Caffeine Inhibition of Aflatoxin Synthesis: Probable Site of Action

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Received 27 February 1984/Accepted 2 March 1984

Aflatoxin production by pregrown cultures of *Aspergillus parasiticus* was completely inhibited by incorporation of 2 mg of caffeine per ml into the medium. This was accompanied by a decrease in glucose utilization and an inhibition of oxygen uptake and carbon dioxide evolution. Enzyme analyses indicated no significant differences in specific activities on glucose-6-phosphate dehydrogenase, mannitol dehydrogenase, phosphofructokinase, fructose-1,6-diphosphatase, pyruvate kinase, or malate dehydrogenase. Glucose uptake kinetics indicated a linear dose-related inhibition of glucose uptake. It appears likely that caffeine inhibits aflatoxin synthesis by restricting the uptake of carbohydrates which are ultimately used by the mold to synthesize this family of mycotoxins.

Caffeine (1,3,7-trimethylxanthine) has been studied extensively during the past several years and has been shown to produce a wide range of effects in a variety of biological systems. Among the effects noted, it has been demonstrated that caffeine inhibits growth and polyketide mycotoxin production by a number of *Aspergillus* and *Penicillium* species (7, 8, 10). It has been concluded that this activity, at least in part, accounts for the resistance of cocoa and coffee beans to contamination by aflatoxins (14, 18), and has been hypothesized that the role of caffeine in these commodities is that of a naturally occurring fungistatic agent (8).

We have been studying the mechanism by which caffeine inhibits aflatoxin production to gain insights into the bioregulatory processes that control the synthesis of this family of mycotoxins. It has been previously established that inhibition by caffeine is highly specific, in that a variety of compounds similar to caffeine are ineffective (6, 7, 9, 10), and that the process does not appear to involve an inhibition of cyclic AMP phosphodiesterase (9). It was also observed that the inhibition of growth and of aflatoxin production may represent separate effects, since the inhibition of growth could be partially overcome by supplementation with adenine or guanine, but these purines had little effect on aflatoxin synthesis (9). The objective of the current study was to further investigate the inhibition of aflatoxin production by caffeine by examining the effect of caffeine on glucose utilization in *Aspergillus parasiticus*.

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 (20) and diluted to contain 10^6 conidia per ml.

Media. The mold was initially cultured in YES medium (2% yeast extract, 6% sucrose) (11) supplemented with a trace metal stock solution (9) at a level of 1.0 ml/liter. Subsequent culturing employed YEG medium, which had the same composition as YES medium except that the sucrose was replaced by glucose on a per gram basis. Appropriate amounts of caffeine were added to the YEG medium before sterilization by autoclaving.

Culture techniques. One-liter Erlenmeyer flasks containing 300 ml of YES medium were each inoculated with 2.0 ml of spore suspension to achieve an inoculum of 6×10^3 conidia per ml. The flasks were incubated for 72 h at 28°C on a rotary shaker (150 rpm). Mycelial pellets were then collected on cheesecloth, rinsed with sterile 0.85% KCl, and transferred to a sterile blender jar containing 450 ml of 0.85% KCl. After homogenization at high speed for ca. 1 min, the mycelium was recollected on cheesecloth and rinsed well with 0.85% KCl. It was then transferred in 20-g portions to 1.0-liter Erlenmeyer flasks containing 500 ml of YEG medium and incubated for 24 h at 28°C on a rotary shaker (150 rpm). The mycelium was collected and pooled on cheesecloth, rinsed with 0.85% KCl, and subsequently transferred in 12-g portions to 125-ml Erlenmeyer flasks containing 25 ml of YEG medium with 0 or 2 mg of caffeine per ml. All flasks were then incubated without agitation at 28°C.

