Chapter V

TOXIC SUBSTANCES CONCERNED IN DEGRADATION OF FOODSTUFFS AND THEIR BIOLOGICAL SIGNIFICANCE

The marked effects of the fungi on the different substrates during storage particularly on foodstuffs such as peanuts, rice, corn and 'Rosetti' meal as demonstrated by Asplin and Carnaghan (1961), Austwick and Ayerst (1963) and Sargeant et al (1961) have led to characterisation of a new class of compounds termed 'aflatoxin'.

Butler (1964) has dealt in detail the different aspects and properties of aflatoxin and many of the disease syndromes in the animals are not now attributed to aflatoxin.

The known aflatoxins are four of a complex array of fluorescent compounds of which two are blue in colour and termed aflatoxin B1, B2, M1, M2, B2a and G2a.

Table 14

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Ultraviolet absorption</th>
<th>Fluorescence Emission, maximum (three)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>C_{17}H_{12}O_6</td>
<td>312</td>
<td>13,400 - 21,800</td>
<td>425</td>
</tr>
<tr>
<td>B2</td>
<td>C_{17}H_{14}O_6</td>
<td>314</td>
<td>9,200 - 14,700</td>
<td>425</td>
</tr>
<tr>
<td>G1</td>
<td>C_{17}H_{12}O_7</td>
<td>328</td>
<td>10,000 - 16,100</td>
<td>450</td>
</tr>
<tr>
<td>G2</td>
<td>C_{17}H_{14}O_7</td>
<td>330</td>
<td>11,200 - 19,300</td>
<td>450</td>
</tr>
</tbody>
</table>
Aspergillus flavus is shown to be able to synthesize the aflatoxins when grown on a wide variety of substrates. However, the yields of different aflatoxins vary with the substrate (Hesseltine et al. 1966, Stubblefield et al. 1967, Subrahmanyan and Rao 1974). In contrast to natural contamination where aflatoxin B1 is frequently the largest component (for example, Rosetti meal which causes "Turkey x disease" contained 10 ppm B1 and negligible G1) when grown in culture aflatoxin G1 may be the most abundant. Aflatoxin according to Stewart et al. (1977) is one of the many mould metabolites in contaminated feeds and feed ingredients.

The spectral characteristics of the four aflatoxins have been determined by several investigators (Asao et al., 1965; H de Iongh et al., 1964; Hartley et al., 1963; Van Dorp et al., 1963; Zijden Vander et al., 1962). Earlier workers, namely, Nesbitt et al. (1962) and Sargeant et al. (1963) studied the products of aflatoxin in vitro in synthetic media. Zdena et al. (1976) however obtained maximum yield of aflatoxin on other powdered substrates.

The elaboration and characterisation of the different mycotoxins as evidenced at the beginning were carried out by use of thin layer chromatographic techniques (Scott et al., 1970 and Fishbein and Falk, 1970). Zijden Vander et al. (1962) using chloroform - 1% ethanol separated crystalline material by TLC on Kieselgel G. Using chloroform : methanol de Iongh et al. (1964) resolved the 4 aflatoxins and classified them according to the rf values and colour.
The original bioassay of the fractions of aflatoxin was described by Sargeant et al (1961) and based on the observation that 1-day old khaki Campbell ducklings were extremely sensitive to toxic groundnut meal (Asplin and Carnaghan, 1961). The chloroform methanol extract in propylene glycol was given by a tube to the lower end of the oesophagus on 5 consecutive days and the survivors were killed on the 8th day. The animals that died prior to 8th day, showed massive necrosis of the liver and the survivors a biliary proliferation which was similar to that seen when ducklings were fed toxic meal. Butler (1964) studied effects of single dose of aflatoxin B1 and demonstrated that the biliary proliferation was very rapid, reaching a peak after 3 days and then regressed.

Many species of fish are very susceptible to the aflatoxins. Abedi and Mckinley (1968) suggested the use of Zebra fish larvae (Brachydanio rerio) and demonstrated a sensitivity at a level of 1 mg/ml B1. Chick embryo has been used by Verret et al (1964).

