ABILITY OF VARIOUS CARBON SOURCES TO INDUCE AND SUPPORT AFLATOXIN SYNTHESIS BY *ASPERGILLUS PARASITICUS*

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ABSTRACT

The ability of a wide variety of carbon sources to induce and support aflatoxin synthesis by Aspergillus parasiticus *was examined using mycelia pregrown in a peptone-mineral salts medium that does not support aflatoxin synthesis. Sugars and derivatives of sugars supported widely varying amounts of aflatoxin production. Amino acids and tricarboxylic acid cycle intermediates except aspartate and malate, respectively, did not appear to support* de novo *aflatoxin synthesis. Embden-Meyerhoff pathway intermediates prior to, but not after, 3-phosphoglycerate supported aflatoxin synthesis.*

INTRODUCTION

Abdollahi and Buchanan (1981a) demonstrated that *Aspergillus parasiticus* does not produce aflatoxins when cultured on a peptone-mineral salts medium, but will commence synthesis when transferred to a glucose-mineral salts medium. They further demonstrated that this process could be blocked if either a protein synthesis or RNA synthesis inhibitor was incorporated into the glucose-mineral salts medium, and concluded that glucose was regulating the induction of one or more of the enzymes associated with aflatoxin synthesis. Abdollahi and Buchanan (1981b) observed that a number of other carbohydrates could induce aflatoxin synthesis in this manner. They suggested that aflatoxin synthesis is regulated by a process analogous to carbon catabolite repression (i.e., a carbon catabolite induction), and suggested that this may involve either an alteration in the energy status of the cell or the accumulation of a key intermediate of carbo-

hydrate catabolism. The objective of the present study was to characterize further the influence of carbon source on the regulation of aflatoxin synthesis by expanding the work of Abdollahi and Buchanan (1981b) to include a wider range of carbon sources.

MATERIALS AND METHODS

Microorganism

Aspergillus parasiticus NRRL 2999 was used throughout the study. The mold was maintained on potato dextrose agar (Difco) slants, and spore suspensions were prepared as previously described (Tice and Buchanan 1982).

Media

Yeast extract-sucrose (YES) (Davis *et al.* 1966) and peptone-mineral salts (PMS) (Buchanan and Lewis 1984b) were prepared as previously described. Basal medium for determining the ability of various carbon sources to support aflatoxin synthesis consisted of $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 10 g; $MgSO_4$, 2 g; trace mineral mix (Buchanan *et al.* 1983), 1 ml; and distilled water, 1,000 ml. Carbon sources were added to the basal medium at a concentration of 20 g/l, and the pH then adjust to 5.5 with HCl or NaOH. All compounds tested were obtained from commercial suppliers. All media were sterilized by autoclaving.

Culture Techniques

The ability of various carbon sources to induce aflatoxin synthesis was evaluated by the protocol of Abdollahi and Buchanan (1981a, 1981b) using a modification of the sequential culturing technique of Buchanan and Lewis (1984b). A set of six 1.0-1 Erlenmeyer flasks containing 300 ml of YES Medium were inoculated with 1.0 ml of spore suspension to achieve an initial inoculum of approximately 3×10^3 conidia/ml. All flasks were then incubated for 72 h at 28°C on a rotary shaker (150 rpm). The mycelia were harvested and pooled on cheesecloth, rinsed with sterile 0.85% KCl, and transferred along with 450 ml of 0.85% KCl to a sterile blender. The mycelia were homogenized at high speed for 1.0 min, recollected on cheesecloth, and rinsed thoroughly with 0.85 KCl. The mycelia were then divided into 6 equal portions, transferred to 1.0-1 Erlenmeyer flasks containing 300 ml of PMS, and incubated for 24 h at 28°C on a rotary shaker (150 rpm).

The cultures were collected and pooled on cheesecloth, rinsed thoroughly with 0.85% KCl, and drained. The mycelia were then divided into 2.0-g portions and transferred to 50-ml Erlenmeyer flasks containing 10 ml of basal medium with various carbon sources. All flasks were incubated for 48 h at 28°C without agitation, and then analyzed for aflatoxin production.

Aflatoxin Analyses

Cultures (mycelium + medium) were extracted thrice with 20-ml portions of chloroform. The extracts were pooled, concentrated with a rotary evaporator, and cleaned up using the silica gel cartridge technique of McKinney (1981). Aflatoxins ($B_1 + B_2 + G_1 + G_2$) were then separated and quantitated by high pressure liquid chromatography using a linear gradient of 0.04 M formic acid/methanol (1:1) to methanol (Buchanan *et al.* 1983). The lower limit of detection was approximately 0.01 μg aflatoxin/culture. All samples were also examined by thin-layer chromatography to determine if there was any significant production of interfering compounds (Buchanan and Houston 1982).

