

# Regulation of Aflatoxin Biosynthesis: Effect of Glucose on Activities of Various Glycolytic Enzymes

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Received 2 April 1984/Accepted 23 May 1984

**Catabolism of carbohydrates has been implicated in the regulation of aflatoxin synthesis. To characterize this effect further, the activities of various enzymes associated with glucose catabolism were determined in *Aspergillus parasiticus* organisms that were initially cultured in peptone-mineral salts medium and then transferred to glucose-mineral salts and peptone-mineral salts media. After an initial increase in activity, the levels of glucose 6-phosphate dehydrogenase, mannitol dehydrogenase, and malate dehydrogenase were lowered in the presence of glucose. Phosphofructokinase activity was greater in the peptone-grown mycelium, but fructose diphosphatase was largely unaffected by carbon source. Likewise, carbon source had relatively little effect on the activities of pyruvate kinase, malic enzyme, isocitrate-NADP dehydrogenase, and isocitrate-NAD dehydrogenase. The results suggest that glucose may, in part, regulate aflatoxin synthesis via a carbon catabolite repression of NADPH-generating and tricarboxylic acid cycle enzymes.**

Aflatoxins have been among the most intensely studied of the large variety of polyketide compounds synthesized by various fungal species. These mycotoxins are typically produced during a period of rapid glucose utilization (3, 8, 23), and Hsieh and Mateles (14) concluded that aflatoxins are synthesized extramitochondrially from acetyl coenzyme A derived from the catabolism of glucose. Tice and Buchanan (25) reported that cyclic AMP increased aflatoxin production, and they hypothesized that this was due to a stimulation of carbohydrate catabolism. Buchanan and Lewis (9) reported that caffeine inhibited aflatoxin synthesis by depressing the rate of glucose utilization.

Recently, Abdollahi and Buchanan (1, 2) reported that aflatoxins were not produced when *Aspergillus parasiticus* was cultured in a peptone-mineral salts medium, but synthesis did occur if the mycelia were transferred to a medium containing an appropriate carbohydrate. They further found that synthesis could be blocked by incorporation of cycloheximide or actinomycin D into the carbohydrate-containing medium and hypothesized that the induction of one or more enzymes of the aflatoxin synthase pathway is regulated by the active catabolism of carbohydrates. Since aflatoxin synthesis is closely associated with carbohydrate catabolism, the objective of the current study was to characterize the effect of glucose on the activities of enzymes associated with carbohydrate utilization. This was achieved by employing a nutritional shift protocol: transferring *A. parasiticus* from a carbohydrate-free medium that does not support aflatoxin production to a carbohydrate-containing medium that does support aflatoxin synthesis.

## MATERIALS AND METHODS

**Microorganism.** *A. parasiticus* NRRL 2999 was used throughout the study. Stock cultures were maintained on potato dextrose agar (Difco Laboratories) slants stored at 4°C. Spore suspensions were prepared as previously described (25) and diluted to contain approximately  $10^6$  conidia per ml.

**Media.** YES (2% yeast extract plus 6% sucrose) medium (10) was used for initial culturing of the mold. Subsequent

culturing employed peptone-mineral salts medium (PMS) and glucose-mineral salts medium (GMS). PMS consisted of: peptone (Difco), 60 g;  $(\text{NH}_4)_2\text{SO}_4$ , 2 g;  $\text{MgSO}_4$ , 2 g;  $\text{KH}_2\text{PO}_4$ , 10 g; metal mix, 1.0 ml; and water, 1,000 ml. GMS was identical, except that peptone was replaced by glucose. Both media were adjusted to pH 4.5 with 10 N HCl before sterilization by autoclaving for 10 min at 15 lb/in<sup>2</sup>. The metal mix was a modification of that of Mateles and Aday (19) and has been previously described (7).

