Effect of Some Inhibitors on Aflatoxin Production in a Synthetic Medium and on the Incorporation of Acetate-1-^{14}C into Aflatoxins by Resting Mycelia of *Aspergillus parasiticus*

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Toxic metabolities produced by fungi have been well demonstrated to be public health hazards. Of the many chemical carcinogens known today, aflatoxins, the metabolities of Aspergillus flavus are among the most potent inducers of tumors. Many programmes have been initiated to control the propogation of environmental toxicants. Many investigators have made conserted efforts in reducing the incidence and severity of aflatoxin contamination by the knowledge of the cultural and environmental conditions but till now no head-way has been made. Delineation of biosynthesis of aflatoxin would go a long way in the prevention of the contamination by this toxin. There are few reports regarding the effect of different inhibitors on aflatoxin production (DAVIS and DIENER, 1967; DETROY and HESSELTINE, 1969; BASAPPA et al. 1970).

In order to understand the relative importance of different metabolic pathways and enzymes for the synthesis of aflatoxins, the effect of a number of inhibitors on acetate incorporation into aflatoxins has been studied in the present investigation, by using resting mycelia of Aspergillus parasiticus suspended in phosphate buffer. Also, selected levels of a number of inhibitors were incorporated in the growth medium and the changes in aflatoxin production were investigated.

MATERIALS AND METHODS

Aspergillus parasiticus NRRL 3240 obtained from Northern Regional Research Laboratory, Peoria, Illinois, U.S.A. was used in the present study. It was preserved in sterile soil and stored at 4°C. A spore suspension in sterile double distilled water was prepared from 5 to 6 days old cultures grown on glucose-peptone-agar and distributed equally to 100 ml of the synthetic medium contained in 500 ml Erlenmeyer flasks. The synthetic medium (SLS medium) used for the present study had the following composition: Sucrose, 85 g; asparagine, 10 g; $(NH_4)_2SO_4$, 3.5 g; KH_2PO_4, 10 g; $MgSO_4 \cdot 7H_2O$, 2 g; $CaCl_2 \cdot 2H_2O$, 75 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 5 mg;

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ammonium molybdate.$4H_2O$, 2 mg; $Na_2B_4O_7$, 2 mg; and $FeSO_4.7H_2O$, 2 mg made upto 1 liter with double distilled water. The pH of the medium was adjusted to 4.5.

The fungus was grown at 26±1°C for four days for all suspension experiments. The mycelium was then washed with double distilled water and cut into two equal parts. Each part of the mycelium was transferred into a 250 ml Erlenmeyer flask containing 50 ml of sterile phosphate buffer, pH 5.8 and 5 uC of acetate-1-^{14}C of specific activity 4.68 mC/mM. After incubating for 2 hours, aflatoxins were extracted from the mycelium with chloroform. In experiments in which the effect of different inhibitors on growth and aflatoxin production was studied, A. parasiticus was grown as stationary culture in SLS medium for 8 days in the presence of different inhibitors. Aflatoxins were extracted with chloroform from the mycelium and the medium separately. They were separated by thin layer chromatography on silica gel G with an initial run in ether and then in 2% methanol in chloroform, were eluted with methanol and estimated by spectrophotometry as described by NABNEY and NESBITT (1965). Aflatoxins B_1 and B_2 were estimated together as aflatoxin B and aflatoxins G_1 and G_2 were estimated as aflatoxin G, using the extinction coefficients for aflatoxins B_1 and G_1 respectively. The methanol eluates were evaporated to dryness and the radioactivity was determined using gas flow counter (Model PCS 14C of Electronics Corporation of India Ltd., India). The efficiency of the counter was 17 per cent for 14_C. Radiopurity of the aflatoxins was checked as reported by GUPTA et al.(1974). The experiments were carried out in duplicate and the data presented are the average of two separate experiments.

RESULTS AND DISCUSSION

It is interesting to note the increase in specific activities of aflatoxins in presence of malonic acid, iodoacetic acid, sodium arsenite, 2:4 dinitrophenol, sodium fluoride, EDTA and p-aminosalicylic acid at low concentrations(Table1). Sodium arsenate inhibited the incorporation of acetate-^{14}C only at high levels. At 20 mM level, Malonic acid, Sodium assenite caused an inhibition in the incorporation to the extent of 80-86 per cent, where EDTA at the same level stimulated the incorporation by 63 per cent. Sodium fluoride and

sodium arsonate at 100 mM level caused an inhibition in the incorporation to the extent of 63 and 38 per cent respectively. Nitrobenzoic acid and dehydroacetic acid decreased the specific activities of aflatoxins at all the levels.

