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Some Cultural Conditions That Control Biosynthesis of Lipid and Aflatoxin by *Aspergillus parasiticus*

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Synthesis of total lipid and aflatoxin by *Aspergillus parasiticus* as affected by various concentrations of glucose and nitrogen in a defined medium and by different incubation temperatures was studied. Maximal yields of lipid and aflatoxin were obtained with 30% glucose, whereas mold growth, expressed as dry weight, was maximal when the medium contained 10% glucose. Maximal mold growth occurred when the medium contained 3% $(NH_4)_2SO_4$; however, 1% $(NH_4)_2SO_4$ favored maximum accumulation of lipid and aflatoxin. Growth of mold and synthesis of lipid and toxin also varied with the incubation temperature. Maximal mold growth occurred at 35 C, whereas most toxin appeared at 25 C. Maximal production of lipid occurred at 25 and 35 C but production was more rapid at 35 C. Essentially all glucose in the medium (5% initially) was utilized in 3 days at 25 and 35 C but not in 7 days at 15 and 45 C. Patterns for formation of lipid and aflatoxin were similar at 15 and 25 C when a complete growth medium was used and at 28 C when the substrate contained various concentrations of glucose or $(NH_4)_2SO_4$. They were dissimilar when the mold grew at 35 or 45 C. At these temperatures lipid was produced preferentially and only small amounts of aflatoxin appeared.

Aflatoxins are secondary metabolites produced primarily by some strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Because of their pronounced toxicity and extreme carcinogenicity in many animal species, aflatoxins have been, and continue to be, extensively investigated (13, 23). Results of limited tests (6, 7, 16) have revealed a close relationship between biosynthesis of lipid and aflatoxin. However, it has not been demonstrated that this relationship prevails under all cultural conditions. Experiments in our laboratory showed that the pattern for synthesis of total lipids and aflatoxin was similar when *A. parasiticus* grew with different degrees of aeration (C. N. Shih and E. H. Marth, Biochim. Biophys. Acta, accepted for publication). Detroy and Hesseltine (6) suggested that synthesis of aflatoxin may be controlled through changes in the environment that can direct precursors into lipid rather than toxin.

This study was designed to define more completely the relationship between synthesis of lipid and aflatoxin by *A. parasiticus*. To accomplish this, the mold was grown in a synthetic medium so that variability in natural substrates was eliminated. Then the concentration of glucose and nitrogen in the medium and incubation temperature were varied. These factors influence synthesis of lipids by some other molds (4, 9, 15), but their importance for synthesis of fat by toxigenic aspergilli has not been defined nor has it been established that the similarity between synthesis of lipid and aflatoxin prevails over a variety of cultural conditions. Results in this communication provide some of the missing information.

MATERIALS AND METHODS

Organism. *A. parasiticus* NRRL 2999, a toxigenic strain obtained from Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., was used throughout this investigation. Stock cultures were maintained at 5 C on slants of mycological agar (Difco).

Preparation of spore suspension. The mold was grown on slants of mycological agar for 7 days at 28 C. Spores were harvested by adding sterile distilled water and a drop of Leconal wetting agent (Laboratory Equipment Co., St. Joseph, Mich.) to the slants. Each spore suspension was adjusted to an optical density value of 0.5 at 550 nm (Spectronic 20, Bausch and Lomb, Rochester, N.Y.) before it was used as inoculum.

Media. A synthetic growth medium was developed

on the basis of earlier reports (5, 11, 14). The medium contained (per liter): 50 g of glucose, 6 g of $(NH_4)_2SO_4$, 5 g of KH_2PO_4, 6.4 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2 g of glycine, 2 g of glutamic acid, 10 mg of $FeSO_4 \cdot 7H_2O$, 5 mg of $ZnSO_4 \cdot 7H_2O$, and 1 mg of $MnSO_4 \cdot H_2O$. The finished medium was prepared by aseptically adding a sterile glucose solution to the sterile glucose-free salts solution when both were cooled. The pH of the medium was 6.4 to 6.5. This medium was used to prepare the inoculum and to study how temperature during growth affected lipid and toxin production.

