Possible Reactions for Aflatoxin Detoxification

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The reactions of the four aflatoxins with a series of selected reagents were observed for possible usefulness in detoxification procedures. Loss of fluorescence and/or change of R_f on TLC were the principal reaction indicators. Chick embryo and tissue culture bioassays were used to provide evidence of detoxification. The reactions appear to be primarily addition and oxidation involving the olefinic double bond of the terminal furan ring and oxidation involving the phenol formed on opening of the lactone ring. Benzoyl peroxide, osmium tetroxide, and I^-/I_2 react with aflatoxins B_1 and G_1, but not with B_2 and G_2. $Ce(NH_4)_2(SO_4)_3$, NaOCl, $KMnO_4$, $NaBO_3$, and 3% H_2O_2 + $NaBO_2$ (1 + 1) react with all four aflatoxins. Detoxification, after contact with gaseous chlorine, chlorine dioxide, and nitrogen dioxide and after treatment with 5% NaOCl solution, was confirmed by bioassay.

Aflatoxins, a group of toxic metabolites of *Aspergillus flavus* and some other fungi, have been described as the most potent carcinogens yet known (Butler and Barnes, 1963). Although human response to the toxins has not been determined, all practical steps should be taken for protection of personnel working with aflatoxins. Therefore, the authors studied chemical reactions that might be useful for detoxification.

Irreversible alteration of aflatoxins by strong alkali and strong oxidizing agents has been reported in the literature (Dorp *et al.*, 1963; Zijden *et al.*, 1962). This information and the possible influence of reactive groups in the proposed structures (Asao *et al.*, 1965) (Figure 1) directed the choice of reagents.

A combination of a typical blue or green fluorescence and the chromatographic pattern on silica gel thin-layer plates is generally used for initial screening and assay (AOAC, 1966). Loss of either or both of these characteristics was taken as presumptive evidence of aflatoxin modification. The reactions eventually chosen for detoxification were established as effective by bioassay with the chick embryo (Verrett *et al.*, 1964), and with cultured embryonic lung cells (Legator and Withrow, 1964).

Procedures and Results

The first survey experiments were performed by dissolving 1.25 μg. of a crude aflatoxin preparation (containing 12.5% each of B_1 and G_1 and smaller amounts of B_2 and G_2) in the aqueous reagents listed below. After 1 hour, the aqueous solutions were extracted with chloroform, and the chlorform extracts were examined by thin-layer chromatography on silica gel G-HR (Brinkmann Instruments, Inc.).

No effect on any of the four aflatoxins was observed with the following reagents: $AgNO_3$ (3.6*M*), $CuSO_4$ (0.63*M*), $FeCl_3$ (1.7*M*), KIO_3 (0.14*M*), $NaNO_2$ (5.9*M*), $NaHSO_3$ (0.5*M*), Na_2SO_3 (0.5*M*), $(NH_2)_2H_2SO_4$ (5%), $NaHCO_3$ (0.41*M*), $SnCl_2$ (2.6*M*), and H_2O_2 (9.0*M*).

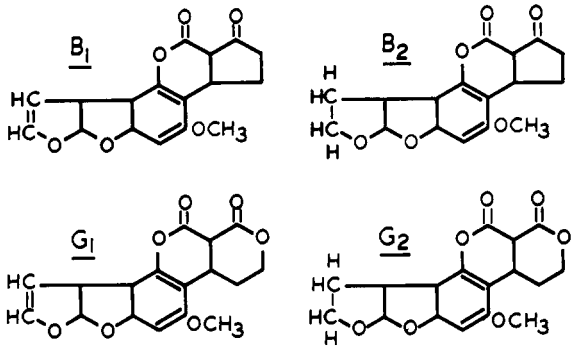

Figure 1. Proposed structure of aflatoxins B_1, B_2, G_1, and G_2 (Asao *et al.*, 1965)

None of the aflatoxins was recovered after treatment with the following (results were verified with larger amounts of aflatoxins B_2 and G_2): NaOCl (6.8*M*), $KMnO_4$ (0.63*M*), $Ce(NH_4)_2(SO_4)_3$ (0.63*M*), and Na_2CO_3 (0.34*M*).

