3	78	0	0	+++
4	75	0.31	8.5	+++
5	63	1.39	117.0	++
6	61	0.87	122.0	-
7	76	1.74	155.5	-

[1)] All values are the average of duplicate flasks.

the availability of β-alanine in this medium when the nitrification process commenced. Once again, very high yields of nitrate were obtained in the peptone + β-alanine medium.

Replacement of the peptone medium of actively nitrifying cultures with buffered solutions of aspartate or β-alanine resulted in the formation of small, but significant, amounts of nitrite (Table 4). Nitrate was not detected, but

TABLE 4

The appearance of nitrite in replacement cultures supplied with different amino acids[1)]

	Nitrite (μgN/ml) appearing after time (hours)		
Nitrogen source	5	22	46
Control	.09	.07	0.24
Aspartate	.09	.09	1.59
Aspartate + D-serine	.07	.05	0.03
β-Alanine	.06	.05	1.38
β-Alanine + D-serine	.07	.06	1.63

[1)] All values are the average of duplicate treatments.

this may be the result of the relatively lower sensitivity of the analytical procedure. The nitrifying activity of these cultures obviously is very low. The results in Table 4 clearly show, however, that D-serine blocks the formation of nitrite from aspartate in the replacement cultures while nitrite formation from β-alanine was not inhibited.

The inhibition by D-serine of nitrification when aspartate is supplied to the fungus, along with the lack of inhibition when β-alanine is supplied, suggests that an α-decarboxylation of aspartate to yield β-alanine may be an early step in the nitrification sequence. Manometric studies with the intact mycelium of actively nitrifying cultures suspended in buffer showed an appreciable endogenous respiration, the R.Q. being slightly less than unity (0.92). The addition of aspartate increased the yield of CO_2, while O_2 uptake remained unaltered. The results indicate a slow, but significant, decarboxylation of aspartate and reveal that this process is inhibited by D-serine (Fig. 2). Using mycelium of a similar age on another occasion, the addition of aspartate resulted in a slight increase in the O_2 uptake. However, there was still a significant decarboxylation of the aspartate (R.Q. = 2.81, the endogenous R.Q. being 0.85) and a marked inhibition of this decarboxylation by D-serine

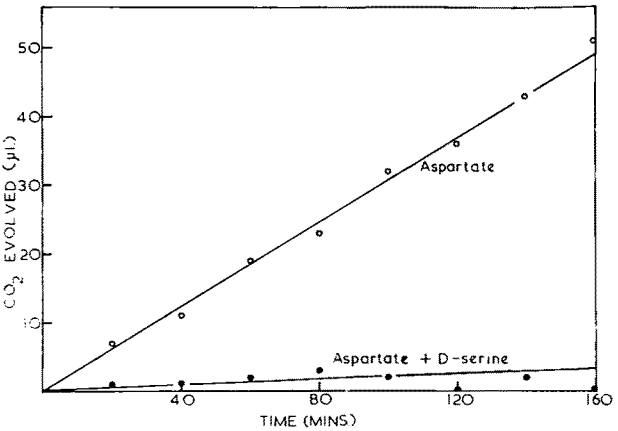

Fig. 2. The production of carbon dioxide from aspartate in the presence and absence of D-serine by the intact mycelium of *Aspergillus flavus*.

(R.Q. = 1.74). β-Alanine was not detected in the suspending medium at the conclusion of these experiments.

DISCUSSION

β-Alanine is not present initially in media containing either peptone, α-alanine, aspartate or ammonium as the source of nitrogen. Consequently if it is involved in the biosynthesis of nitrate by *A. flavus*, β-alanine must be produced from some precursor present in the fully grown culture. The most logical precursor is aspartic acid which is readily synthesized from the nitrogen source supplied. A decarboxylation of aspartate by the mycelium of *A. flavus* has been demonstrated in the present investigation. Because of the observed inhibition by D-serine, this was probably an α-decarboxylation of the aspartate to yield β-alanine. It has been shown with *Flavobacterium* sp. (Durham and Milligan, 1962) and with *Erwinia* sp. (Grula and Grula, 1963) that the α-decarboxylation of aspartate is inhibited by D-serine, while the β-decarboxylation to yield α-alanine apparently is not inhibited in *Flavobacterium* sp. (Kudlac et al., 1964; Durham, Jacobs and Ferguson, 1964). The failure to demonstrate the presence of β-alanine on completion of the respiration experiment or in replacement cultures supplied with aspartate is not surprising, as the β-alanine is probably metabolized rapidly and fails to accumulate.

The inhibition by D-serine of both aspartate decarboxylation and nitrification in a peptone-containing medium suggests that β-alanine is involved in nitrate biosynthesis. It was anticipated that D-serine would inhibit nitrification when either aspartate or α-alanine were supplied to the fungus as these

amino acids are present in peptone, but the inhibition of nitrate production in a β-alanine medium was surprising. It must be emphasised, however, that the products of nitrification do not appear until the cessation of fungal growth, regardless of the composition of the medium (Marshall and Alexander, 1962). When β-alanine was employed as the nitrogen source, it was all metabolized during the growth of the fungus and any resynthesis of β-alanine at the commencement of the nitrification phase was blocked by the D-serine. Where β-alanine + peptone were used in the medium, the peptone probably was metabolized selectively during the growth phase leaving sufficient β-alanine in the medium to allow nitrification to proceed even in the presence of the inhibitor. This difference in availability of β-alanine in these two media at the onset of nitrification was confirmed by chromatographic examination of the respective culture filtrates. The consistently higher yield of nitrate in the β-alanine + peptone medium is undoubtedly a reflection of the same mechanism, whereby a much higher effective concentration of β-alanine is made available for nitrification. The inhibition of nitrification by D-serine in an aspartate + peptone medium emphasises the significant role played by β-alanine in the nitrification system.

If β-nitropropionate is involved in nitrification by *A. flavus*, then it is likely that the pathway of β-nitropropionate synthesis in this fungus differs from that in *Penicillium atrovenetum*. The evidence presented by Birch et al. (1960) indicates that β-alanine is not concerned in the biosynthesis of β-nitropropionate by *P. atrovenetum*, while it appears that β-alanine may be involved in this process in *A. flavus*. Further investigations of both fungal systems are required, particularly at the cell-free enzyme level.

The author is indebted to Miss Sue Stanier for excellent technical assistance.

Received 26 *May* 1965

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