The medium used to study the effect of initial glucose concentration on formation of total lipid and toxin was prepared by eliminating glucose, glycine, and glutamic acid from the substrate just described and by adding various amounts of glucose, 0.5, 1, 5, 10, 20, 30, and 50% (wt/vol), to the solution. When various concentrations of nitrogen were desired, all nitrogen sources [glycine, glutamic acid, and $(NH_4)_2SO_4$] were eliminated from the growth medium and different amounts of $(NH_4)_2SO_4$, 0.05, 0.1, 0.5, 1, 3, 5, and 10% (wt/vol), were added to the nitrogen-free solution.

Cultural conditions. In experiments to determine how the initial glucose and nitrogen concentrations affected biosynthesis of lipid and toxin, 2 ml of the spore suspension (7×10^6 to 8×10^6 spores/ml) were incubated quiescently with 100 ml of medium in a 500-ml Erlenmeyer flask at 28 C for 6 days. To study the effect of various temperatures on production of lipid and toxin, 2 ml of the spore suspension was incubated quiescently with 100 ml of the synthetic growth medium in a 500-ml Erlenmeyer flask at 25 C for 2 days. After this initial incubation, some flasks were allowed to remain at 25 C, whereas others were moved to storage at 15, 35, or 45 C. Molds at various temperatures were then incubated quiescently for up to 7 days. This procedure eliminated the problem of delayed spore germination at some of the temperatures. Samples were analyzed for toxin, lipid, glucose concentration, and mycelial dry weight after 3, 5, and 7 days of incubation. All experiments were done in duplicate and results are reported as average values.

Glucose determination. Glucose was measured by the anthrone test (21).

Determination of mycelial dry weight. Samples of the culture were filtered using vacuum and four layers of cheesecloth on a Buchner funnel. After washing three times with cold distilled water, the mycelium was dried at 50 C for 24 h and weighed.

Aflatoxin determination. A 20-ml amount of filtrate was extracted in a separatory funnel with 40 ml of chloroform, and this procedure was repeated three times. Aflatoxin in the mycelium was extracted by blending it with 50 ml of chloroform, 100 ml of methanol, and 40 ml of water in a Waring blender for a few minutes. Then 50 ml of chloroform and 50 ml of water were added to the mixture and it was blended again. After filtration, chloroform was removed from the mixture and the remaining methanol-water fraction was then extracted twice with chloroform (19). The combined chloroform extracts from either broth or mycelium were evaporated separately in a flash evaporator in preparation for aflatoxin analysis. Aflatoxins were separated on thin-layer chromatographic plates which were developed with chloroform-methanol-water (98:1:1, vol/vol/vol). The concentration of aflatoxins was measured using procedures described by Shih and Marth (18). Data in the figures represent the total aflatoxin (B_1, B_2, G_1, and G_2) present in the broth and mycelium.

Determination of total lipid. A portion of the chloroform extract was used to determine total lipid produced by the mold (1). The chloroform extract was dried by filtration over Na_2SO_4, and then chloroform was removed by flash evaporation. After chloroform was removed, lipid was transferred to a tared 50-ml Erlenmeyer flask with 4 to 5 ml of chloroform, which was then removed under a stream of nitrogen. The residue in the flask was dried over calcium chloride anhydride in a vacuum desiccator and weighed.

RESULTS

Initial concentration of glucose and synthesis of lipid and toxin. Data in Fig. 1 show that both the highest total lipid and the highest total aflatoxin concentrations were obtained with 30% glucose, although dry weight of the mold was maximal when the medium contained 10% glucose and it was nearly similar when the medium contained 5 or 20% glucose. Increases in initial concentrations of glucose up to 30% regularly resulted in greater amounts of lipid and aflatoxin produced by the mold. However, when the glucose concentration was increased from 30 to 50%, synthesis of both lipid and toxin was diminished and mycelial dry weight was reduced.

Residual glucose in the medium increased markedly as the initial concentration was raised beyond 5%. Hence, the efficiency of glucose utilization by the mold decreased as the concen-

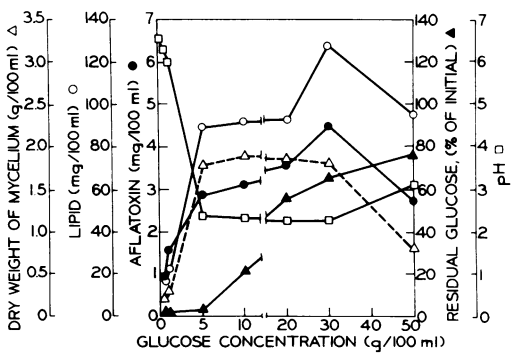

FIG. 1. *Growth, glucose uptake, changes in pH, and formation of aflatoxin and lipid by Aspergillus parasiticus in a chemically defined medium with various concentrations of glucose.*

tration was increased. The mold did not produce appreciably more acid when the medium contained more than 5% glucose.

