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CHARACTERIZATION OF THE MODE OF ACTION OF BENZOIC ACID ON AFLATOXIN BIOSYNTHESIS BY ASPERGILLUS FLAVUS.

The Ohio State University, Ph.D., 1977
Microbiology

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CHARACTERIZATION OF THE MODE OF ACTION OF BENZOIC ACID ON AFLATOXIN BIOSYNTHESIS

BY ASPERGILLUS FLAVUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University

1977

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ADKNOLEDGEMENTS

The author wishes to express unqualified gratitude to his adviser, Professor John R. Chipley, for his encouragement and guidance. His thoughtful approach to research has been the source of my inspirations.

Sincere appreciation is extended to Professors Bruno J. Kolodziej and Michael O. Garraway for their counseling and cooperation throughout this investigation.

The author also acknowledges the assistance of C. Weisenberger and C. Cothel for characterization of the yellow compound through NMR and Mass Spectral Analyses.

To my mother, I am deeply grateful for her moral and financial support through most of the difficult stages of my education.
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### CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>4</td>
</tr>
<tr>
<td><strong>Biosynthesis of Aflatoxins.</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>The Structure and Chemistry of Aflatoxins</strong></td>
<td>8</td>
</tr>
<tr>
<td><strong>Metabolites of Aflatoxins</strong></td>
<td>9</td>
</tr>
<tr>
<td><strong>Incidence of Aflatoxins</strong></td>
<td>11</td>
</tr>
<tr>
<td><strong>Metabolism of Aflatoxins</strong></td>
<td>13</td>
</tr>
<tr>
<td><strong>Toxicology of Aflatoxins</strong></td>
<td>17</td>
</tr>
<tr>
<td><strong>Biochemical and Intracellular Effects of Aflatoxins.</strong></td>
<td>21</td>
</tr>
<tr>
<td><strong>The Assay of Aflatoxins</strong></td>
<td>24</td>
</tr>
<tr>
<td><strong>Factors Affecting Aflatoxin Production</strong></td>
<td>28</td>
</tr>
<tr>
<td><strong>Control of Aflatoxin Production</strong></td>
<td>33</td>
</tr>
<tr>
<td><strong>Detoxification of Aflatoxin</strong></td>
<td>35</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>38</td>
</tr>
<tr>
<td><strong>Organism.</strong></td>
<td>38</td>
</tr>
<tr>
<td><strong>Growth Media.</strong></td>
<td>38</td>
</tr>
<tr>
<td><strong>Aflatoxin Analysis.</strong></td>
<td>40</td>
</tr>
<tr>
<td><strong>Analysis of Metabolites</strong></td>
<td>41</td>
</tr>
<tr>
<td><strong>Physical and Chemical Properties of Compound.</strong></td>
<td>42</td>
</tr>
<tr>
<td><strong>Preparation of Cell-Free Extract.</strong></td>
<td>43</td>
</tr>
<tr>
<td><strong>Protein Determination</strong></td>
<td>43</td>
</tr>
<tr>
<td><strong>Enzyme Assay.</strong></td>
<td>44</td>
</tr>
<tr>
<td><strong>Protein Isolation</strong></td>
<td>45</td>
</tr>
<tr>
<td><strong>Disc-Gel Electrophoresis.</strong></td>
<td>45</td>
</tr>
<tr>
<td><strong>Marking of Column</strong></td>
<td>46</td>
</tr>
</tbody>
</table>
## CONTENTS (continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>47</td>
</tr>
<tr>
<td>Effects of Various Acids and Salts on Growth and Aflatoxin Production</td>
<td>47</td>
</tr>
<tr>
<td>Characterization of Yellow Compound</td>
<td>68</td>
</tr>
<tr>
<td>Conversion of Yellow Compound to Aflatoxin</td>
<td>71</td>
</tr>
<tr>
<td>Factors Affecting the Production of Aflatoxin in Cell-Free System</td>
<td>71</td>
</tr>
<tr>
<td>Protein Characterization</td>
<td>76</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>77</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>79</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>86</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>Synthetic medium for <em>A. flavus</em></td>
</tr>
<tr>
<td>2</td>
<td>Effects of 2-nitrobenzoate (NBA) on growth and production of aflatoxin by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>3</td>
<td>Effects of para-aminobenzoic acid (PABA) on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>4</td>
<td>Effects of benzocaine (ethyl amino-benzoate) on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>5</td>
<td>Effects of ethyl benzoate on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>6</td>
<td>Effects of methyl benzoate on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>7</td>
<td>Effects of benzoic acid and sodium benzoate on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>8</td>
<td>Effects of salicylic acid (0-hydroxybenzoic acid) on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>9</td>
<td>Effects of sodium chloride on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>10</td>
<td>Effects of sodium acetate on growth and production of aflatoxins by <em>Aspergillus flavus</em></td>
</tr>
<tr>
<td>11</td>
<td>Effects of protein concentration on the conversion of yellow compound to aflatoxin B₁</td>
</tr>
<tr>
<td>12</td>
<td>Effects of benzoic acid on cell-free system</td>
</tr>
<tr>
<td>13</td>
<td>Effects of barium chloride on cell-free system</td>
</tr>
</tbody>
</table>
### FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distribution of $^{14}$C in aflatoxin $B_1 - ^{14}$C from radioactive precursors</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Structural formulae of aflatoxins and related compounds</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Calibration of aflatoxin $B_1$, $B_2$, $G_1$, and $G_2$ standards</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>Standard curve for Lowry protein determination</td>
<td>82</td>
</tr>
<tr>
<td>5</td>
<td>Calibration curve of Bio-Gel A 1.5 column</td>
<td>84</td>
</tr>
<tr>
<td>6</td>
<td>The effects of sodium malonate and malonic acid on aflatoxin production by <em>Aspergillus flavus</em></td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>The effects of benzoic acid or sodium benzoate on production of yellow compound</td>
<td>64</td>
</tr>
<tr>
<td>8</td>
<td>The effects of benzoic acid or sodium benzoate on aflatoxin $B_1$ production</td>
<td>66</td>
</tr>
<tr>
<td>9</td>
<td>Infrared spectrum of yellow compound</td>
<td>69</td>
</tr>
</tbody>
</table>
Aflatoxin, a toxic metabolite produced by certain strains of *Aspergillus flavus* and *A. parasiticus*, is one of the most potent carcinogens known. There is ample evidence that aflatoxins are widely distributed in the environment. Their ubiquitous nature suggests a potential hazard of human intoxication, either directly by consumption of moldy foods or indirectly by ingestion of animal products previously intoxicated as a result of animal consumption of mold-infested feeds. Ingestion of such moldy food or feeds causes a pathological disorder called aflatoxicosis.