RESULTS

Aflatoxin production by *A. parasiticus* cultured sequentially in YES and PMS, and then transferred in 2-g portions to the various carbon source media is summarized in Table 1. The glucose and peptone cultures depict a typical positive and negative response, respectively,

Table 1. Aflatoxin production on various carbon sources after transfer of *A. parasiticus* precultured on peptone-mineral salts medium

Carbon Source	n[a]	Aflatoxin/Culture (μg)						%B[c]	$\bar{X}/\bar{X}o$[d]
		B_1	B_2	G_1	G_2	Total[b]			
None	4	1.11	0.03	0.07	ND[e]	1.22	(±0.05)	93	-
Amino Acids									
Peptone	19	0.81	0.02	0.02	ND	0.85	(±0.11)	98	0.7
Glutamic Acid	4	1.69	0.07	0.45	0.02	2.22	(±0.36)	79	1.8
Aspartic Acid	4	2.85	0.05	1.06	<0.01	3.94	(±0.38)	74	3.2
Asparagine	4	0.68	0.02	0.07	ND	0.76	(±0.19)	92	0.6
Alanine	4	0.63	0.03	0.07	ND	0.73	(±0.21)	90	0.6
Proline	4	1.26	0.05	0.40	0.06	1.76	(±0.30)	74	1.4

Table 1. Continued

Embden Meyerhoff Intermediates and Products									
Glucose	20	72.65	6.12	10.20	0.55	89.52	(±8.55)	88	73.4
Glucose-6-phosphate	4	16.29	0.46	9.69	0.04	26.47	(±11.16)	63	21.7
Fructose-6-phosphate	4	11.68	0.25	9.32	0.11	21.35	(±5.18)	56	17.5
Fructose-1,6-diphosphate	4	2.38	0.05	1.37	0.08	3.88	(±0.47)	63	3.2
3-Phosphoglycerate	4	4.24	0.36	1.10	0.04	5.75	(±2.12)	80	4.7
2,3-Diphosphoglycerate	4	0.66	0.06	ND	ND	0.72	(±0.14)	100	0.6
Phosphoenolpyruvate	4	0.79	0.04	ND	ND	0.83	(±0.16)	100	0.7
Pyruvate	4	1.27	ND	0.11	ND	1.37	(±0.30)	93	1.1
Lactate	4	0.09	ND	0.08	ND	0.17	(±0.11)	53	0.1
Acetate	4	0.75	0.07	ND	ND	0.82	(±0.21)	100	0.7
Sugars									
Dihydroxyacetone	8	5.26	0.11	1.06	0.05	6.47	(±2.04)	83	5.3
Glyceraldehyde	4	0.29	ND	ND	ND	0.29	(±0.07)	100	0.2
Ribose	4	56.45	4.60	11.77	0.33	73.14	(±6.76)	83	60.0
Fructose	4	175.52	9.85	31.19	1.05	217.61	(±37.21)	85	178.4
Galactose	4	4.70	0.14	2.00	ND	6.83	(±2.09)	71	5.6
Rhamnose	4	7.67	0.53	1.05	0.14	9.38	(±2.02)	87	7.7
Arabinose	3	7.28	0.10	0.08	ND	7.46	(±1.37)	99	6.1
Maltose	4	94.10	7.82	25.78	0.80	128.49	(±38.8)	79	105.3
Sucrose	3	49.48	1.35	8.92	0.05	59.81	(±14.47)	85	49.0
Trehalose	4	22.17	1.79	5.62	0.21	29.78	(±7.99)	80	24.4
Cellobiose	4	4.00	0.26	2.35	0.04	6.60	(±0.48)	65	5.4
Lactose	4	0.85	0.01	0.24	ND	1.10	(±0.42)	77	0.9
Melezitose	4	15.55	0.97	6.73	0.31	23.56	(±5.67)	68	19.3
Starch, Soluble	4	0.68	0.02	ND	ND	0.70	(±0.28)	100	0.6
Sugar Alcohols, Acids, and Amines									
Glycerol	4	123.13	7.26	10.62	1.26	142.27	(±39.76)	92	116.6
Adonitol	4	33.77	2.78	6.39	0.46	43.40	(±2.61)	84	35.6
Xylitol	4	1.65	0.06	0.10	ND	1.80	(±0.41)	95	1.5
Ducitol	4	33.17	1.96	4.59	0.43	40.15	(±3.12)	87	32.9
Sorbitol	4	13.07	0.46	0.38	ND	13.91	(±4.10)	97	11.4
Mannitol	4	4.16	0.36	0.37	0.01	4.90	(±1.00)	92	4.0
Inositol	4	3.06	0.24	0.75	0.03	4.07	(±1.14)	81	3.3
Glucono-delta-lactone	4	2.84	0.07	0.14	0.01	3.06	(±0.58)	95	2.5
Gluconic Acid	4	3.24	0.28	0.31	ND	3.82	(±0.44)	92	3.1
Glucosamine	3	2.42	0.23	0.17	0.01	2.82	(±0.64)	94	2.3
Tricarboxylic Acid Cycle Intermediates and Related Compounds									
Citrate	4	1.71	0.08	0.47	0.08	2.35	(±0.23)	76	1.9
Isocitrate	4	1.81	0.19	0.40	0.01	2.40	(±0.51)	83	2.0
α-Ketoglutarate	4	0.73	ND	0.07	ND	0.80	(±0.32)	91	0.7
Succinate	3	1.63	0.13	0.63	ND	2.39	(±0.50)	74	2.0
Fumarate	4	0.99	0.04	0.23	ND	1.26	(±0.23)	82	1.0
Malate	8	4.37	0.33	2.07	0.02	6.80	(±1.31)	69	5.8
Oxaloacetate	4	0.23	0.02	ND	ND	0.25	(±0.10)	100	0.2
Malonate	4	0.78	0.05	0.40	ND	1.23	(±0.42)	67	1.0
Glyoxylate	4	0.04	ND	ND	ND	0.04	(±0.02)	100	<0.1

