NITRATE REPRESSION OF AVERUFIN AND AFLATOXIN BIOSYNTHESIS

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ABSTRACT.—Biosynthesis of averufin, a nontoxic early precursor of aflatoxin, by a blocked mutant of *Aspergillus parasiticus*, was found to be regulated mainly by the nitrogen source used for growth. Nitrate exerted a negative effect, whereas ammonium ion was a favored nitrogen source for averufin biosynthesis. The suppressive effect of nitrate is not caused by pH changes nor by an increased energy requirement to reduce nitrate to ammonium, but is due to repression of enzyme(s) involved in averufin formation. Aflatoxin synthesis by the parent culture was also suppressed by nitrate.

Considerable information is available on the production, analysis, biosynthetic pathway, and toxicology of aflatoxin, but almost nothing is known about metabolic regulation at its formation. We are investigating the control mechanism(s) that regulates formation of this carcinogenic mycotoxin. Understanding of this mechanism(s) might lead to methods of preventing aflatoxin synthesis in feed and foodstuffs, as well as of improving production in the laboratory.

For ease and safety, most of our work was done with *A. parasiticus* ATCC 24551, a mutant that does not produce aflatoxin but does produce averufin, an early intermediate of aflatoxin biosynthesis. Averufin is neither toxic nor mutagenic.

EXPERIMENTAL

STRAINS.—The cultures used in this investigation include the aflatoxin-producing wild type, *Aspergillus parasiticus* ATCC 15517, and its averufin-producing mutant ATCC 24551 (1).

CULTIVATION METHODS.—Spores of both strains were prepared by growth on mycological agar (Difco) in Blake bottles for six days at 27°. Spores were washed off the agar with 50 ml of sterile 0.01% sodium dodecyl sulfate per Blake bottle. The strains were incubated as stationary cultures for six days at 30° in the following medium modified from Ayde and Mateles (2): yeast extract, 0.01 g; sucrose, 40.0 g; $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 10.0 g; $MgSO_4 \cdot 7\ H_2O$, 2.0 g; $Na_2B_4O_7 \cdot 10\ H_2O$, 0.7 mg; $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 0.5 mg; $Fe_2(SO_4)_3 \cdot 6\ H_2O$, 10.0 mg; $CuSO_4 \cdot 5\ H_2O$, 0.3 mg; $MnSO_4 \cdot H_2O$, 0.11 mg; $ZnSO_4 \cdot 7\ H_2O$, 17.6 mg. The medium was brought to one liter with distilled water, and adjusted to pH 4.5 with HCl. Portions of 50 ml were added to each 250-ml Erlenmeyer flask, sterilized by autoclaving and inoculated with 0.3-ml spore suspension.

All time course studies were conducted with multiple flasks and were done at least twice. At each time point, two flasks were removed and assayed.

Experiments using washed mycelial suspensions were carried out as follows. After three days of growth, mycelia were harvested by filtration and washed with pH 7.0 phosphate buffer. They were then suspended in phosphate buffer containing 100 μg cycloheximide per ml and incubated for 24 h. Assays for cell dry weight and averufin were done at 0 and 24 h. Cycloheximide was not necessary for growth prevention but was used to prevent protein synthesis from endogenous reserves during the 24-h incubation. Cycloheximide was noninhibitory to averufin formation under these conditions.

EXTRACTION AND ESTIMATION OF AVERUFIN.—The mycelium was separated from the broth by decantation and extracted four times each with 50 ml acetone. After collecting the acetone extracts by filtration, the pooled extract was concentrated by evaporation to a volume of 100 ml. Quantitative analysis of averufin (1) was done in a spectrophotometer at 450 nm (molecular weight: 408; molecular extinction coefficient: 10.5×10^3).

ISOLATION AND ESTIMATION OF AFLATOXIN.—An equal volume of chloroform was added to the whole broth, and the mixture was treated in a Waring blender at low speed for 2 min. The chloroform

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phase was separated from the aqueous phase in a separatory funnel. This extraction was done three times. After drying over Na_2SO_4, the combined chloroform extracts were evaporated to dryness and then resuspended in 3 ml chloroform. An aliquot of the extract was applied to a thin-layer plate (silica gel) and developed with chloroform-acetone (9:1). Aflatoxin spots (uv) were scraped off the plate, eluted from the silica gel with 5 ml methanol, and measured quantitatively (2) on a spectrophotometer at 363 nm (molecular weight of aflatoxin B_1:312; molar extinction coefficient: 21,800). With strain ATCC 15517 and the conditions used, the other aflatoxins constituted only a minor proportion of the total; thus, only a slight error in determination of total aflatoxins was introduced by assuming all of the absorption at 363 nm to be due to aflatoxin B_1.

