Aflatoxin Formation in Sterilized Feedlot Manure and Fate During Simulated Water Treatment Procedures

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Toxigenic strains of the Aspergillus flavus-oryzae species aggregate are readily isolated from stockpiled feedlot manure. Nevins (1) found that 5-10 percent of isolates obtained from cattle feedlot manure, and tentatively identified as A. flavus, produced appreciable amounts of aflatoxin in a medium containing 2 percent yeast extract and 20 percent sucrose. Nevins also demonstrated that fresh feedlot manure provides sufficient nutrients for fungal growth and detectable aflatoxin formation under laboratory conditions. It is not known whether toxin formation occurs in nature during storage of feedlot manure. However, considering the vast quantity of this material generated and stored at commercial feedlots, and in view of the marked carcinogenicity of the aflatoxins, the formation of these agents in nature presents a possible public health hazard. Aflatoxins, if formed in stored manure, may reach human populations following contamination of surface and ground water supplies by storm run-off and leaching. In the present studies, we have evaluated feedlot manure in various stages of decomposition as substrates for aflatoxin formation. In addition, we report on the fate of aflatoxin during simulated water treatment procedures and assess the probable human exposure following contamination of surface and ground water supplies.

MATERIALS AND METHODS

Aspergillus flavus (ATCC#15517) was obtained from the American Type Culture Collection. The organism was grown on the Moyer sporulation medium described by Raper and Fennel (2). Spores were harvested on sterile sand and stored at 5C. When needed for inocula, the spores were suspended in sterile water containing 0.03 percent Tween 80 to obtain approximately 4 X 10^8/ml. Each growth flask contained 25 gm of manure substrate, mixed with water when required to bring the moisture content to about 75 percent, and was inoculated with 8 X 10^8 spores after being autoclaved for 15 minutes at 121C. Three types of feedlot manure were examined

Bulletin of Environmental Contamination & Toxicology,
Vol. 6, No. 6, 1971, published by Springer-Verlag New York Inc.

as substrates for aflatoxin formation. Fresh manure was collected immediately following deposition on the feedlot floor. Partially decayed manure was that accumulating in feedlot pens over a period of several months. Stockpiled manure was that removed from feedlot pens and was undergoing storage for subsequent disposition by land spreading. Pooled samples of each manure type were mixed thoroughly, distributed in plastic bags, and stored at 15C. Manure substrates were evaluated by incubating triplicate stationary flasks at the appropriate temperature. All experiments were repeated three times.

Aflatoxin formed during growth of A. flavus on the various manure types was extracted by adding two volumes of chloroform and placing the flasks in darkness at 21C for 24 hours. The chloroform extracts were clarified and dehydrated by filtration through anhydrous sodium sulfate, then evaporated to near dryness in a rotary evaporator. Residues were dissolved in chloroform and analyzed by thin layer chromatography on silica gel G in a solvent system of chloroform : acetone (9:1). Aflatoxin concentrations were estimated by visual comparison of fluorescence against analytical standards of aflatoxin B_1 and G_1 obtained from the Southern Utilization and Development Division of the United States Department of Agriculture, New Orleans, Louisiana. Identification of aflatoxins was confirmed biologically with the brine shrimp bioassay (3), and chemically with the derivative test described by Stoloff (4). Aflatoxins B_1 and G_1 were purchased from CalBiochem, Los Angeles, California.

The critical procedures used in standard municipal water purification plants (6) were simulated in laboratory scale studies to examine the fate of aflatoxin during water treatment. Aqueous solutions of aflatoxin B_1 and G_1 were subjected to simulated chlorination, floculation, and sand filtration. Residual chlorine concentrations of 0.5 to 20 ppm determined with the orthotolidine method (7), were obtained by addition of appropriate amounts of sodium hypochlorite. Floc forming agents ($Al_2(SO_4)_3 \cdot 18\ H_2O$ and $Ca(OH)_2$) were added to aqueous aflatoxin solutions and, following a 6 hour settling period, the clear upper layers were sampled for analysis. Seven samples of silica sand were obtained from three different municipal water filtration plants; two samples were obtained from commercial sand and gravel companies. The sands were evaluated for their ability to remove aflatoxins B_1 and G_1 from aqueous solutions both in batch and continuous processes. Soil samples, representing four common soil types, were obtained from the Department of Agronomy, Colorado State University. Transformation or removal of aflatoxin from aqueous solutions during simulated water purification procedures was monitored

by thin layer chromatography, following chloroform extraction, for observing altered R_f values or disappearance of fluorescent spots corresponding to aflatoxins B_1 and G_1.

RESULTS

A. Aflatoxin formation on manure substrates

Maximum aflatoxin formation occurred after 7 days incubation when fresh or partially decayed manures were used as substrates and incubation temperature was 28C. An incubation period of 21 days was required for maximum aflatoxin yields when stockpiled manure was the substrate. Average aflatoxin yields (B plus G) of 2.9, 0.21, and 0.01 mg/kg substrate were obtained on fresh, partially decayed, and stockpiled manures respectively. The aflatoxin B/G ratio was 2.6 on fresh and 25 on partially decayed manure. No detectable amounts of aflatoxin G were produced on the stockpiled manure substrate. Table 1 summarizes the results obtained from experiments evaluating the three manure types as substrates for aflatoxin formation.

TABLE 1

Aflatoxin Production on Autoclaved Manure Substrates

Type of manure	Aflatoxin, mg/kg manure	
	B	G
Fresh	2.1	0.8
Partially decayed	0.2	0.008
Stockpiled	0.01	<0.003

On the fresh manure substrate, growth of A. flavus occurred at temperatures of 15C, 28C, and 37C but not at 10C or 41C. Aflatoxin yields were highest during incubation at 15C and 28C; however, the formation of aflatoxin G_1 was favored at the lower temperature causing the B/G ratio to shift from 2.6 to 0.8. The influence of incubation temperature on average aflatoxin yields in manure substrates is presented in Table 2.