**Culture techniques.** Cultures were grown by using a modification of the technique described by Niehaus and Dilts (21). Six 1.0-liter Erlenmeyer flasks containing 250 ml of YES medium were each inoculated with 1.0 ml of spore suspension to achieve an initial inoculum of approximately  $4 \times 10^3$  conidia per ml. All flasks were incubated on a rotary shaker (150 rpm) at 28°C for 72 h. Mycelial pellets were collected and pooled on cheesecloth, rinsed with sterile 0.85% KCl, and transferred to a sterile blender containing 450 ml of 0.85% KCl. The mycelia were homogenized at high speed for 1 min and then recollected on cheesecloth. After being rinsed thoroughly with 0.85% KCl, the mycelia were divided into equal portions and transferred to eight 1.0-liter Erlenmeyer flasks containing 300 ml of PMS. The flasks were then incubated for 24 h at 28°C on a rotary shaker (150 rpm).

The mycelia were collected and pooled on cheesecloth, rinsed thoroughly with 0.85% KCl, and divided into 57 3.6-g portions. Three portions were immediately frozen in liquid N<sub>2</sub>. The remaining portions were transferred to 50-ml Erlenmeyer flasks containing 15 ml of either PMS or GMS. All flasks were incubated without agitation at 28°C.

After 1, 5, 23, 29, 47, 53, 70, 77, and 143 h, triplicate flasks of each medium were removed and the pH was determined. Each mycelium was collected on cheesecloth, with the medium being collected and frozen for subsequent aflatoxin analysis. The mycelium was then rinsed thoroughly and immediately frozen in liquid N<sub>2</sub>. All frozen mycelium samples were subsequently freeze-dried, weighed to determine dry weight, and stored at -80°C pending enzyme analyses.

**Aflatoxin analysis.** The levels of extracellular aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> were used as an estimate of total aflatoxin production (1, 9). A 5.0-ml portion of the collected medium

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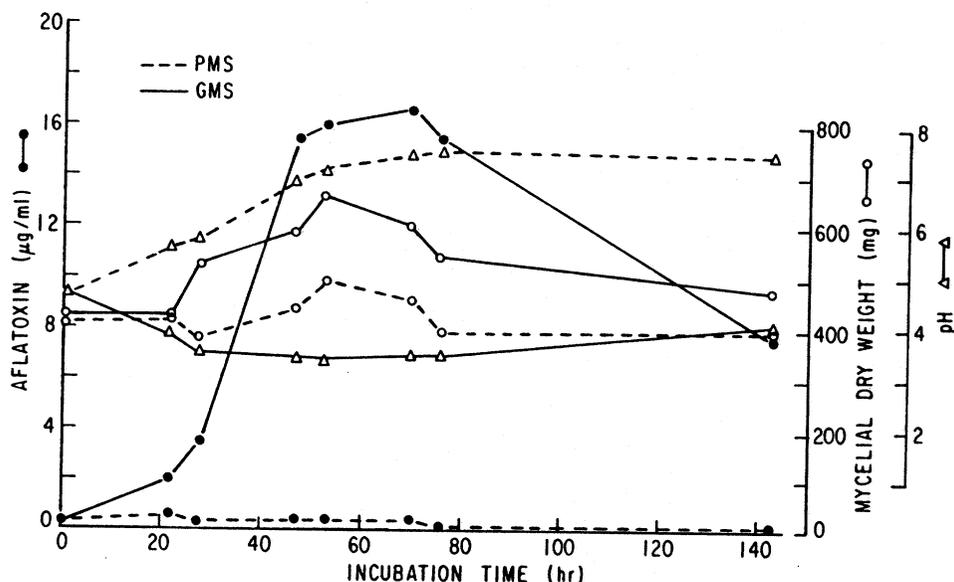


FIG. 1. Mycelial dry weight, pH, and extracellular aflatoxin concentration of PMS-cultured *A. parasiticus* after transfer to GMS and PMS.

was extracted thrice with 5.0-ml portions of chloroform. The chloroform extracts were pooled and cleaned by the silica gel cartridge technique of McKinney (20), and aflatoxins were quantitated by high-pressure liquid chromatography as previously described (7).