DRY CELL WEIGHT.—Mycelia were dried at 80° for 24 h after extraction and were then weighed.

RESIDUAL SUCROSE.—Broth samples were hydrolyzed with HCl to convert residual sucrose to reducing sugars according to AOAC method 31.026. Reducing sugars were assayed as described by Miller (3).

RESULTS

CARBON SOURCES.—The influence of different carbon sources at 40 g/liter concentration on growth and averufin (4) production was studied first. All carbon sources tested supported fair to good growth, except lactose. Sucrose was best for averufin production (figure 1). Fair averufin production was observed with glucose, fructose, and sorbitol, whereas galactose, xylose, mannitol, and lactose were extremely poor. The pH values were similar during fermentation in the different media. The optimal concentration of sucrose was found to be 40 g/liter (data not shown). Higher concentrations yielded lower levels of averufin per gram of mycelia produced.

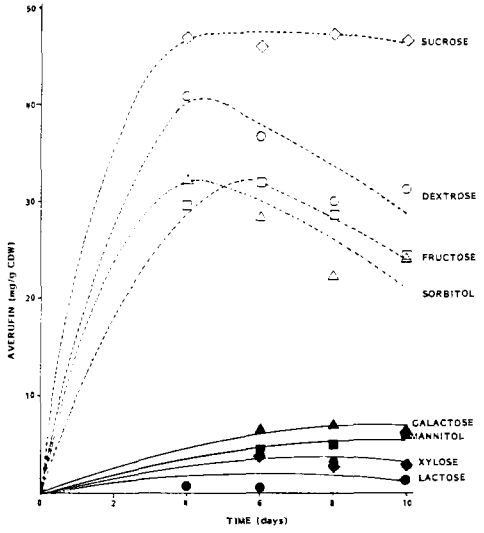

FIGURE 1. Influence of different carbon sources on averufin synthesis by *Aspergillus parasiticus* ATCC 24551.

To determine whether any of the poor carbon sources inhibit averufin production, they were added to cultures containing a suboptimal (50 g/liter) sucrose concentration. However, they were no more inhibitory than additional sucrose itself.

PHOSPHORUS SOURCES.—The effect of different phosphorus sources in various concentrations on growth and averufin synthesis was examined. All tested compounds supported growth, but pyrophosphate and acetylphosphate became toxic at concentrations greater than 10 mM. Further experiments showed that the toxicity of acetylphosphate is due to the acetyl moiety. At a concentration of 0.1 mM, acetylphosphate was

the best phosphorus source for production of averufin on a specific basis, *i.e.*, per unit weight of cells; however, its toxicity limited the volumetric titer of averufin attained. With this exception, KH_2PO_4 was the best source of phosphorus, and none of the compounds appeared to have marked regulatory effects, *i.e.*, inhibition or stimulation of averufin production.

NITROGEN SOURCES.—Different N-containing substances were tested as replacements for $(NH_4)_2SO_4$ and could be divided into three groups according to their influence on averufin production: (a) N-sources without significant influence; (b) stimulatory N-sources; and (C) inhibitory N-sources. The compounds in group a were ammonium nitrate, ammonium acetate, glycine, DL-serine, L-asparagine, and L-tryptophan. Tables 1 and 2 show the results with substances of groups b and c, respectively. Ammonium phosphate was the best nitrogen source for averufin formation. Compounds that showed an inhibtory effect in the absence of $(NH_4)_2SO_4$ (table 2) were

TABLE 1. N-sources with a stimulatory effect on averufin synthesis.[a]

Added N-source	Concentration (N g)/liter)	Cell dry weight (g/liter)	Averufin production (mg/g CDW)
None	—	1.2	20
$(NH_4)_2SO_4$	0.6	6.6	43
	1.2	9.0	47
	2.4	8.4	49
	4.8	8.2	41
NH_4Cl	0.6	6.4	32
	1.2	8.4	48
	2.4	8.0	47
	4.8	7.2	43
$(NH_4)_2HPO_4$	0.6	5.4	30
	1.2	11.4	55
	2.4	10.6	59
	4.8	10.2	66
NH_4 oxalate	0.6	4.8	26
	1.2	11.0	32
	2.4	11.8	62
	4.8	10.6	59
L-cysteine	0.6	2.6	49
	1.2	2.2	32
	2.4	3.4	29
	4.8	2.2	24
L-glutamine	0.6	5.2	22
	1.2	10.0	27
	2.4	14.0	41
	4.8	15.0	36