Partial loss of aflatoxin B_1 fluorescence was effected by hydrazine hydrochloride (5% in 95% EtOH) and by pH 10 buffer (Beckman 3505); G_1 was not affected by the former, but its fluorescence was completely destroyed by the latter solution. The fluorescence of aflatoxins B_1 and G_1 was completely destroyed, but B_2 and G_2 were unaffected by OsO_4 (0.01*M*), which is a specific oxidant for olefinic bonds, or by benzoyl peroxide in chloroform (0.1*M*).

Based on results of these experiments, a second group of reagents was selected and tested under conditions designed to determine effectiveness for short contact times. Portions (0.25 μg.) of the same crude toxin were spotted and developed on silica gel thin-layer plates. Under ultraviolet illumination, a drop of each reagent was added to the aflatoxin B_1 and G_1 spots, and the time required for the disappearance of fluorescence was noted (Table I). Results were identical for aflatoxins B_1 and G_1.

Gaseous reagents were examined by displacement of the air in vials, each of which contained 250 μg. of the

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crude aflatoxin. The stoppered vials were held at room temperature overnight. The gases were then displaced by addition of 1 to 2 ml. of chloroform and evaporation of the chloroform on a steam bath under a stream of nitrogen. The residues, dissolved in chloroform, were examined by thin-layer chromatography on silica gel plates. No aflatoxins were found after contact with chlorine, chlorine dioxide, or nitrogen dioxide.

Sulfur dioxide or hydrogen cyanide when dry did not have an observable effect on the aflatoxins; however, the generated gases containing water and acid constituents produced fluorescent substances chromatographically different from B_1 and G_1.

Bioassays [chick embryo (Verrett *et al.*, 1964) and tissue culture (Legator and Withrow, 1964)] of the samples exposed to the above gases and of crude aflatoxin treated with 5% sodium hypochlorite solution confirmed the TLC and fluorescence evidence of aflatoxin destruction.

On the basis of the foregoing data and in an attempt to approximate the oxidation potential(s) of aflatoxin B_1, a number of reduction couples were added to a mixture of toxins. Solutions (0.01*M*) of each reagent were allowed to remain in contact with the aflatoxin for 5 minutes before the aflatoxin was extracted with chloroform and examined by thin-layer chromatography. The effect on the aflatoxins, based on the presence or absence of the typical fluorescent spots, is given in Table II.

Table I. Time for Disappearance of Fluorescence of Aflatoxins B_1 and G_1 after Addition of One Drop of the Noted Reagents to 0.03-μG. Spots on Silica Gel G-HR (Brinkmann) Thin-Layer Plates

Reagent	Concn.	Time, Sec.
NaOCl	(5%)	0[a]
NaOCl	(2.5%)	0
NaOCl	(1.25%)	0
NaOCl	(0.63%)	5
NaOCl	(0.31%)	10
$KMnO_4$	(1%)	0
$KMnO_4$	(0.1%)	120
Phenol	(92%)	0
Phenol	(9.2%)	0
Phenol	(0.92%)	120
Chlorohydroquinone	(10%)	0
Chlorohydroquinone	(1%)	120
Resorcinol	(10%)	0
Resorcinol	(1%)	120
$Na_2S_2O_3$	(0.2*M*)	120
$NaBO_3$	(0.1*M*)	15
$NaBO_2$	(0.2*M*)	120
H_2O_2 (3%) + $NaBO_2$	(0.1*M*)	15–30
H_2O_2 (3%) + $NaBO_2$	(0.01*M*)	45–60
H_2O_2(3%) + HCl	(2.0*N*)	30–45
H_2O_2 (3%) + HCl	(0.2*N*)	120
HCl	(2.0*N*)	120
NaOH	(1.0*N*)	30–45
NaOH	(0.1*N*)	120

[a] Reaction was instantaneous when time is given as 0 seconds.

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Discussion

Aflatoxin G_1 is identical to B_1 except that a 5-valerolactone ring is substituted for the cyclopentanone ring. Aflatoxins B_2 and G_2 are the dihydro addition products of the terminal dehydrofuran ring of B_1 and G_1, respectively.