The toxic preparation of aflatoxin varied with the type of animals, age, sex and the duration of exposure. The first lesion of aflatoxicosis is hepatic damage and ducklings considered to be most susceptible show these lesions within three days of feed (Osuna, 1977).

In the present investigation day-old black Australorp chick, albino mice and bacteria cultures of Staphylococcus aureus and Escherichia coli were used for bioassay of aflatoxin
in the mixed form as well as after separation into individual components.

**Preparation:**

Preparation and purification of toxin was followed according to the method described by Zdena et al (1976).

250 ml Erlenmeyer's flasks with 25 g rice powder was mixed 50 ml Czapek's media in each were autoclaved at 15 lb for 20 mts., cooled, inoculated with *A. flavus* and incubated at 37°C for 10 days. The mycelial mats with the substrates were then transferred to large beakers and extracted with methanol 100 ml per 25 g. of the mat. The extract was filtered under pressure and concentrated to 1/20 the total volume.

The resultant toxic mixture was analysed chromatographically using T.L.C. in a series of solvents in N Butanol: Acetic acid: Water (4:1:4) was found to give the best resolution and hence was used in all the chromatographic studies. The plates on examination under U.V. showed two major fluorescent compounds. The blue and the yellow green having rf values of 0.63, 0.82 and 0.95 and thus were found to be akin to aflatoxins B and G (Plate 8).

**Feeding experiment with day old chick**

For feeding experiments, toxin was prepared by chromatographic separation and extraction of the compounds with methanol. The methanol was evaporated off completely and the residue was taken up in a small quantity of sterile distilled
Plate 8

Thin layer chromatography of Aflatoxin.

Rf values -  
0.63 G
0.82 B
0.95 B

Solvent system - N Butanol : Acetic Acid : Water
(4 : 1 : 4)
water (0.5 mg/0.5 ml.).

0.5 ml. of the aqueous extract was fed orally to 2 groups of day old Australorp chickens taking 5 chicks in each group. The chicks were provided with feed and water as usual. 2 chicks were kept as control on feed and water only without feeding toxin.

The treated chicks were found to lose weight gradually and die in about 2 weeks (Table 15; Fig. 8 and Plate 9).

Table 15

<table>
<thead>
<tr>
<th>Mean concentration in mg/ml.</th>
<th>No. of chick in the group tested</th>
<th>Average initial weight in grams</th>
<th>Average change in wt. in grams</th>
<th>Mortality</th>
<th>Time exposed in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg/0.5 ml. Aflatoxin B</td>
<td>5</td>
<td>34</td>
<td>+37</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>0.5 mg/0.5 ml. Aflatoxin G</td>
<td>5</td>
<td>34</td>
<td>+43</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>34</td>
<td>+100</td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>

Pathological changes in the experimental birds were studied after death according to the method followed by Culling (1974). Sections of liver of treated chicken showed congestion of blood vessels with focal haemorrhages in the parenchyma (Plate 10A). Congestion and degenerative changes in the kidney were also apparent (Plate 10B).
Fig. 8. LOSS OF WEIGHT OF DAY OLD CHICK TREATED WITH AFLATOXIN-B AND AFLATOXIN-G.
**Plate 9**

Effect of purified aflatoxin on day-old-chick

A - Day-old-chick (stunted) treated with aflatoxin

B - Treated chick showing drowsiness
Feeding Experiment with Albino Mice

3 groups of albino mice numbering 10 in each group were taken for the study. The first group of 10 mice was fed with purified extracts of the toxin at the rate of 0.3 ml. per mouse per day. The second group of 10 mice was fed with crude extract at the rate of 0.3 ml/mice per day. The third group was kept on normal feed and water without toxin. The experiment was continued for 14 days; results recorded in Table 16.