[a]The basal medium for this experiment contained no $(NH_4)_2SO_4$ but did contain yeast extract; duration was 6.5 days.

then tested for their possible inhibitory effect in its presence. These substances were added to basal medium containing 3 g/liter $(NH_4)_2SO_4$ (= 0.6 g N/liter) to give final concentrations of 2.4 and 6.0 g N/liter. Table 3 shows that, although none of the amino acids inhibited averufin biosynthesis in the presence of ammonium sulfate, $NaNO_3$ did show a significant inhibition. Fermentations were next conducted with NH_4^+, NO_3^-, and a combination of the two. The ability of NO_3^- to support growth and its suppression of averufin synthesis can be seen in figure 2.

Up to this point, all experiments had been done in unbuffered media. Figure 2 shows that the pH rose to 6.0 in sodium nitrate-containing cultures, while with

TABLE 2. N-sources with an inhibitory effect on averufin synthesis.[a]

Added N-source	Concentration (N g/liter)	Cell dry weight (g/liter)	Averufin production (mg/g CDW)
None	—	1.2	20
L-valine	0.6	3.6	20
	1.2	6.4	14
	2.4	7.8	13
	4.8	8.0	12
L-phenylalanine	0.6	3.2	5.6
	1.2	3.4	3.5
	2.4	3.8	2.8
	4.8	4.2	1.3
L-lysine	0.6	4.0	14
	1.2	5.8	13
	2.4	5.0	12
	4.8	3.8	15
L-leucine	0.6	3.4	3.2
	1.2	3.8	2.6
	2.4	4.2	2.4
	4.8	5.2	2.4
L-methionine	0.6	4.0	12
	1.2	4.6	6.5
	2.4	8.6	4.6
	4.8	7.0	4.9
L-isoleucine	0.6	3.6	13
	1.2	6.0	9.2
	2.4	7.6	6.3
	4.8	9.4	5.6
L-tyrosine	0.6	3.0	14
	1.2	5.0	8.6
	4.8	7.4	5.2
$NaNO_3$	0.6	6.4	8.5
	1.2	10.0	7.6
	2.4	8.8	11
	4.8	7.0	13

[a]The basal medium for this experiment contained no $(NH_4)_2SO_4$ but did contain yeast extract.

$(NH_4)_2SO_4$, it dropped to 2.9. Thus, it was necessary to determine whether the inhibitory effect of NO_3^- on averufin formation was merely a pH effect. Three buffer systems used (citric acid/Na citrate; fumaric acid/NaOH, and phthalic acid/NaOH) kept the pH relatively constant at 4.5 throughout the fermentation. Although all the buffers interfered with averufin production, their ability to stabilize pH allowed us to determine that pH was not involverd in the nitrate effect, *i.e.*, in the presence of buffers, nitrate still had a marked suppressive effect on averufin synthesis. In a later experiment without buffers, pH was controlled manually by adjusting the pH to 4.6 every 4-10 h with 0.2 M HCl or NaOH, using pH indicator paper as a guide. NH_4^+-containing cultures synthesized 52 mg averufin per gram CDW whereas NO_3^--grown cells produced only 8.8 mg/g CDW. Again, these results confirm that pH is not involved in the nitrate effect.

It is possible that the energy required to reduce nitrate to the ammonium ion leaves less sucrose available for averufin synthesis. If this is true, addition of excess sucrose should negate the nitrate effect. Thus, increasing sucrose concentrations were tested in the presence of $NaNO_3$ and a combination of $NaNO_3$ and $(NH_4)_2HPO_4$; the residual sugar concentrations were measured at the end of the fermentation. Figure 3 shows that greater sugar utilization occurred in the presence of nitrate. However, at all initial suc-