The unsaturation of aflatoxins B_1 and G_1 is most likely responsible for some of the chemical reactivity of these molecules, especially towards oxidizing agents. The selectivity of the olefinic bond reagents, the I^-/I_2 couple, 0.01*M* osmium tetroxide, and 0.1*M* benzoyl peroxide in chloroform, towards B_1 and G_1 tends to strengthen that theory. The ion I^- alone (aqueous KI) had no effect on aflatoxin B_1—i.e., did not reduce it. The susceptibility of the terminal dehydrofuran ring of aflatoxin B_1 or G_1 to electrophilic attack can be rationalized by the resonance form A:

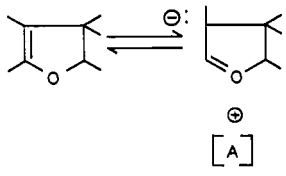

Table II. Effect of 5-Minute Contact with Selected Redox Systems (0.01*M*) on Aflatoxins

E°	Couple	TLC Observations
+0.126[a]	$Pb \rightleftharpoons Pb^{+2} + 2e^-$	No reaction
−0.070	$Sn^{+2} \rightleftharpoons Sn^{+4} + 2e^-$	No reaction
−0.346[a]	$Cu \rightleftharpoons Cu^{+2} + 2e^-$	No reaction
−0.536	$2I^- \rightleftharpoons I_2 + 2e^-$	B_1 and G_1 absent
−0.699[a]	$C_6H_4(OH)_2 \rightleftharpoons C_6H_4O_2 + 2H^+ + 2e^-$ (Hydroquinone) (quinone)	All toxins absent
−0.740[a]	$Se + 3H_2O \rightleftharpoons H_2SeO_3 + 4H^+ + 4e^-$	No reaction
−0.770	$Fe^{+2} \rightleftharpoons Fe^{+3} + e^-$	No reaction
−0.800	$Ag \rightleftharpoons Ag^+ + e^-$	No reaction
−0.940	$Cl^- + 2OH^- \rightleftharpoons ClO^- + H_2O + 2e^-$	All toxins absent
−1.087	$2Br^- \rightleftharpoons Br_2 + 2e^-$	All toxins absent
−1.45	$Cl^- + 3H_2O \rightleftharpoons ClO_3^- + 6H^+ + 6e^-$	No reaction
−1.61	$Ce^{+3} \rightleftharpoons Ce^{+4} + e^-$	All toxins absent
−2.05	$2SO_4^{-2} \rightleftharpoons S_2O_8^{-2} + 2e^-$	No reaction

[a] Only the oxidant of the couple was used.

Probably in aqueous systems the lactone linkage common to all the aflatoxins partially hydrolyzes to the phenol form (and carboxylate group) which would be very susceptible to most oxidizing agents, especially alkaline agents. This could account for the observation of the instability of aflatoxins in protonic solvents such as water and methanol—i.e., oxidation of the phenol form by dissolved oxygen may occur. Thus, aflatoxins B_2 and G_2 (in which the terminal furan ring is saturated) were affected by some aqueous solutions of oxidants that also destroy B_1 and G_1—namely, $Ce(NH_4)_2(SO_4)_3$, NaOCl, $KMnO_4$, $NaBO_3$, and 3% H_2O_2 + $NaBO_2$ (1 + 1).

Two (and perhaps more) oxidation reactions can occur with B_1. In some instances, the reactions may occur simultaneously at individual rates, the relative magnitudes of which would be determined by factors such as solvent, pH, and type of oxidizing agent. The oxidation mechanism is probably a major factor, since there is no apparent relation to redox potential.

Selected oxidation reactions could be useful for aflatoxin detoxification, and reagents such as sodium hypochlorite, alkaline sodium perborate, and chlorine gas have already been applied effectively (Fischbach and Campbell, 1965; Stoloff and Trager, 1965).

However, loss of fluorescence and harmlessness to the chick embryo and embryonic lung cells have been the indices of effectiveness used up to now. Experiments with warm-blooded animals are needed to establish better the absence of toxic residues and, if the treatments have been applied to commodities, the integrity of the nutrient properties.

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