Synthesis of Aflatoxins by the Non-growing Mycelia of *Aspergillus parasiticus* and the Effect of Inhibitors

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INTRODUCTION

A chemically defined medium giving high yields of aflatoxins has been reported from this laboratory (Reddy, Viswanathan & Venkitasubramanian, 1971). Many workers have studied the incorporation of [^{14}C]acetate into aflatoxins (Adye & Mateles, 1964; Donkersloot, Hsieh & Mateles, 1968; Detroy & Hesseltine, 1969; Hsieh & Mateles, 1970, 1971). Labelled acetate is efficiently incorporated into aflatoxins when added directly to cultures of *Aspergillus parasiticus*. When studying the effects of different factors on aflatoxin production with such a system, it is always possible that the results will be influenced by the growth of the mycelium. Therefore, attempts were made to develop a system capable of synthesizing aflatoxins under completely resting conditions, and this system was used to study the effect of different inhibitors on aflatoxin production.

METHODS

Aspergillus parasiticus NRRL3240, from the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A., was used, and was maintained as a soil culture. Radioactive acetate was obtained from the Bhabha Atomic Research Centre, Bombay, India. Spore suspensions in sterile, double distilled water were prepared from 5- to 6-day-old cultures grown on 50 ml glucose-peptone agar and distributed equally between five 500 ml Erlenmeyer flasks each containing 100 ml sterile medium. The synthetic medium (SLS medium) had the following composition: 85 g sucrose, 10 g asparagine, 3.5 g $(\text{NH}_4)_2\text{SO}_4$, 10 g KH_2PO_4 , 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 75 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2 mg $(\text{NH}_4)_2\text{MoO}_4 \cdot 4\text{H}_2\text{O}$, 2 mg $\text{Na}_2\text{B}_4\text{O}_7$ and 2 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, made up to 1 l with double distilled water. The pH of the medium was adjusted to 4.5.

The flasks were incubated at $26 \pm 1^\circ\text{C}$ as stationary cultures for four days. The mycelium was then washed with double distilled water and cut into two equal portions. Each portion was transferred to a 250 ml Erlenmeyer flask containing 50 ml of sterile suspension medium or 0.02 M-phosphate buffer pH 5.8 plus 10 μCi [^{14}C]acetate (specific activity 4.68 mCi/mmol) and incubated.

Inhibitors at different concentrations were added to the suspension buffer. Diphenylamine was added together with 50 mM-ethanol, and the percentage change in specific activity calculated by comparison with a control containing 50 mM-ethanol. At the end of the experiment the mycelium and medium were separated, and aflatoxins were extracted with chloroform and separated by thin-layer chromatography on silica gel G (irrigation by

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Table 1. *Change in specific activity of aflatoxins by mycelia grown in SLS medium and suspended in different buffers*

Suspension medium	Aflatoxin in suspended mycelium (mg/flask)		Change in specific activity of aflatoxin* (%)	
	B	G	B	G
Tris-HCl buffer (0.02 M, pH 7.2)	5.4	1.3	+3	+113
Phosphate buffer (0.02 M, pH 5.8)	5.0	1.4	+32	+109
Phthalate buffer (0.02 M, pH 4.5)	4.8	1.1	-38	-44
Maleate buffer (0.02 M, pH 4.5)	5.2	1.2	-50	-35
Citrate-phosphate buffer (0.02 M, pH 2.8)	5.7	1.4	+15	+64
KCl-HCl buffer (0.02 M, pH 2.0)	5.6	1.3	+1	+101

* Compared with the control using SLS suspension medium.

ether and then by 2 % methanol in chloroform). Aflatoxins were eluted with methanol and estimated spectrophotometrically (Nabney & Nesbitt, 1965). Since the amounts of aflatoxins B₂ and G₂ were very low, B₁ and B₂ were estimated together as aflatoxin B, and G₁ and G₂ as aflatoxin G, using the extinction coefficients for aflatoxins B₁ and G₁ respectively. For determining radioactivity, methanol eluates were evaporated to a small volume and counts were made with a PCS 14C model gas flow counter (Electronics Corporation of India Ltd). The efficiency of the counter was about 17 % for ¹⁴C. Radio-purity of the aflatoxins was checked by the method of Gupta *et al.* (1974). All experiments were done in duplicate, and the variation between them was less than 5 %.

RESULTS AND DISCUSSION

In contrast to Reddy *et al.* (1971), we observed that the addition of large amounts of KH₂PO₄ and MgSO₄ to a fresh culture of *A. parasiticus* in their SL medium, increased aflatoxin levels and [1-¹⁴C]acetate incorporation. We therefore used a medium with a high salts content (SLS medium). Since maximum incorporation occurred on the 4th day of culture, 4-day-old mycelia were used for all suspension experiments. In view of the extremely low incorporation of [1-¹⁴C]acetate into extracellular aflatoxins in the medium, only mycelial aflatoxins were studied. A number of different buffers were tried for suspending the mycelium. The specific activities of aflatoxins synthesized in phosphate (pH 5.8), tris-HCl (pH 7.2), citrate-phosphate (pH 2.8) and KCl-HCl (pH 2.0) buffers were higher than those in SLS medium, while in phthalate and maleate buffers specific activities were much lower (Table 1). The synthesis of aflatoxins by non-growing mycelia was clearly demonstrated, and the amount and type of aflatoxin produced was dependent on the nature of the suspension medium (Table 1).