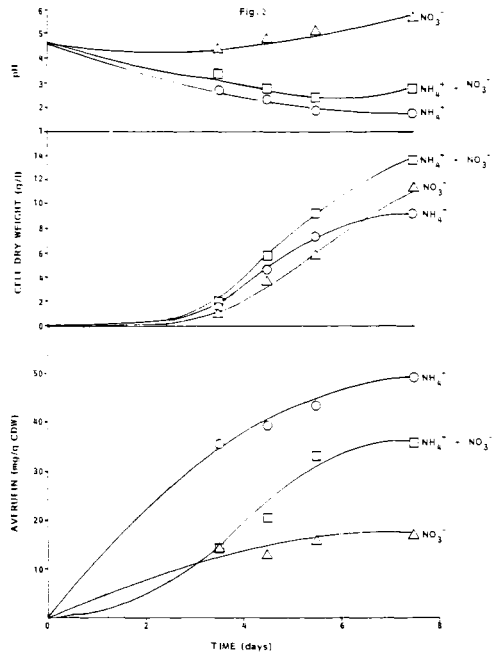

FIGURE 2. Effect of ammonium sulfate, sodium nitrate and a combination on growth, pH and averufin production. All flasks contained 2.4 N g/liter, exclusive of yeast extract.

TABLE 3. Effect of inhibitory N-sources on averufin synthesis in the presence of ammonium sulfate.

N-sources added to 3 g/liter $(NH_4)_2SO_4$[a]	Concentration (N g/liter)[b]	Cell dry weight (g/liter)	Averufin production (mg/g CDW)
None	0.6	8.4	40
$(NH_4)_2SO_4$	2.4	7.8	46
	6.0	7.4	44
L-tyrosine	2.4	8.0	56
	6.0	7.8	42
L-lysine	2.4	8.6	47
	6.0	9.6	44
L-methionine	2.4	7.8	45
	6.0	12.6	41
L-phenylalanine	2.4	9.4	43
	6.0	13.2	35
L-leucine	2.4	11.0	50
	6.0	13.2	37
L-isoleucine	2.4	10.2	52
	6.0	12.6	41
L-valine	2.4	10.0	49
	6.0	15.4	39
$NaNO_3$	2.4	12.8	29
	6.0	13.0	29

[a]3 g/liter $(NH_4)_2SO_4$ is equivalent to 0.6 N g/liter.
[b]The basal medium for this experiment also contained yeast extract as a N source.

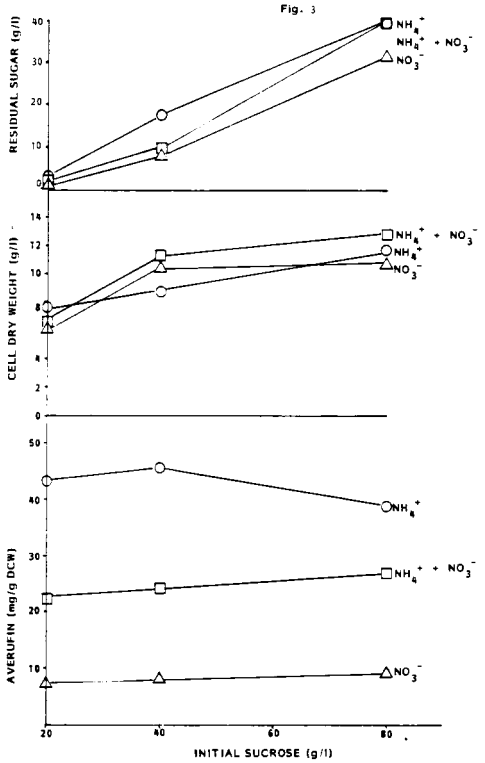

FIGURE 3. Nitrate suppression of averufin formation in the presence of excess sugar. No ammonium salt was in the basal medium. In all cases, the N concentration was 1.2 g/liter, exclusive of yeast extract.

rose levels, sugar remained at the end of the fermentation and the negative effect of nitrate on averufin production was seen in all cases. Therefore, sugar depletion is not the basis of the nitrate effect.

Because neither pH change nor sugar depletion was responsible for the nitrate effect, nitrate was probably exerting a true regulatory effect by inhibiting or repressing averufin formation. To decide between these two possibilities, cell suspensions were washed and incubated in buffer in the presence of cycloheximide. NH_4^+, NO_3^-- or no nitrogen source was added to these resting cells. Averufin production at the expense of endogenous carbon reserves was monitored for 24 h. As shown in table 4, nitrate had no negative effect under these conditions, suggesting that nitrate does not act by inhibiting preformed enzymes of averufin biosynthesis.

TABLE 4. Lack of nitrate effect when added to washed mycelial suspensions.[a]

N-source in suspension (0.01 M)	Cell dry weight (g/liter)		Averufin (mg/g CDW)		
	0 h	24 h	0 h	24 h	Δ
None	2.6	2.4	18	35	+17
$NaNO_3$	3.8	3.0	20	37	+17
$(NH_4)_2HPO_4$	3.8	2.6	20	35	+15

[a]Cells were grown for three days in medium containing $(NH_4)_2SO_4$. Incubation done in pH 7.0 phosphate buffer containing cycloheximide.