TABLE 1

Effect of some inhibitors on the incorporation of acetate-1-^{14}C into aflatoxins by resuspended mycelia

Inhibitor added	Aflatoxin	Per cent change in specific activity of aflatoxin — Concentration of the added inhibitor (mM)				
		0.01	0.10	0.50	1.00	10.0
Malonic acid	B	- 6	+ 1	+28	+ 97	-54
	G	+ 5	0	+43	+142	-35
Iodoacetic acid	B	+13	+18	-31	- 73	-95
	G	+ 3	+23	-51	- 76	-90
Sodium arsenite	B	- 6	+60	+10	- 24	-57
	G	- 3	+43	+29	- 20	-62
2:4 Dinitrophenol	B	+16	-10	-80	- 77	
	G	+42	- 7	-85	- 86	
Sodium fluoride	B	+69	+41	-25	- 39	-68
	G	+53	+58	-13	- 7	-38
Ethylenediamine tetraacetic acid	B		+ 2		+ 43	+39
	G		+ 4		+ 43	+60
Sodium arsenate	B		+ 4		- 4	- 7
			- 3		- 15	-10
p-Nitrobenzoic acid	B	-27	-22	-31	- 45	-50
	G	-36	-40	-49	- 44	-69
p-Aminosalicylic acid	B	+24	+55	+34	+ 26	-48
	G	+ 9	+44	+23	- 8	-69
Dehydroacetic acid	B	- 4	-11	-50	- 60	-70
	G	- 1	-20	-59	- 70	-76

Malonic acid and DNP have been reported to stimulate succinic dehydrogenase at low concentrations (KEARNEY, 1957; SUSHEELA and RAMASARMA, 1972). Iodoacetic acid is known to inhibit glycolysis at concentrations of 0.1 to 0.5 mM in many systems (WEBB, 1966 b). This could have the effect of reducing the amount of

endogenously formed acetyl CoA, which may normally be diluting the incorporation of ^{14}C-acetate. Sodium chloride also may be stimulating the specific activity of aflatoxins in a similar manner. On the other hand, malonate and arsenite inhibit citric acid cycle at low concentrations (WEBB, 1966 a,b). This may reduce the extent of oxidation of ^{14}C-acetate and make more of it available for the synthesis of aflatoxins. EDTA may be either chelating some heavy metal impurity which is inhibiting aflatoxin biosynthesis or it may be stimulating the uptake of acetate by the fungus by altering the permiability of the cell membrane (SRIPRAKASH and RAMAKRISHNAN, 1970). Evidence for the inhibition of aflatoxin formation by heavy metal impurities has been reported by REDDY et al., 1971).

The decrease in the specific activity of aflatoxins observed at higher concentrations of these inhibitors may be due to the inhibition of a number of enzymes at these levels. For example, iodoacetate inhibits a number of sulphydryl enzymes at higher concentrations (WEBB, 1966 b). It has also been reported to inhibit fatty acid biosynthesis in yeast (WEBB, 1966 b). Arsenate and DNP probably act by uncoupling oxidative phosphorylation and thus reducing the supply of ATP. The decrease in specific activity of aflatoxins in the presence of p-nitrobenzoic acid even at low concentrations is probably due to interference with p-aminobenzoic acid and folic acid metabolism. A reduction in the synthesis of p-aminobenzoic acid would lead to lowered levels of coenzymes A and folic acid may be involved in the provision of the O-methyl groups of aflatoxins.

Effect of addition of selected levels of these inhibitors to the synthetic medium on growth and aflatoxin production is presented in Table 2.

Abbreviations used:- DNP - 2:4 Dinitrophenol,
EDTA - Ethylenediamine tetraacetic acid.