Initial concentration of nitrogen and synthesis of lipid and toxin. The influence of several concentrations of $(NH_4)_2SO_4$ on biosynthesis of lipid and toxin is shown in Fig. 2. Maximal mycelial dry weight occurred when the medium contained 3% $(NH_4)_2SO_4$. However, 1% $(NH_4)_2SO_4$ favored maximum accumulation of total lipid and toxin. The results indicate that concentrations of nitrogen somewhat suboptimal for maximal growth (greater carbon-to-nitrogen ratio) enhanced lipid synthesis and production of aflatoxin.

Presence of 5% or less $(NH_4)_2SO_4$ permitted use of all glucose (5%) in the medium during the incubation of 6 days. Nearly 50% of the glucose remained unused when 10% $(NH_4)_2SO_4$ was in the medium. Acid production by *A. parasiticus* was decreased when the medium contained more than 1% $(NH_4)_2SO_4$. This coincided with the decrease in the amount of lipid and toxin that was produced.

Incubation temperature and synthesis of lipid and toxin. Data in Fig. 3 indicate that maximal mycelial dry weight was produced at 35 C. However, maximal concentrations of total lipid appeared at 25 and 35 C in 2 to 5 days, and maximal concentration of aflatoxin appeared at 25 C in 5 days. Both lipid and aflatoxin were produced when *A. parasiticus* grew at 15 and 25 C (Fig. 3). At higher temperatures, 35 and 45 C, the mold continued to form lipid, but produced only negligible amounts of toxin.

Results of these experiments demonstrate that a temperature which was somewhat suboptimal for growth of *A. parasiticus* permitted maximal production of both lipid and toxin. Furthermore, biosynthesis of aflatoxin by the mold was essentially completely suppressed at or above 35 C, whereas production of total lipid prevailed under these conditions.

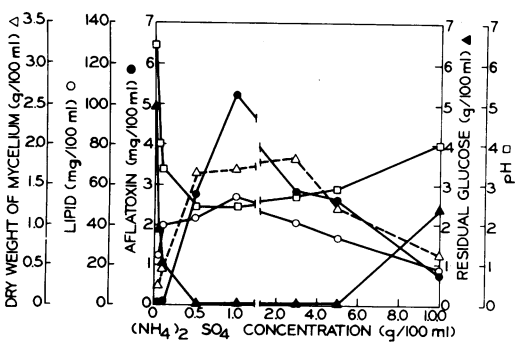

FIG. 2. *Growth, glucose uptake, changes in pH, and formation of aflatoxin and lipid by Aspergillus parasiticus in a chemically defined medium with various concentrations of ammonium sulfate.*

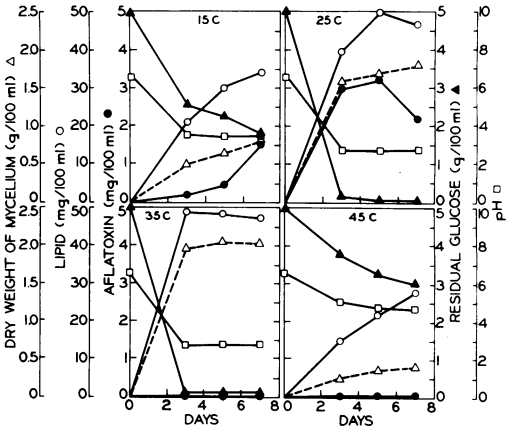

FIG. 3. *Growth, glucose uptake, changes in pH, and formation of aflatoxin and lipid by Aspergillus parasiticus in a chemically defined medium during incubation at various temperatures.*

DISCUSSION

Results from this study define some of the conditions under which synthesis of lipid and aflatoxin have a similar or dissimilar pattern. The close relationship between biosynthesis of lipid and aflatoxin also has been suggested in limited studies by other investigators (6, 7, 16). However, they found that certain cultural conditions supported good production of aflatoxin but were unfavorable for synthesis of lipid. The reverse also was true. These observations led to the general conclusion that formation of more aflatoxin or more lipid can be controlled by manipulating the environment.