After 0, 1, 2, 4, 22, 28, 46, 52, and 70 h, duplicate flasks of each caffeine concentration were removed, and the mycelia were collected on individual filter papers (Whatman no. 1). The medium was collected in plastic tubes and immediately stored in a −20°C freezer for later analyses for aflatoxins and residual glucose. Each harvested mycelium was then rinsed with 0.85% KCl, frozen in liquid nitrogen, and immediately freeze-dried. Dried mycelial samples were stored at −80°C while awaiting analyses for enzyme activities.

Mycelia designated for dry-weight determinations were cultured in a similar manner except that after the removal of the medium, the mycelium from each flask was extracted for 30 min with 75 ml of acetone. The solvent was removed by filtration through a preweighed filter paper, and each mycelium was rinsed with distilled water. The filters were then placed in an 85°C oven for 18 h, and mycelial dry weights were determined gravimetrically.

Aflatoxin and residual glucose analyses. A 5.0-ml portion of the collected medium was transferred to a 15-ml conical tube, and 5.0 ml of chloroform was added. After thorough mixing, the layers were allowed to separate, and the chloroform layer was transferred to a 20-ml vial with a Pasteur pipette. The extraction was repeated twice, and the chloroform layers were pooled. The solvent was then evaporated under nitrogen, and the samples were cleaned up by the method of McKinney (16) and analyzed for aflatoxins by previously described techniques (9). After initial experimentation indicated that caffeine did not affect the release of

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aflatoxins by the mycelium, the sum of extracellular aflatoxins B_1, B_2, G_1, and G_2 was used as the estimate of total aflatoxin production.

Using 20-μl portions of 10^{-1} and 10^{-2} dilutions of the collected medium, we determined residual glucose concentrations with oT-V glucose analysis kits (StanBio Laboratory, Inc.).

Enzyme determinations. Cell extracts were prepared by a modification of the procedure of Niehaus and Dilts (17). Freeze-dried mycelium (50 mg) was rehydrated in 1.5 ml of pH 7.2 buffer (50 mM K_2 HPO_4, 1 mM EDTA, 2 mM mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) and disrupted with a Teflon-on-glass homogenizer. The resultant suspension was centrifuged (7,000 × *g*) for 5 min at 4°C, and the supernatant was used immediately for enzyme analyses.

Enzyme reaction rates were determined by monitoring at 340 nm the oxidation or reduction of pyrimidine nucleotides with a spectrophotometer (model DU-6; Beckman Instruments, Inc.) equipped with kinetics software in conjunction with 1.0-cm disposable polystyrene cuvettes. Glucose-6-phosphate dehydrogenase, phosphofructokinase, and fructose-1,6-diphosphatase were assayed by the techniques of Gancedo and Gancedo (12), except that the buffer system was adjusted to pH 8.2. Mannitol dehydrogenase was assayed by the procedure of Niehaus and Dilts (17) modified to employ a 50 mM imidazole buffer (pH 9.2). Malate dehydrogenase and pyruvate kinase were assayed by the procedures outlined in the *Worthington Enzyme Manual* (23).

Glucose uptake determination. The mold was sequentially cultured in YES and YEG media as described above. The washed mycelium was then transferred to a 500-ml Erlenmeyer flask containing 300 ml of a mineral solution (1,000 ml of water, 10 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 2 g of $MgSO_4$, 1.0 ml of trace metal mix [9]; adjusted to pH 4.5 with HCl) and incubated for 1.0 h at 28°C on a rotary shaker (150 rpm). The mycelium was then harvested on cheesecloth, rinsed with 0.85% KCl, and transferred in 100 mg portions to sterile tubes (150 × 20 mm) containing 3.0 ml of the mineral solution with caffeine concentrations of 0.0, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/ml. All tubes were incubated in a rack on a rotary shaker (100 rpm) for 30 min at 28°C.