That fungi can produce toxic substances has been recognized for centuries. Ergotism, known a thousand years ago as St. Anthony's Fire, was a disease which killed many thousands of people in Europe (Berger, 1931). The disease was caused by a fungus, *Claviceps purpurea*, which grows on rye. Japanese research workers have been involved in mycotoxin research for many years and their work on "yellowish" rice in the 1940's can probably be considered the fore-runner of the research concerning carcinogenic mycotoxins.

The Japanese discovery of mycotoxins, although published in the Western world in the 1950's, had little global impact until 1960 when work on other carcinogenic...
mycotoxins was undertaken. Most of this research occurred as a result of the "turkey X" disease which killed thousands of turkeys in Britain. The etiological agent was blue fluorescing substances produced by A. flavus, and hence called aflatoxins. It was found that several domestic animals, including poultry, pigs, and cattle, were susceptible to this toxin. Reports of laboratory studies indicated that the mode of action of aflatoxin involved not only hepatoxic effects, but also hepatocarcinogenic effects producing liver tumors in rats, trout, ducklings, and pigs. Recently, impetus was given to this idea by subsequent findings which indicated that aflatoxin was not only a carcinogen, but that it was the most potent carcinogen known to man. A few micrograms are enough to produce tumors in a high percentage of test animals.

Since the discovery of the aflatoxin problem in 1960, a vast and continually expanding amount of literature has been published. Various difuranocoumarin compounds have been isolated and characterized. The four which are most familiar are aflatoxins B_1, B_2, G_1, and G_2 based on their respective blue and green fluorescence and R_f values on thin-layer chromatograms. In view of the extreme toxicity and carcinogenic activity of aflatoxin-contaminated materials, much effort has been devoted to achieving effective control of aflatoxin likely to contaminate foodstuffs.