[a]Number of replicate cultures analyzed

[b]$B_1+B_2+G_1+G_2$: $\bar{X}$ (±SEM)

[c]$(B_1+B_2)/(B_1+B_2+G_1+G_2)$

[d]$\bar{X}$ for cultures with no carbon source

[e]None detected

and are in agreement with previous studies (Buchanan and Lewis 1984b; Buchanan *et al.* 1984). Glucose supported abundant production, while the peptone cultures did not support *de novo* aflatoxin synthesis. Low levels of aflatoxins detected in the peptone controls are attributable to a small "carry-over" from the initial growth of the mold in YES (Buchanan and Lewis 1984b; Buchanan *et al.* 1984). Cultures transferred to medium with no available carbon source displayed a response similar to that observed with peptone.

Due to the small carry-over of aflatoxins, cultures containing less than twofold the level of detectable toxin observed with the no-carbon source cultures ($\bar{X}/\bar{X}o \leqq 2.0$) were arbitrarily defined as being toxin-negative. Using this criterion, a wide range of carbon sources were capable of inducing aflatoxin synthesis, though the amounts of aflatoxins produced differed greatly among the various compounds. These responses were readily differentiated into nonsupporters and low, intermediate, and high producers using the criteria of 0-2, 2-10, 10-40, and >40 μg aflatoxin/culture, respectively.

Amino acids were generally nonsupporters, except aspartic acid which appeared to support low levels of aflatoxin synthesis. Similarly, intermediates of the tricarboxylic acid cycle did not support aflatoxin production, except for the malic acid which yielded low levels of synthesis.

The various sugars examined supported widely differing amounts of aflatoxin production. Glucose, ribose, fructose, sucrose, and maltose supported high levels of toxin accumulation, while trehalose and melezitose gave intermediate values. Low levels of production were observed with dihydroxyacetone, rhamnose, arabinose, galactose, and cellobiose. *De novo* aflatoxin synthesis was not apparent with lactose, soluble starch, and glyceraldehyde.

Sugar alcohols, acids, and amines also supported varying amounts of aflatoxin production. Glycerol yielded high levels of production, while ducitol, adonitol, and sorbitol gave intermediate values. Low levels of aflatoxin production were observed with mannitol, inositol, gluconic acid, glucono-delta-lactone, and glucosamine. Aflatoxin production was not observed with xylitol.

Comparison of the ability of intermediates and products of the Embden-Meyerhoff pathway to induce and support aflatoxin synthesis indicated a general pattern of decreasing toxin production for intermediates between glucose and 3-phosphoglycerate. This latter compound appeared to represent a critical breakpoint since *de novo* aflatoxin synthesis was not evident with 2,3-diphosphoglycerate, phosphoenolpyruvate, and various potential products of the EM pathway.