Each tube then received 20 μl of a solution of [*U*-^{14}C]glucose (125 μCi/ml; 4.4 mCi/mmol). After 15 and 60 min of incubation, three tubes of each caffeine concentration were removed, and the mycelia were collected on 0.45-μm pore-size filters. After being rinsed well with distilled water, the filters were air dried for ca. 30 min, and the mycelia were subsequently transferred to plastic scintillation vials containing 4.5 ml of Aquasol (New England Nuclear Corp.). Radioactivity was measured with a liquid scintillation detector (model LS8100; Beckman Instruments, Inc.). Glucose uptake was linear over the test period, and glucose uptake rates were determined by calculating the slope of the least-squares regression.

Respirometry. The mold was sequentially cultured in YES and YEG media as described above. The mycelium was then transferred in 1.0-g portions to 10 Warburg flasks containing 2.0 ml each of YEG medium and to a second set of 10 flasks containing YEG medium with 2 mg of caffeine per ml. Five flasks of each set were used to determine oxygen uptake, and the remaining five flasks were used to estimate CO_2 evolution. All flasks were incubated without agitation at 28°C. After 5, 24, 30, 49, 54, 73, and 78 h, the flasks were connected to a respirometer (model G20; Gilson, Inc.), and oxygen uptake and CO_2 evolution were monitored for 20 min by standard techniques (22).

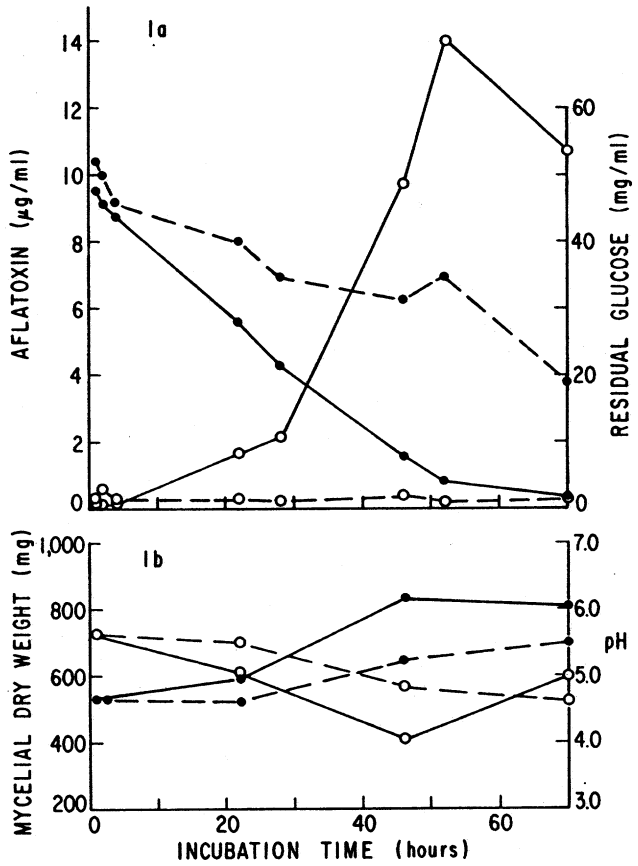

FIG. 1. (a) Production of aflatoxins (○) and residual glucose concentration (●); (b) mycelial dry weight (●) and pH (○) of *A. parasiticus* cultured in YEG medium containing 0 (——) or 2 (- -) mg of caffeine per ml.

RESULTS AND DISCUSSION

When caffeine is added to YES and YEG medium at a concentration of 2 mg/ml and then inoculated with conidia of *A. parasiticus*, it typically causes an 80 to 90% inhibition of growth, while decreasing aflatoxin production by >98% (7,