There are few published reports on detoxification of aflatoxin-contaminated substances. Applegate and Chipley
(1973) reported that growth and sporulation of toxigenic strains of A. flavus were greatly reduced in both wheat and synthetic medium by exposure to 300 krad with complete inhibition from exposure to 400 or 600 krad of gamma irradiation. However, there was an increase in aflatoxin production when toxigenic spores were irradiated with 50, 100, or 200 krad of gamma irradiation. During an extensive screening of microorganisms, Cieger et al. (1966a) found that Flavobacterium aurantiacum and different Rhizopus species were potent aflatoxin-degrading organisms.

There has been a limited amount of research involving the use of benzoic acid or its derivatives to inhibit growth and aflatoxin production of toxigenic strains of A. flavus in foodstuffs (Davis and Diener, 1967). The purpose of this study was to determine the levels of benzoic acid and benzoic acid derivatives necessary to inhibit growth and aflatoxin production by an aflatoxin-producing strain of A. flavus and to elucidate the reasons for their effectiveness in inhibiting aflatoxin production.
REVIEW OF LITERATURE

Biosynthesis of Aflatoxins

Several experimental approaches have been employed by different investigators for the elucidation of the pathway for aflatoxin biosynthesis. Based on the structural characteristics of aflatoxins and possible analogs from the synthesis of other fungal secondary metabolites, several plausible pathways were advanced with no experimental evidence (Moody, 1964; Holker and Underwood, 1964; Heathcote et al., 1965; Thomas, 1965). Particularly notable was the derivation of the substituted coumarin moiety from the polyketide hypothesis:

acetate $\rightarrow$ anthraquinone $\rightarrow$ xanthones $\rightarrow$ coumarins

and the involvement of averufin, versicolorin, and sterigmatocystin as biosynthetic intermediates (Thomas, 1965; Mateles and Wogan, 1967; Biollaz et al., 1970; Moss, 1972).

Biollaz et al. (1970) determined the label distribution in the molecule of $^{14}$C-labeled aflatoxin synthesized from [1-$^{14}$C] - and [2-$^{14}$C] acetate and Hsieh and Mateles (1971) provided evidence for the polyketide hypothesis. They suggested the possible involvement of a C$_{18}$ polyhydroxynaphthacene compound and averufin in the biosynthesis of aflatoxin.
Aflatoxin $B_1^{14}C$ (Figure 1) was produced by resting cell cultures of $A. \text{flavus}$ (NRRL 3145) from sodium acetate precursors (Mabee et al., 1972). In a similar experiment, Steyn et al. (1975) observed aflatoxin $B_1$ biosynthesis from acetate via two oxidative fissions of a preformed aromatic precursor established by $^{13}C$ NMR spectra of derivatives enriched with [2-$^{13}C$] – [1, 2-$^{13}C$] – acetate. Incorporation of [1-$^{14}C$] acetate and [2-$^{14}C$] malonate into aflatoxin by resting mycelia of $A. \text{parasiticus}$ suspended in different buffers was investigated by Gupta et al. (1975). They reported that mycelia took up comparatively more acetate than malonate, but more malonate (4.3%) entering mycelia was incorporated into aflatoxins than was acetate (1.6%). Furthermore, the addition of unlabeled acetate reduced the incorporation of label from [1-$^{14}C$] acetate by 75% but from [2-$^{14}C$] malonate by only 25%. Their results suggested that malonate was an intermediate in aflatoxin biosynthesis and that it can be incorporated without prior conversion to acetate.

The use of mutant strains of $A. \text{parasiticus}$ impaired in aflatoxin biosynthesis led to the finding that averufin and norsolorinic acid (Lee et al., 1971) were accumulated when ability to produce aflatoxins diminished. These findings indicated that the two compounds produced by $A. \text{parasiticus}$ mutant strains were intermediates in the aflatoxin $B_1$ biosynthetic pathway. Other compounds, such as storigmatocystin and averufin, could be readily converted into aflatoxin.
Source of Carbon Atoms

△ - Acetate-1-$^{14}$C
□ - Acetate-2-$^{14}$C
○ - Methionine (-CH$_3$)

Figure 1. Distribution of $^{14}$C in aflatoxin B$_1$-$^{14}$C from radioactive precursors.
B₁ by the wild type *A. parasiticus* (Lin et al., 1973; Hsieh et al., 1973).