TABLE 2

Aflatoxin Production on Autoclaved Fresh Manure

Incubation temperature (degrees C)	Aflatoxin, mg/kg manure B	G
10	<0.003*	<0.003*
15	1.8	2.3
28	2.1	0.8
37	0.003	<0.003
41	<0.003*	<0.003*

*No visible growth

B. Fate of aflatoxin during simulated water treatment and soil leaching

As anticipated, the extent of aflatoxin inactivation during simulated chlorination depended on the residual chlorine concentration, the aflatoxin concentration, and the time of contact. For example, a residual chlorine concentration of 1 ppm produced essentially complete inactivation in a solution containing 0.005 µg aflatoxin/ml within 15 minutes, but only 40 percent inactivation in a solution containing 1 µg aflatoxin/ml. A residual chlorine concentration of 0.5 ppm, a value typical of some municipal water supplies, provided complete inactivation of aflatoxins at a concentration of 0.5 µg/ml within 12 hours contact. In general, aflatoxin inactivation was rapid and complete provided that the residual chlorine : aflatoxin ratio was at least 2:1.

The action of floculating agents in removing or inactivating aflatoxins from aqueous solutions was tested at normal and 5 X-normal concentrations. The normal floculation treatment was without detectable effect; approximately 50 percent removal of aflatoxins was effected by the floc forming agents when used at elevated concentrations.

Initial studies on sand filtration of aqueous aflatoxin solutions demonstrated that 90-100 percent of the added aflatoxin was removed during passage through a column packed with brown silica sand. Since the sand column was so strikingly effective in removal of aflatoxin from aqueous solutions, the phenomenon was further characterized. Eight of the nine sand

samples tested efficiently adsorbed aflatoxin B_1 or G_1 from aqueous solutions. The non-adsorbing sample was a highly polished white silica sand with uniform particle size, a type rarely used in the construction of rapid sand filters. The adsorption was rapid, depended partially on particle size and partially on the nature of the minor chemical constituents present, was not related to the presence of organic materials nor microbial flora, and was essentially irreversible. The adsorption limit of sand samples was about 30 μg aflatoxin/gm. The eluant discharged from a model rapid sand filter contained only 2 percent of the added aflatoxin.

The fate of aflatoxin during simulated leaching was examined, using samples of four different soil types. The filtrates (simulated leachates) obtained following addition of soils to aqueous aflatoxin B_1 or G_1 solutions contained no detectable toxins.

DISCUSSION

Aflatoxin yields obtained during growth of A. flavus in sterilized fresh, partially decayed, and stockpiled manure substrates apparently reflected the extent to which the manure had undergone microbial decomposition. Although substantial amounts of aflatoxin B (0.2-2.1 mg/kg) were formed in fresh and partially decayed manures, much smaller amounts (0.01 mg/kg) were produced when stockpiled manure was the substrate. Furthermore, the B/G ratio of aflatoxins formed in the three types of manure was substrate-dependent; the formation of aflatoxin G being decreased in partially decayed manure and non-detectable in stockpiled manure. Incubation temperature also influenced the B/G ratio; formation of aflatoxin G was stimulated at 15C. The lower temperature limit permitting aflatoxin synthesis in an autoclaved fresh manure substrate was 10-15C; the upper limit was 37-41C. These findings are in general agreement with those reported by other workers using various natural substrates and artificial media (5).

Decomposing manure, under natural conditions, contains a varied microflora that would interact with A. flavus in a complex manner to modify fungal growth and toxin formation. The use of autoclaved manure substrates precluded complications accompanying mixed culture studies, but also left unanswered the possible role of microbial antagonism or synergism in altering the ability of A. flavus to grow and produce toxin in stored feedlot wastes. The detection of minute amounts of aflatoxin in stockpiled manure under field conditions is complicated by environmental variables and difficulties with isolation and purification procedures. However,

the laboratory studies do demonstrate that, under appropriate conditions, stored feedlot wastes may serve as a substrate for aflatoxin formation.

If aflatoxins were formed in stockpiled feedlot wastes, they may contaminate water destined for domestic use through runoff and leaching. Inactivation or removal of aflatoxin from water supplies would have to occur at municipal water treatment plants in order to protect consumers from a potential health hazard. Both chlorination and rapid sand filtration proved effective in destroying or removing aflatoxin from aqueous solutions. The inactivation of aflatoxin by chlorine is not surprising since hypochlorites are standard agents for decontaminating aflatoxins in the laboratory. However, the finding that a residual chlorine concentration of 1 ppm effectively transforms aflatoxin at a concentration of 0.005 μg/ml within 15 minutes is of importance in view of the fact that 38.9 percent of the population in this country consume water treated by chlorination only (6).

The efficient adsorption of aflatoxin during rapid sand filtration was an unexpected phenomenon. This finding allows the prediction that contaminating aflatoxins could not survive the treatment procedures used at most large municipal plants. Furthermore, it is unlikely that aflatoxin formed in decomposing feedlot wastes could contaminate ground water by leaching through the soil if the aflatoxin adsorbing behavior of the samples tested is typical.

It is improbable that aflatoxins B and G, if formed during natural decomposition of stockpiled manure or other agricultural wastes, could reach consumers of water that has undergone standard purification procedures. The necessary precautionary measures to protect consumers of aflatoxin-contaminated water are sand filtration and chlorination.

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