Table 16

EFFECTS OF FEEDING MICE WITH AFLATOXIN ON THE WEIGHT AND MORTALITY

<table>
<thead>
<tr>
<th>Mean concentration</th>
<th>No. in groups</th>
<th>Average initial weight in grams</th>
<th>Post feeding in days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Control without toxin</td>
<td>10</td>
<td>19</td>
<td>23.5</td>
</tr>
<tr>
<td>Purified extract</td>
<td>10</td>
<td>19</td>
<td>22.9</td>
</tr>
<tr>
<td>Crude extract</td>
<td>10</td>
<td>19</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Mice were killed at 7 days interval and examined for histopathological changes. (Liver of the treated mice was fixed in 10% formalin and sections were stained with haematoxylin and eosin before examination). Mice treated with purified extracts for 7 days showed congestion of blood vessels, focal haemorrhages in the parenchyma, cytoplasmic vacuolation and degenerative and necrotic changes in the hepatocytes (Plate 11A). There was no perceptible changes in the other
Plate 10 and Plate 11

Histopathological study in day-old-chick treated with purified aflatoxin

10. A - Congestion of blood vessels with focal haemorrhages in the parenchyma (H E x 560)

   B - Congestion and degenerative changes in the kidney (H E x 560) in albino mice

11. A - Degenerative and necrotic changes in the hepatocytes (H E x 640)

   B - Biliary cell proliferation around the portal areas with formation of new bile ducts (H E x 640)
organs. After 14 days of treatment mice showed a sign of biliary cell proliferation around the portal areas with formation of new bile ducts (Plate 11B). The experiment on Albino Rats has been shown separately (Annexure-I).

The effect of metabolites of *A. flavus* was also investigated to find out if there is inhibition of pathogenic bacteria, like *Staphylococcus aureus* and *Escherichia coli*. Thom and Raper (1945) found some strains of *A. flavus* producing antibiotic like substance, "Flavicin".

Tadashi et al (1966) found that antimicrobial activity of the purified toxin against various strains of Actinomycetes increased and this crude toxin was identical with the purified aflatoxin B₁. The antimicrobial spectrum of aflatoxin is narrow and limited inhibiting only various strains belonging to the family Actinomycetes. They further found that antimicrobial activity of crude preparation of aflatoxin inhibits the growth of bacteria like *Staph. aureus* and *E. coli*.

Glasby (1976) studied the metabolic product of *A. flavus*, which is penicillin-like substance. The formula of Aspergillic acid is \( C_{19}H_{20}O_2N_2 \); MP 97.9°C. The media on which *A. flavus* was grown was tryptone medium on Czapek's Dox.

Sensitivity test was carried out by single disk diffusion method as per method of Bauer et al (1966). Disks were prepared at the following concentrations, 300 mcg., 150 mcg. and 75 mcg. per disk.
Staph. aureus and E. coli cultures were used for the sensitivity test. The above organisms were grown in nutrient broth for 12 hours at 37°C and then seeded on Nutrient agar plates and kept for half an hour at 37°C. The excess of the culture fluid was pipetted out and 6.5 millimeter dia disk of whatman filter paper containing different concentrations of aflatoxin mentioned above were placed in order, keeping a blank disk as the negative control in the centre and sulphathiazole, 300 mcg. per disk as positive control for Staph. aureus and E. coli respectively. The plates were incubated at 37°C for 24 hours and results were recorded by measuring the inhibition zones against each concentrations of aflatoxin as well as both the controls in mm. The test was repeated three times and the average of the three were presented in Table 17 (Plate 12A and B).

**Table 17**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Disk concentrations in microgram</th>
<th>Inhibition zone (in mm) Staph. aureus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>12.0</td>
<td>11.0</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>13.5</td>
<td>12.5</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>16.0</td>
<td>16.0</td>
</tr>
<tr>
<td>4</td>
<td>Blank</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Plate 12

Effect of aflatoxin on gram positive and gram negative bacteria

Effect on E. coli

A. (1) Aflatoxin in micrograms 0.75 mcg - Zone - 11 mm
(2) 0.150 " - " - 12.5 mm
(3) 0.300 " - " - 16.0 mm

Furazolidine
Negative (B) Blank control - No inhibition

B. Effect on S. aureus

(1) 0.75 mcg - Zone - 12 mm
(2) 0.150 " - " - 13.5 mm
(3) 0.300 " - " - 16.0 mm

Sulphatriad
Negative (B) Blank control - No inhibition