**Enzyme analyses.** Cell extracts were prepared by using a modification of the procedure of Niehaus and Dilts (21). Approximately 50 mg of freeze-dried mycelium was rehydrated in 1.5 ml of cold pH 7.2 buffer (50 mM  $K_2HPO_4$ , 1 mM EDTA, 2 mM mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride), and immediately disrupted with a Teflon-on-glass homogenizer. The suspension was then centrifuged ( $7,000 \times g$ ) for 10 min at  $4^\circ C$ , and the supernatant was employed for enzyme analyses. Enzyme analyses were performed on mycelia from at least three, and in most instances six, replicate cultures. The protein content of the extracts was determined by the method of Lowry et al. (17), using bovine serum albumin as a standard.

The specific activities of evaluated enzymes were assayed by monitoring the oxidation or reduction of the appropriate pyridine nucleotide at 340 nm, using a spectrophotometer (model DU-6, Beckman Instruments, Inc.) equipped with kinetics software. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), phosphofructokinase (EC 2.7.1.11), and fructose-1,6-diphosphatase (EC 3.1.3.11) were assayed by the techniques of Gancedo and Gancedo (12), modified by adjusting the buffer system to pH 8.2. Mannitol dehydrogenase (EC 1.1.1.138) was assayed according to Niehaus and Dilts (21), except that a 50 mM imidazole buffer (pH 9.2) was employed. Published protocols were used for the analyses of isocitrate-NADP dehydrogenase (EC 1.1.1.42) (16), isocitrate-NAD dehydrogenase (EC 1.1.1.41) (16), malic enzyme (EC 1.1.1.40) (15), pyruvate kinase (EC 2.7.1.40) (26), and malate dehydrogenase (EC 1.1.1.37) (26).

## RESULTS

The dry weights, pH, and aflatoxin production by *A. parasiticus* transferred from PMS to PMS and GMS are shown in Fig. 1. The posttransfer mycelia incubated in PMS varied little in dry weight, but the GMS cultures increased in dry weight by approximately 50% between 22 and 53 h of

incubation. The PMS cultures increased in pH, but there was a depression of pH in the GMS cultures. Aflatoxin production was evident in the GMS cultures after 23 h of incubation, and the concentration of extracellular aflatoxins increased rapidly thereafter. Maximal levels were detected between 47 and 70 h and then decreased by approximately 50% after 143 h of incubation. Aflatoxin production was not evident in the PMS cultures over the course of the incubation. Analysis of pretransfer mycelia indicated that the trace levels of aflatoxins detected in the posttransfer PMS cultures could be attributed to a small "carry-over" from the initial growth of the mold in YES.

The effects of posttransfer incubation in PMS and GMS on the specific activities of selected enzymes associated with glucose catabolism are summarized in Fig. 2A to I.

Glucose 6-phosphate dehydrogenase (Fig. 2A) and mannitol dehydrogenase (Fig. 2B) had similar activity patterns, being elevated immediately after transfer and then declining upon further incubation. The activity of both enzymes was consistently higher with the PMS-cultured samples.

Phosphofructokinase (Fig. 2C) activity in GMS-cultured mycelia was low and declined slightly with incubation. Activity in the PMS-cultured samples was roughly equivalent to that observed with GMS during the initial phases of posttransfer incubation. However, increased activity was observed in the 53-, 72-, and 77-h samples. Fructose diphosphatase (Fig. 2D) activity was low and unaffected by carbon source.

The activities of pyruvate kinase (Fig. 2E), malic enzyme (Fig. 2G), and isocitrate-NAD dehydrogenase (Fig. 2I) were low, relatively constant, and approximately equivalent in PMS and GMS samples. Isocitrate-NADP dehydrogenase (Fig. 2H) activity was highly variable among replicates; however, there appeared to be a trend in the PMS cultures toward elevated activity immediately after transfer, followed by decreasing activity upon further incubation. The GMS-cultured mycelia tended toward increasing activity until 29 h, depressed activity from 53 to 77 h, and elevated activity at 143 h.