To determine whether nitrate acts *via* enzyme repression, cells were grown in the presence of increasing levels of either NH_4^+ or NO_3^-. They were then tested for averufin production in buffer plus cycloheximide but without any additional source of nitrogen. As shown in table 5, growth in nitrate markedly repressed continuing averufin biosynthesis in resting cells.

TABLE 5. Repression of averufin synthesis by growth in the presence of nitrate.[a]

N source in growth medium (N g/liter)	Cell Dry Weight (g/liter)		Averufin (mg/g CDW)		
	0 h	24 h	0 h	24 h	Δ
$(NH_4)_2HPO_4$					
0.6	3.2	1.8	20	35	+15
1.2	1.6	1.8	19	31	+12
2.4	2.4	1.4	17	31	+14
$NaNO_3$					
0.6	1.6	1.6	8.0	8.0	0
1.2	2.8	2.2	10	14	+ 4
2.4	1.2	1.2	5.0	6.8	+ 1.8

[a]Incubation done in pH 7.0 phosphate buffer containing cycloheximide but no N source.

The nitrate effect was also examined with the aflatoxin-synthesizing wild-type culture. The culture was grown with either NH_4 or NO_3 under the same conditions as in averufin fermentations. Figure 4 shows that much less aflatoxin was produced in NO_3^--containing media as compared with that produced in NH_4^+. Thus, the regulatory effect of nitrate on averufin formation results in repression of aflatoxin biosynthesis.

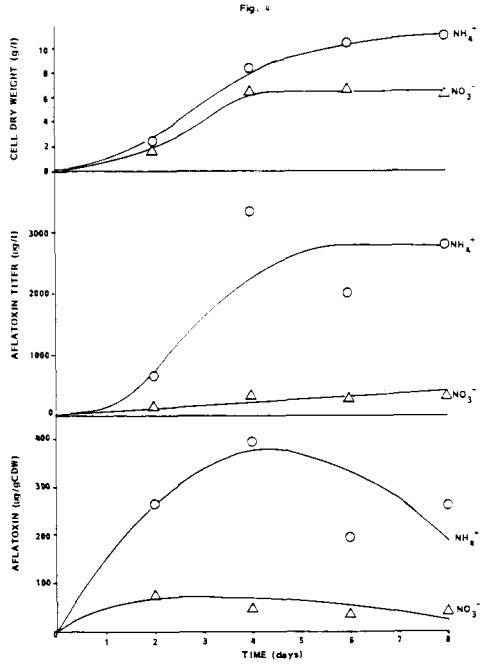

FIGURE 4. Influence of ammonium phosphate and nitrate on aflatoxin production by *Aspergillus parasiticus* ATCC 15517.

DISCUSSION

Although little is known about the regulation of aflatoxin biosynthesis, scattered reports in the literature suggest that nitrogen sources may exert significant control. Davis et al. (5) reported that, although KNO_3 and $NaNO_3$ supported good growth of *Asperigillus flavus,* organic nitrogen was necessary for significant aflatoxin biosynthesis. (Ammonium salts were not tested.) In media containing inorganic nitrogen sources, asparagine markedly increased aflatoxin formation by *A. parasiticus* (6); but because asparagine also improved growth, it is not clear whether regulation *per se* is involved in this effect. Shih and Marth (7) reported that aflatoxin production by *A. parasiticus* was optimal at a concentration of $(NH_4)_2SO_4$ that was suboptimal for growth. Bennett et al. (8) found that versicolorin A and C production by a blocked mutant of *A. parasiticus* was completely inhibited by use of $NaNO_3$ as a nitrogen source. On the other hand, $(NH_4)_2SO_4$ and NH_4NO_3 supported greater growth and versicolorin production. Versicolorin A is an intermediate of aflatoxin biosynthesis.

The present study shows that certain nitrogen sources exert a crucial effect on averufin and aflatoxin synthesis whereas carbon and phosphorus sources exert only minor effects. The most repressive nitrogen source was nitrate, and its effect was not caused by pH changes nor by sugar depletion.

A few carbon sources (galactose, mannitol, and xylose) that supported good growth but only poor averufin synthesis showed no unusual degree of inhibition when added to the best carbon source, sucrose. Similarly, a number of amino acids supported fair growth and inhibited averufin synthesis in the absence of NH_4^+, but failed to inhibit in the presence of NH_4^+.

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