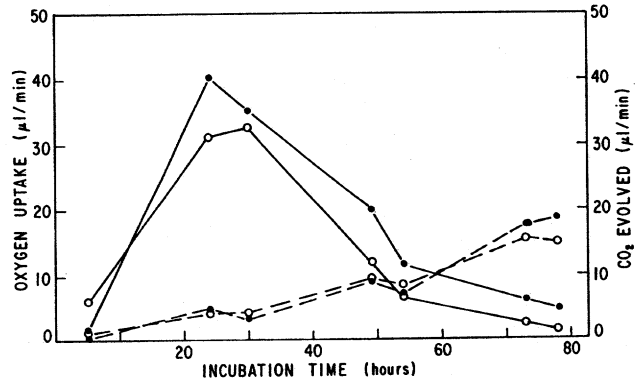

FIG. 2. Oxygen uptake (●) and CO_2 evolved (○) by *A. parasiticus* cultured in YEG medium containing 0 (——) or 2 (—— ——) mg of caffeine per ml.

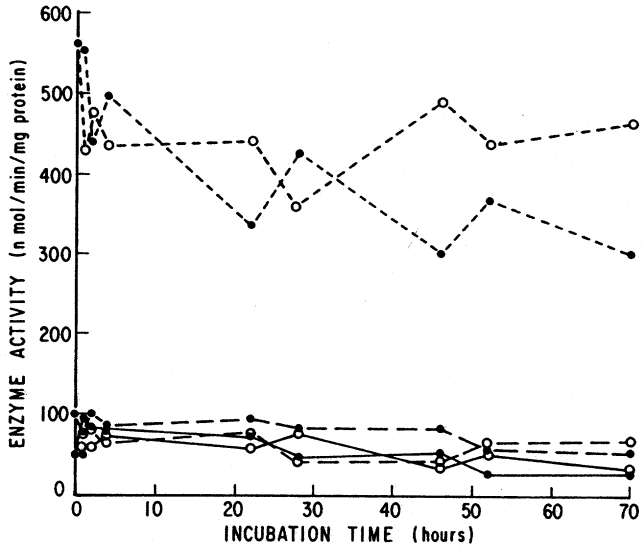

FIG. 3. Specific activities of malate dehydrogenase (– – –), glucose-6-phosphate dehydrogenase (——), and mannitol dehydrogenase (—— ——) in *A. parasiticus* cultured in YEG medium containing 0 (●) or 2 (○) mg of caffeine per ml.

9). This inhibition of growth makes it difficult to directly study the effect of caffeine on aflatoxin synthesis; since aflatoxins are secondary metabolites, the production of these mycotoxins is dependent on achieving a degree of growth. To avoid this problem, we used a sequential culturing technique in which the mold was initially cultured in caffeine-free medium. The mycelium was then transferred (at high density, to limit further growth) into medium containing either no caffeine or 2 mg of caffeine per ml. The effects of caffeine on the mycelial dry weight, aflatoxin production, pH, and residual glucose concentration of *A. parasiticus* cultures grown in this manner are depicted in Fig. 1.

Both caffeine-treated and caffeine-free cultures increased in dry weight by a relatively small degree; the increase was

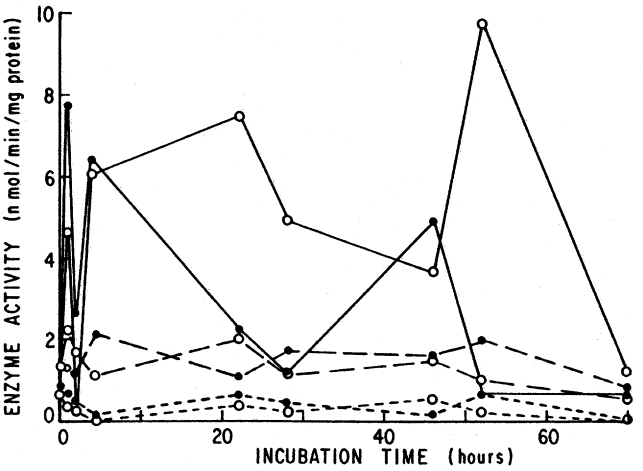

FIG. 4. Specific activities of pyruvate kinase (——), phosphofructokinase (—— ——), and fructose-1,6-diphosphatase (– – –) in *A. parasiticus* cultured in YEG medium containing 0 (●) or 2 (○) mg of caffeine per ml.