Of the various enzymes analyzed, malate dehydrogenase (Fig. 2F) displayed the greatest differential with regard to the

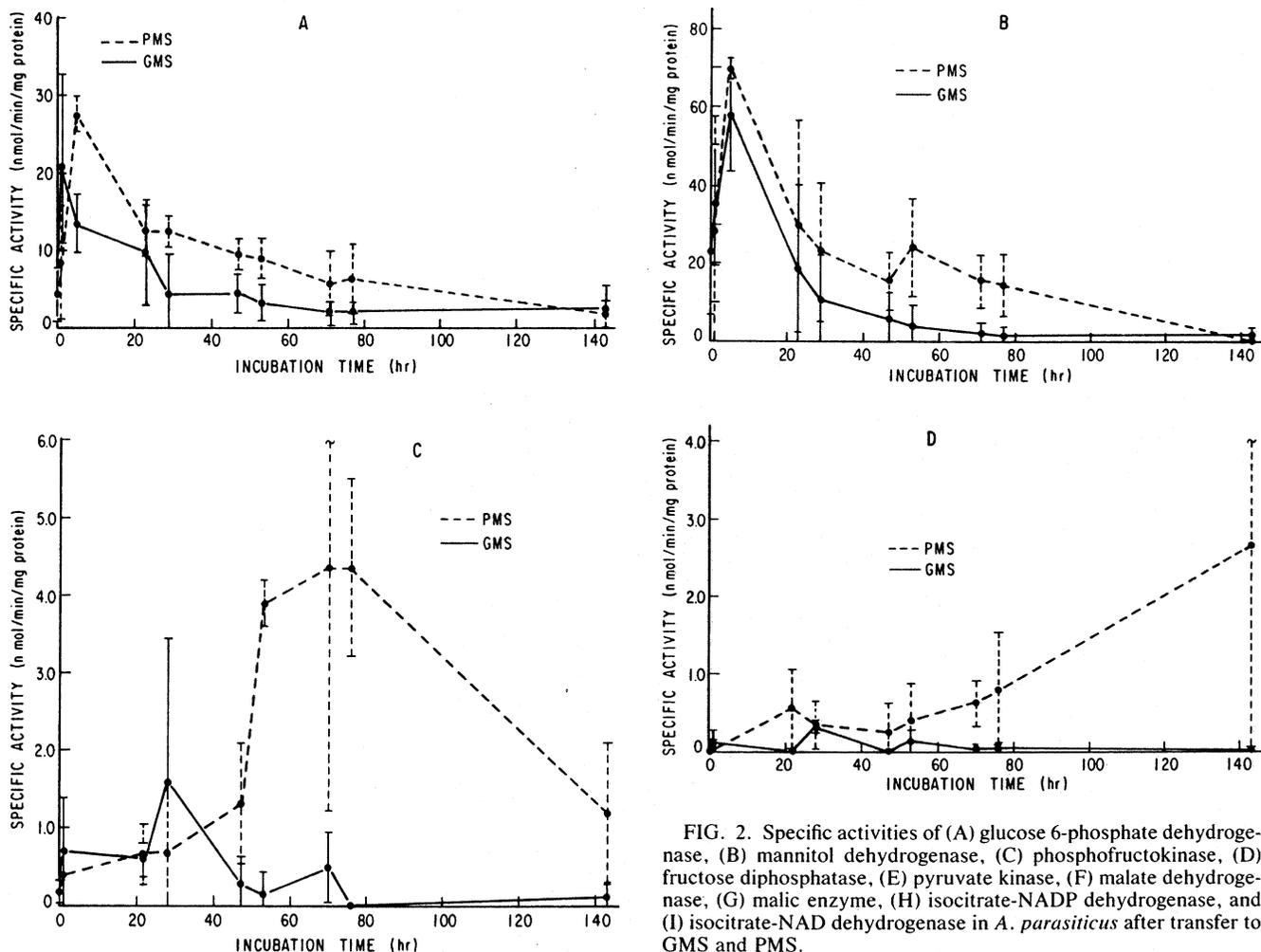


FIG. 2. Specific activities of (A) glucose 6-phosphate dehydrogenase, (B) mannitol dehydrogenase, (C) phosphofructokinase, (D) fructose diphosphatase, (E) pyruvate kinase, (F) malate dehydrogenase, (G) malic enzyme, (H) isocitrate-NADP dehydrogenase, and (I) isocitrate-NAD dehydrogenase in *A. parasiticus* after transfer to GMS and PMS.

two carbon sources. With PMS, activity was elevated in the 23-h samples and remained elevated throughout the course of the incubation. The GMS-cultured mycelia had approximately constant activity through 29 h and then declined with further incubation. During the period of active aflatoxin synthesis (i.e., 23 to 53 h), there was approximately a two- to sevenfold differential in malate dehydrogenase activity between the GMS- and PMS-cultured mycelia.

#### DISCUSSION

One difficulty in studying aspects of primary metabolism that influence synthesis of a secondary metabolite is distinguishing events associated with growth of the microorganism from those directly related to secondary metabolism. This is particularly true when attempting to compare cultures from media that support different rates of growth. The current study largely avoided this difficulty by employing a sequential culturing technique that included transferring the mold at high mycelial densities to restrict posttransfer growth. The relatively small increase in mycelial dry weights observed with the GMS cultures is assumed to largely represent an accumulation of glucose-derived secondary metabolites (i.e., polyketides, lipids, etc.). However, even if the differential between the PMS and GMS cultures totally represents differences in growth, it is small compared with that expected if conidium-inoculated cultures had been employed (1, 8).

The pattern of aflatoxin production observed in the present study was in agreement with earlier reports that established that *S. parasiticus* does not produce aflatoxins in PMS but commences synthesis if transferred to GMS (1) or if glucose is added to PMS (1, 8). The pH patterns of the PMS and GMS cultures were distinctly different and reflected the metabolic orientations of the cultures (carbohydrate versus amino acid catabolism). It is possible that other effects observed could result partly from this pH differential. Buchanan and Ayres (5) did observe that aflatoxin production was depressed in *A. parasiticus* cultures having an initial pH of  $>7.0$ ; however, there was not a complete lack of synthesis as observed with the PMS culture. Using PMS supplemented with various levels of glucose, Buchanan and Houston (8) reported that aflatoxin production was a function of the glucose concentration and that production occurred despite elevated pH levels due to the catabolism of the peptone. This suggests that the differential aflatoxin production in PMS and GMS in the present study cannot be attributed to pH differences, but instead reflects the effect of carbon source.

Of the various enzymes assayed, malate dehydrogenase was most affected by the carbon source of the posttransfer medium. The pattern observed is consistent with either glucose-reducing activity via a carbon catabolite repression or peptone-stimulating activity by induction. It is generally assumed that aflatoxin synthesis occurs during a period of