Results of our investigation lead to another conclusion—manipulating the environment does not always favor production of one at the expense of the other. We noted a similar pattern for synthesis of lipid and aflatoxin at all concentrations of glucose and $(NH_4)_2SO_4$ in the medium and when incubation was at 15 and 25 C. A dissimilarity was noted only when the mold grew at 35 and 45 C; production of lipid continued unhindered but formation of aflatoxin was suppressed.

Maximal formation of lipid and aflatoxin by *A. parasiticus* was favored by a high concentration of glucose (30%) that did not support formation of maximal mycelial dry weight. A somewhat suboptimal (for maximal growth) concentration of ammonium sulfate (1%) yielded maximal production of lipid and aflatoxin. These conditions also were found

necessary for maximal production of lipid by other molds or by yeast (4, 10, 15, 24).

It was reported earlier that an increase in the carbohydrate concentration of the medium resulted in the synthesis of more total lipid by other molds (4, 12, 15, 24). Lockwood et al. (12) obtained most total lipid when *Penicillium javanicum* grew in a medium with 30% glucose. A high concentration of carbohydrate is believed necessary to supply the carbon and energy needed for maximal synthesis of lipid (4, 9, 15). Since our results show that the highest concentration of aflatoxin appeared in the medium that also supported maximal synthesis of total lipid, a similar explanation may apply for aflatoxin synthesis, at least when proper environmental conditions prevail. That a high concentration (20%) of sucrose supports maximal production of aflatoxin has been reported (5).

It has also been shown that an abundant supply of glucose in the medium caused a marked impairment of respiratory capability and concurrent development of an anaerobic mode of metabolism by many microorganisms (22). In other studies (20; Shih and Marth, Biochim. Biophys. Acta, accepted for publication), we showed that conditions bordering on anaerobiosis favored synthesis of aflatoxin. That observation also may help to explain why the maximal yield of aflatoxin appeared in a medium with a high concentration of glucose since the mold probably developed a less aerobic mode of metabolism in response to the excess glucose.

According to Bu'Lock (2), the concentration of nitrogen in the medium is critical for synthesis of secondary metabolites. We observed that a nitrogen concentration somewhat suboptimal for maximal growth produced maximal yields of lipid and aflatoxin. This also was found true for other molds when conditions for maximal yield of lipid alone were studied (4, 10, 15). Since a similar C:N ratio is optimal both for synthesis of aflatoxin and lipids, aside from the similar precursor acetate (6, 7), perhaps another condition(s) is common to the synthesis of both products.

Formation of lipid and aflatoxin varied with the temperature of growth used after an initial 2-day incubation at 25 C allowed conidia to germinate. Maximal synthesis of aflatoxin occurred at 25 C and of lipid at 25 and 35 C, whereas maximal mycelial dry weight was produced by *A. parasiticus* at 35 C. The observations made in these trials verify those of Schindler et al. (17), who studied only aflatoxin production and reported that highest yields were obtained at 24 C, whereas maximum mold growth occurred at 29 or 35 C, depending on the particular isolate.

At each of the lower temperatures, 15 or 25 C, we observed that formation of both products followed a somewhat similar pattern. At higher temperatures, 35 or 45 C, lipid was produced preferentially and only small amounts of aflatoxin appeared. Previous studies by Ciegler et al. (3) and by Diener and Davis (8) also showed that aflatoxin formation was limited by high temperatures. Our observation and those of others suggest that a key enzyme responsible for aflatoxin synthesis was inactive above 35 C, and thus it is possible to regulate aflatoxin production through control of temperature.

Our results demonstrate that similarities exist between synthesis of lipid and aflatoxin under a series of different conditions. This was not observed by other workers. Our data also indicate that certain conditions (e.g., incubation at 35 or 45 C) favor lipid synthesis and suppress production of aflatoxin. These observations suggest that formation of both products shares some similar biosynthetic steps. Perhaps a key enzyme responsible for synthesis of either lipid or aflatoxin is regulated by certain cultural conditions and hence the condition(s) determines the amount of the final products that will appear. Data from another study in our laboratory (C. N. Shih and E. H. Marth, unpublished data) support the idea that some similar biosynthetic steps are involved in formation of both lipid and aflatoxin. These data suggested that the same pathway is used initially in the biosynthesis of lipid and aflatoxin through condensation of acetate and malonate units and that formation of polyacetate precedes production of aflatoxin.

ACKNOWLEDGMENTS

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