TABLE 2

Effect of addition of some inhibitors to the growth medium on aflatoxin production by A. parasiticus NRRL 3240

Inhibitor added	Concentration (mM)	Mycelial dry weight g/100 ml medium	Aflatoxins mg/100 ml medium			
			in medium		in mycelium	
			B	G	B	G
Nil		3.0	6.1	4.2	12.1	3.5
Malonic acid	1.0	3.1	5.8	3.1	12.8	3.6
	10.0	3.2	6.5	4.7	12.0	4.1
Iodoacetic acid	0.1	2.6	2.4	1.1	5.2	1.5
	1.0	2.7	1.7	0.6	2.6	0.5
2:4 Dinitrophenol	0.01	2.8	4.7	2.8	14.1	4.4
	0.1	3.0	3.4	1.3	6.0	1.2
Sodium fluoride	1.0	2.4	6.1	4.2	4.1	2.2
Ethylenediamine tetraacetic acid	1.0	2.0	6.1	1.4	4.2	0.9
Potassium arsenate	1.0	2.8	2.9	2.0	0.9	0.2
p-Nitrobezoic acid	1.0	2.7	8.1	5.5	12.9	3.9
p-Aminosalicylic acid	1.0	2.6	5.6	3.7	14.1	5.2
Dehydroacetic acid	1.0	2.9	2.1	0.8	2.6	0.6

In contrast to increase in the specific activities of aflatoxins observed in presence of malonic acid, iodoacetic acid, sodium arsenite, DNP, EDTA and p-aminosalicylic acid at low concentrations in the suspension medium, addition of same levels of malonic acid, DNP and p-aminosalicylic acid to the growth medium did not have any appreciable effect on either growth or toxin production. Addition of iodoacetic acid, EDTA and sodium arsenite markedly decreased both growth and toxin production.

The inhibition of growth and aflatoxin production in the presence of EDTA can be ascribed to the cheltion of essential trace elements whereas inhibition in presence of sodium fluoride is probably due to inhibition of enolase and hence of glycolysis. DAVIS and DIENER (1967) have reported the effect of fluoride on a sucrose-yeast extract medium. They found no effect on growth and a 25 and 30 per cent reduction in toxin production at fluoride levels of about 1.0 and 10 mM. This is a very mild effect when compared with its effects in the present study. Dehydroacetic acid had very little effect on the growth, but reduced toxin production drastically at 1.0 mM concentration. In the suspension system also, there was a marked decrease in acetate incorporation into aflatoxins. It has been reported that dehydroacetic acid is toxic to fungi. It appears that the production of aflatoxins is more affected by dehydroacetic acid than the growth of the fungus.

SUMMARY

The effect of a number of metabolic inhibitors on the incorporation of acetate-1-^{14}C into aflatoxins was investigated, using resting mycelia of Aspergillus parasiticus suspended in phosphate buffer. Malonate, iodoacetate, sodium arsenite, 2:4 dinitrophenol, sodium fluoride and p-aminosalicylate stimulated the incorporation at low concentrations and inhibited the same at high concentrations. p-Nitrobenzoic acid was inhibitory at all the concentrations tried. Fluoride, arsenite, arsenate and iodoacetate inhibited both growth and aflatoxin production when added directly to the growth medium. In general, there was a greater inhibition in growth medium than with the suspended mycelia.

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REFERENCES

BASAPPA, S. C., V. SREENIVASAMURTHY, and H. A. B. PARPIA: J. Gen. Microbiol., 61, 81 (1970).

DAVIS, N. D., and U. L. DIENER: Appl. Microbiol. 15, 1517, (1967).

DETROY, R. W., and C. W. HESSELTINE: Developments in Industrial Microbiology, 10, 127 (1970).

GUPTA, S. R.: Metabolism of Aspergillus with reference to aflatoxin production. Ph.D. thesis, University of Delhi, Delhi-7, India (1973).

GUPTA, S. R., H. R. PRASANNA, L. VISWANATHAN, and T. A. VENKITASUBRAMANIAN: J. Gen. Microbiol. 80, 31 (1974).

KEARNEY, B. E.: J. Biol. Chem. 229, 363 (1957).

NABNEY, J. and B. F. NESBITT: Analyst 90, 155 (1965).

REDDY, T. V., L. VISWANATHAN, and T. A. VENKITASUBRAMANIAN: Appl. Microbiol. 22, 363 (1971).

SRIPRAKASH, K. S. and T. RAMAKRISHNAN: J. Gen. Microbiol. 60, 125 (1970).

SUSHEELA, L., and T. RAMASARMA: Biochem. Biophys. Res. Commun. 46, 2087 (1972).

WEBB, J. L.: Enzyme and metabolic inhibitors. Vol. II Academic Press, New York, U.S.A. (1962).

WEBB, J. L.: Enzyme and metabolic inhibitors. Vol. III Academic Press, New York, U.S.A. (1966 b).