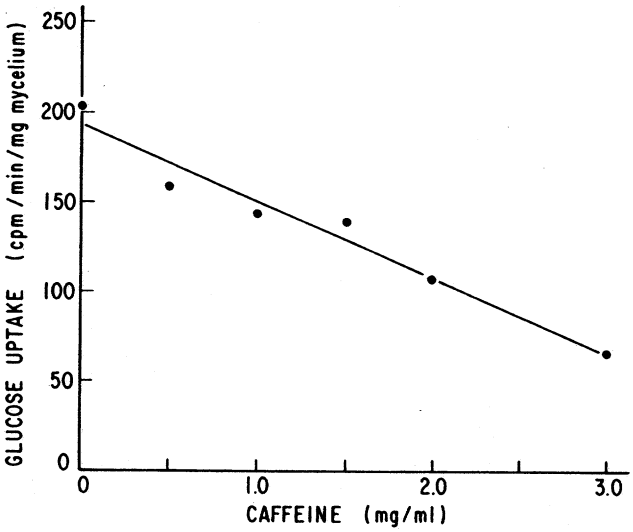

FIG. 5. Effect of caffeine on the rate of [U-^{14}C]glucose uptake by *A. parasiticus*.

greater in the untreated cultures. However, the differential between the caffeine-treated and caffeine-free cultures was relatively small, representing a ca. 22% difference at its greatest. With this preculturing protocol, problems with attempting to compare treated and untreated cultures having large differences in mycelial mass could be avoided.

Extracellular aflatoxin levels, used as an indicator of total aflatoxin production, show that caffeine strongly affected aflatoxin synthesis in the pregrown cultures. In caffeine-free cultures, aflatoxin production was detected after 22 h and reached a maximum at 52 h. No de novo aflatoxin production was detected in the caffeine-treated cultures over the course of the 70-h incubation. The low aflatoxin levels detected in the medium of the caffeine-containing cultures could be accounted for by carry-over from the preculturing of the mold in caffeine-free medium before the transfer. These results suggest that the inhibition of aflatoxin production by caffeine cannot be completely attributed to insufficient mycelial development, in agreement with Buchanan et al. (8, 9), who hypothesized that inhibition of growth and of mycotoxin production are not directly related.

Caffeine also depressed glucose utilization, based on the measurement of residual glucose concentrations in the medium. In caffeine-free cultures, glucose was used rapidly and was largely depleted by 52 h. This period of rapid glucose utilization coincided with that for aflatoxin production and pH depression. In caffeine-treated cultures, the rate of glucose utilization was reduced, and ca. one-third of the glucose was still available after 70 h of incubation. The pHs of these cultures also decreased at a slower rate.

Caffeine treatment also produced an inhibition of respiration in the mold (Fig. 2). In caffeine-free medium, both oxygen uptake and carbon dioxide evolution by the mold increased rapidly during the first 24 h of incubation, followed by a gradual decline upon further incubation. This initial burst of respiratory activity was not evident in the caffeine-treated cultures. Instead, both oxygen uptake and carbon dioxide production were low during the initial phase of the incubation and gradually increased over time. The maximal rate of respiration in the caffeine-treated cultures was only half of that observed in the caffeine-free cultures.

Aflatoxin synthesis has been shown to be highly dependent on the catabolism of an appropriate carbohydrate source. Production typically occurs during a period of rapid glucose utilization (3, 19), and Hsieh and Mateles (13) concluded that aflatoxins are synthesized extramitochondrially from acetyl coenzyme A derived from glucose. Abdollahi and Buchanan (1, 2) have hypothesized that the induction of one or more of the enzymes of the aflatoxin synthesis pathway is dependent on the catabolism of a suitable carbohydrate. Restricting carbohydrate catabolism in *A. parasiticus* would be expected to decrease its ability to synthesize its mycotoxins. The initial results obtained in the present study were consistent with the inhibition of aflatoxin synthesis by caffeine in such a manner.