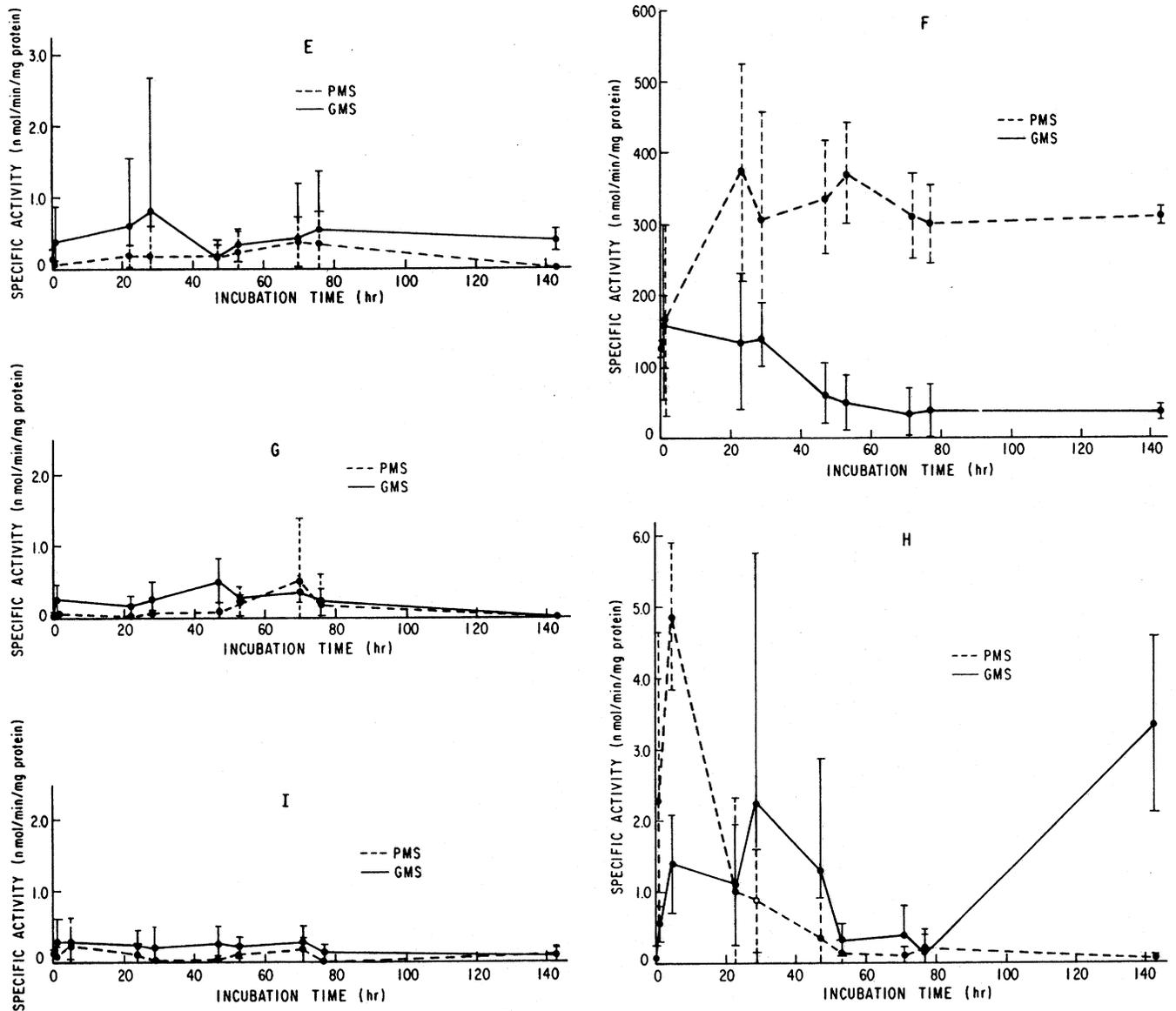


FIG. 2. continued

decreased tricarboxylic acid (TCA) cycle activity, though the specific role this plays is not clear. Gupta et al. (13) and Maggon et al. (18) hypothesized that reduced TCA cycle activity leads to an accumulation of TCA cycle intermediates, which leads to a shunting of acetyl coenzyme A to aflatoxin synthesis. However, Buchanan and Ayres (6) and Shantha and Murthy (22) concluded that any significant accumulation of intermediates increases TCA cycle activity and concomitantly depresses aflatoxin production. The catabolism of peptone to TCA intermediates could have induced increased activity; however, Buchanan and Houston (8) observed aflatoxin production in a peptone-glucose medium, suggesting that the effect observed in the present study results from a carbon catabolite repression.