DISCUSSION

Various investigators have demonstrated that the synthesis of aflatoxins is dependent on the identity (Mateles and Adye 1965; Davis and Diener 1968; Abdollahi and Buchanan 1981b; Prasad 1983) and concentration (Mateles and Adye 1965; Davis *et al.* 1966, 1967; Hsieh and Mateles 1971; Shih and Marth 1974a, 1974b; Applebaum and Buchanan 1979; Buchanan and Houston 1982) of available carbon sources. Abundant aflatoxin production was associated generally with elevated levels of specific carbohydrates, and Abdollahi and Buchanan (1981a, 1981b) hypothesized that the active catabolism of suitable carbohydrates was involved in the regulation of aflatoxin synthesis. Glucose utilization by *A. parasiticus* was typically biphasic when batch cultures were employed (Shih and Marth 1974a; Applebaum and Buchanan 1979; Buchanan and Houston 1982), but linear in precultured mycelia (Buchanan and Lewis 1984a). Tice and Buchanan (1982) reported that exogenously-supplied cyclic AMP stimulated aflatoxin synthesis, and Bennett and Christensen (1983) suggested that this was due to a stimulation of glucose utilization. Conversely, Buchanan and Lewis (1984a) reported that aflatoxin synthesis was strongly depressed when glucose utilization was partially inhibited. They suggested that aflatoxin synthesis is dependent on the mold being capable of rapidly utilizing a carbohydrate. The results of these various investigations have indicated that aflatoxin synthesis is strongly influenced by carbohydrate catabolism.

The results of the present study support further the importance of carbohydrate catabolism as a determinant of aflatoxin synthesis, confirming and expanding the findings of Abdollahi and Buchanan (1981a, 1981b) that a wide variety of carbohydrates can induce and support aflatoxin synthesis after transfer from PMS medium. Abdollahi and Buchanan (1981b) suggested that the active catabolism of an appropriate carbohydrate may alter either the concentration of a key intermediate or the energy status of the cell; this, in turn, regulated the induction of aflatoxin synthesis. Subsequent studies (Buchanan and Lewis 1984b; Buchanan *et al.* 1984) have indicated that the catabolism of glucose may repress key enzymes associated with the pentose phosphate pathway, the mannitol shunt, and the tricarboxylic acid cycle.

The widely differing amounts of aflatoxins produced in conjunction with the sugars and sugar derivatives examined in the present study have been noted by other investigators (Mateles and Adye 1965; Davis and Diener 1968; Abdollahi and Buchanan 1981b; Prasad 1983). The reason for these differences is not known, but could reflect differences

in the mechanisms or relative rates of catabolism among the compounds. Further, the various investigations (including the present study) examining relative aflatoxin production on different carbohydrates have not considered the relative rates of transport of the compounds into the cell. Buchanan and Lewis (1984a) have hypothesized that the rate of carbohydrate transport across the plasma membrane influences aflatoxin synthesis. The lack of production on starch, which has been previously reported to support aflatoxin synthesis (Mateles and Adye 1965; Reddy *et al.* 1979; Prasad 1983), may reflect insufficient amylase levels during the post-transfer incubation.

Comparison of EM intermediates and related compounds indicated decreasing ability to suport aflatoxin synthesis through 3-phosphoglycerate. Subsequent intermediates and products of the pathway were incapable of inducing and/or supporting aflatoxin synthesis. This apparent "breakpoint" suggests that the steps mediating the conversion of 3-phosphoglycerate to phosphoenolpyruvate may be important in regard to the regulation of aflatoxin synthesis. However, it is possible that phosphoenolpyruvate, pyruvate, and the various potential products of the EM pathway are transported into the cell at a rate insufficient to support aflatoxin production.

An exception to the "breakpoint" noted above was the apparent low level aflatoxin synthesis (based on $\bar{X}/\bar{X}o > 2.0$) in conjunction with malate and aspartate. Generally, amino acids and tricarboxylic acid cycle intermediates as sole carbon sources are not considered to support aflatoxin synthesis (Davis and Diener 1968; Maggon *et al.* 1977; Abdollahi and Buchanan 1981b), though specific amino acids have been reported to stimulate aflatoxin production when employed in conjunction with a carbohydrate source (Reddy *et al.* 1979; Payne and Hagler 1983). Mateles and Adye (1965) did not observe aflatoxin production when malate was employed as a sole carbon source; however, this difference may reflect the use of cultures initiated from a conidial inoculum versus the sequential culturing technique employed in the present study. Malate and aspartate have been reported to play a role in fungi both as mediators of transport between the cytosolic and mitochondrial compartments of the cell, and in the generation of NADPH (Hult and Gatenbeck 1976; Osmani and Scrutton 1983), these, in turn, influencing the synthesis of fatty acids and polyketides (Hult and Gatenbeck 1976; Niehaus and Dilts 1982). How malate and aspartate are influencing aflatoxin synthesis by *A. parasiticus* will require further study.

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