Possible modes of action by which compounds may alter glucose utilization are alteration in the synthesis or activity of enzymes associated with carbohydrate catabolism, inhibition of glucose transport, or modification of intracellular energy status. Recently, Tortora et al. (21) reported that caffeine, but not theophylline, affected glucose utilization in *Saccharomyces cerevisiae*. In this microorganism, caffeine depressed the rate of glucose utilization, elevated ATP levels and energy charge, altered the pattern of glycolytic intermediates, and delayed the catabolite inactivation of fructose-1,6-diphosphatase, malate dehydrogenase, and phosphoenolpyruvate carboxykinase. Tortora et al. (21) similarly ruled out caffeine inhibition of cyclic AMP phosphodiesterase as the mode of action and suggested that the compound may alter the regulation of fructose-1,6-diphosphatase, phosphofructokinase, or both.

The possibility that caffeine affects fructose-1,6-diphosphatase, phosphofructokinase, or both was evaluated by determining their activities in *A. parasiticus* cultured in medium containing 0 or 2 mg of caffeine per ml. Additionally, the activities of glucose-6-phosphate dehydrogenase, mannitol dehydrogenase, malate dehydrogenase, and pyruvate kinase were determined (Fig. 3 and 4).

The specific activities of the various enzymes remained relatively constant over the course of the 70-h incubation. Treatment with caffeine had little, if any, effect on the tested enzymes. A possible exception was pyruvate kinase, for which there was a trend toward higher activity in the caffeine-treated mycelium. However, pyruvate kinase activity varied greatly between and among incubation times, and the differences between caffeine-free and caffeine-treated cultures were not significant. Preliminary assessment of the effect of caffeine on the in vitro activity of the tested enzymes also suggested that caffeine does not directly inhibit enzyme activity. These results suggest that caffeine did not inhibit glucose utilization and aflatoxin synthesis in *A. parasiticus* by altering the level or activity of enzymes associated with glucose catabolism.

The alternative possibility that caffeine inhibited glucose utilization by interfering with glucose transport was examined by determining the kinetics of [U-^{14}C]glucose uptake by mycelia preincubated with various levels of caffeine. The results indicate that increases in caffeine concentration produced a linearly related decrease in the rate of glucose uptake (Fig. 5). The 2-mg/ml caffeine level employed throughout the rest of the study caused a ca. 50% decrease in the uptake rate.

This observed inhibition of glucose transport appears to readily account for the ability of caffeine to inhibit aflatoxin synthesis, and it seems likely that this is the site of action for the compound. As already indicated, the synthesis of aflatoxins is highly dependent on carbohydrate catabolism. It has been hypothesized that before the initiation of aflatoxin synthesis, the mold must be capable of catabolizing carbohydrates at a rate rapid enough to lead to an accumulation of intermediates of primary metabolism (4). By sufficient restriction of glucose transport, this accumulation of primary metabolites would be limited, thereby disrupting the supply of intermediates normally used for the synthesis of polyketides.

How caffeine inhibits glucose transport awaits future research. Little information is available concerning the mechanism of glucose transport in *A. parasiticus*, but presumably it is similar to that in *Aspergillus nidulans*, in which glucose is taken up without phosphorylation by an active transport system (5). Potentially, caffeine could interfere with this process either by directly inhibiting the carrier system or by depleting available energy sources. Tortora et al. (21) have reported that caffeine elevated the ATP level and energy charge of *S. cerevisiae*, and Lopez-Saez et al. (15) reported similar results with onion cells. These data suggest that caffeine does not affect glucose transport by depleting available energy sources and that it is more likely that the compound is acting either by directly inhibiting the carrier system or by some as yet unidentified mechanism.

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