A loss of TCA cycle activity would be expected to produce a shunting of acetyl coenzyme A equivalents to anabolic processes such as polyketide or lipid synthesis. The extent to which the overall activity of the TCA cycle is depressed will require more detailed experimentation. The lack of an equivalent effect with isocitrate-NAD dehydroge-

nase or isocitrate-NADP dehydrogenase suggests that not all TCA cycle enzymes are controlled via carbon catabolite repression. The effect observed with malate dehydrogenase may be limited to only a few TCA cycle enzymes, thereby allowing the catabolic function of this pathway to be blocked while sufficient activity is maintained to meet the anabolic needs of the microorganism. Maggon et al. (18) observed differential changes among selected TCA cycle enzymes in *A. parasiticus* as a function of growth phase.

Activities of glucose 6-phosphate dehydrogenase and mannitol dehydrogenase both decreased with incubation time and were consistently greater in the PMS cultures. This suggests that these enzymes are age dependent and carbon catabolite repressible. Comparison of specific activities of these enzymes with activities of malic enzyme and isocitrate-NADP dehydrogenase suggests further that the pentose phosphate pathway and mannitol shunt are the primary sites for NADPH generation of *A. parasiticus*. Further, the decreased activities of glucose 6-phosphate dehydrogenase and mannitol dehydrogenase in the GMS-cultured mycelia

were not compensated by increased activities of malic enzyme or isocitrate-NADP dehydrogenase or both, suggesting that the GMS-cultured cells had depressed levels of NADPH.

The relative concentrations of NADP and NADPH have also been proposed as a factor of primary metabolism that can affect aflatoxin synthesis. Shih and Marth (23) and Maggon et al. (18) have proposed that elevated levels of NADPH stimulate aflatoxin synthesis, and Singh and Hsieh (24) have demonstrated that in vitro conversion of sterigmatocystin to aflatoxin B<sub>1</sub> is NADPH dependent. However, Niehaus and Dilts (21) proposed that an elevated NADPH/NADP ratio favors fatty acid synthesis, whereas a depressed NADPH/NADP ratio favors polyketide formation. The apparent loss of NADPH-generating capacity observed with the toxin-supporting GMS medium in the present study suggests that depressed NADPH/NADP ratios favor aflatoxin synthesis. This effect would be particularly significant considering that both glucose 6-phosphate and mannitol dehydrogenases have also been reported to be strongly inhibited by zinc levels that support high levels of aflatoxin production (21).

Detroy et al. (11) and Maggon et al. (18) have speculated that carbon catabolite repression is one regulatory mechanism controlling aflatoxin synthesis; however, neither identified specific sites affected by the catabolism of carbohydrates. The present study demonstrates that three potential loci regulated by carbon catabolite repression are malate dehydrogenase, glucose 6-phosphate dehydrogenase, and mannitol dehydrogenase. Using a similar experimental protocol, Abdollahi and Buchanan (1, 2) demonstrated that initiation of aflatoxin production after transfer from PMS to GMS required a period of de novo RNA and protein synthesis. They observed this response with a variety of carbohydrates and suggested that a process analogous to, but opposite from, carbon catabolite repression (i.e., a carbon catabolite induction) controls the synthesis of one or more of the enzymes associated with aflatoxin synthesis. They further suggested that this process is regulated by either the accumulation of a common intermediate of carbohydrate catabolism or an alteration in the energy status of the cell. The present study demonstrates that the addition of glucose produces alterations in the specific activities of a number of key enzymes associated with carbohydrate utilization. These changes in enzyme activities may lead to the altered energy status or metabolite accumulation proposed by Abdollahi and Buchanan (1, 2) as the regulator of the induction of aflatoxin production. Additional studies are under way to determine possible relationships among these observed effects.

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