The effect of inhibitors at different concentrations on the incorporation of labelled acetate into aflatoxins is shown in Table 2. The effects of desoxypyridoxine hydrochloride and boric acid were concentration-dependent: low concentrations stimulated aflatoxin synthesis. The exact mode of action of these inhibitors on aflatoxin synthesis is unknown.

Table 2. *Effects of inhibitors on the specific activity of aflatoxins synthesized by mycelia grown in SLS medium and suspended in phosphate buffer*

Compound added	Aflatoxin	Change in specific activity of aflatoxin (%)							
		Concentration of added inhibitor (mm):							
		0.01	0.05	0.1	0.5	1.0	10.0	50.0	100.0
Acetyl pyridine	B	.	.	+11	.	-7	-2	.	-70
	G	.	.	-7	.	+11	-9	.	-62
L-Cycloserine	B	+4	+10	+10	+15	+29	+32	.	.
	G	+3	+5	+7	+12	+12	+17	.	.
Desoxypyridoxine hydrochloride	B	+21	+17	+2	+9	-9	.	.	.
	G	+4	-4	-5	-12	-19	.	.	.
Oxythiamine chloride	B	+2	-14	+7	+21	+31	.	.	.
	G	+2	-2	+17	+19	+23	.	.	.
Salicylic acid	B	+2	-7	-11	-19	-50	-62	.	.
	G	+8	+2	-8	-21	-41	-71	.	.
Sulphanilamide	B	-8	.	-4	-3	-1	-9	.	.
	G	+9	.	0	+2	-7	-5	.	.
Boric acid	B	+14	+22	+7	-10	-24	-52	.	.
	G	+22	+20	+17	-4	-14	-40	.	.
L-Cysteic acid	B	-61	-55	-64	-52	-60	-73	.	.
	G	-73	-54	-60	-49	-65	-83	.	.
Diphenylamine	B	+2	-9	+5	.	.	.	.	.
	G	-7	-2	+3	.	.	.	.	.
<i>o</i> -Iodosobenzoic acid	B	+19	+27	+26	0	.	.	.	.
	G	+31	+32	+33	+1	.	.	.	.
Salicylaldehyde	B	.	.	+2	.	-42	-48	-90	.
	G	.	.	+4	.	-27	-48	-92	.
Sodium azide	B	-12	.	-42	-65	-81	.	.	.
	G	-16	.	-20	-30	-94	.	.	.
Sodium bisulphite	B	.	.	-9	.	-4	-3	-27	.
	G	.	.	-2	.	+15	+7	-27	.

However, the decreases of only 10 and 25 % in specific activities of aflatoxins B and G respectively at high concentrations of desoxypyridoxine hydrochloride (5.0 mm) indicate that pyridoxal phosphate-dependent reactions are not involved in aflatoxin biosynthesis. The specific activities of both aflatoxins were stimulated by oxythiamine chloride, an analogue of thiamine; thiamine is involved in decarboxylation and oxidation of alpha-keto acids. Inhibition of the former reaction would decrease formation of unlabelled acetyl CoA from endogenous substrates which would otherwise dilute the radioactivity of the added [^{14}C]acetate. Also, inhibition of alpha-ketoglutaric acid oxidation may divert [^{14}C]acetate from tricarboxylic acid cycle to aflatoxin biosynthesis.

The inhibition caused by salicylic acid, an antimetabolite of pantoic acid, is probably due to reduced synthesis of pantothenic acid and in turn of CoA (Mass, 1952). Salicylic acid is known to uncouple oxidative phosphorylation and thereby decrease ATP production (Brody, 1956). Addition of sulphanilamide to the suspension medium had no marked effect on specific activities of individual aflatoxins, which is in agreement with the results of Basappa, Sreenivasamurthy & Parpia (1970); however, sulphanilamide added to the growth medium decreases growth and aflatoxin production (Davis & Diener, 1967).

L-Cysteic acid (0.01 to 10 mM) in phosphate buffer markedly inhibited acetate incorporation. Cysteic acid inhibits decarboxylation of aspartic acid and transamination of alanine in *Escherichia coli* (Ravel & Shive, 1946). The presence of aspartic acid, asparagine or alanine is essential for good growth and aflatoxin production on a synthetic medium (Reddy, 1972). The exact role of these amino acids in aflatoxin synthesis is not clear, but it is likely that the inhibition by cysteic acid is due to interference with aspartic acid utilization. *o*-Iodosobenzoic acid (0.01 to 0.1 mM) stimulated incorporation of acetate into aflatoxins. *o*-Iodosobenzoic acid is a sulphhydryl reagent which inhibits succinic dehydrogenase at low concentrations (Singer, Kearney & Bernath, 1956; Stoppani & Brignone, 1956). This would have the effect of reducing oxidation of acetate through the tricarboxylic acid cycle, thus diverting more acetate towards the synthetic pathways. Decreased specific activities of aflatoxins B and G brought about by sodium azide could be due to interference with the electron transport system and hence with ATP production. Detailed studies are in progress to determine the mode of action of these compounds.

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