Biosynthesis of aflatoxin was found to be inhibited by an organophosphorus insecticide dichlorvos (Rao and Harein, 1972; Hsieh, 1973; Schroeder et al., 1974; Yao and Hsieh, 1974) and by benzoic acid and its salt (Uraih and Chipley, 1976). The inhibition of the biosynthesis was accompanied by the accumulation of an orange or yellow pigment which was readily converted into aflatoxin B₁ (Yao and Hsieh, 1974; Uraih and Chipley, 1977). Schroeder et al. (1974) and Yao and Hsieh (1974) have identified the orange pigment as a versiconal-type acetate. These findings indicate that the orange pigment could be another intermediate in aflatoxin biosynthesis.

Hsieh et al. (1976) compared the efficiency of conversion of norsolorinic acid into aflatoxin B₁ to that of proven intermediates such as sterigmatocystin, orange pigment, versicolorin and averufin. They found that while only trace amounts of the four intermediates were converted to aflatoxin B₁, 2% norsolorinic acid was converted to aflatoxin B₁. Based on experimental data, they thus proposed the following pathway for aflatoxin B₁ biosynthesis:

10 acetate → Norsolorinic acid → averufin →
Orange pigment → versicolorin → sterigmatocystin →
aflatoxin B₁.
The Structure and Chemistry of the Aflatoxins

The structural interpretations of aflatoxin are based mainly upon their long-wave ultraviolet (uv) fluorescence on thin-layer chromatography plates (TLC), infrared (IR), mass spectrometry, and nuclear magnetic resonance (NMR) spectra (Asao et al., 1965).

Aflatoxin B\textsubscript{1} and B\textsubscript{2} exhibit blue fluorescence while G\textsubscript{1} and G\textsubscript{2} fluoresce green under ultraviolet light (Nesbitt et al., 1962). Mass spectrometric analysis showed molecular formulae of C\textsubscript{17}H\textsubscript{12}O\textsubscript{6} and C\textsubscript{17}H\textsubscript{12}O\textsubscript{7} for aflatoxins B and G, respectively, based on the molecular weights of 312 (B\textsubscript{1}), 314 (B\textsubscript{2}), 328 (G\textsubscript{1}), and 380 (G\textsubscript{2}) (Chang et al. 1963; Wogan, 1966a). NMR spectra of aflatoxin showed easily discernible peaks due to the acetyl and methoxy protons, and peaks due to the aromatic protons of the synthetic coumarin. Infrared spectra showed absorption bands in the spectrum of natural products and are attributable to coumarin and ketone carboxyl groups.

Toxicities of aflatoxin B\textsubscript{1} and G\textsubscript{1} are higher than those of aflatoxins B\textsubscript{2} and G\textsubscript{2} which indicate that the reduction of the double bond of the dihydrofuran ring decreases the level of toxicity of aflatoxins (Carnaghan et al., 1963; Hartley et al., 1963). Catalytic hydrogenation of aflatoxin B\textsubscript{1} could be stopped after the uptake of one mole of hydrogen to yield aflatoxin B\textsubscript{2} (Van der Merwe et al., 1963; Van Dorp et al., 1963).
Similar results were obtained by Chang et al. (1963) thus confirming that aflatoxin B₂ is dihydroaflatoxin B₁. Due to the structural similarity of aflatoxin G₁ to aflatoxin B₁, Asao et al. (1965) easily deduced the structure of aflatoxin G₁ (Figure 2). Aflatoxin G₂ was reported to be the dihydro-derivative of aflatoxin G₁ (Van der Merwe et al. 1963).

**Metabolites of Aflatoxins**

Aflatoxins B₂a and G₂a are structural derivatives of aflatoxins B₁ and G₁ (Pohland et al., 1968). Ciegler and Peterson (1968) found their ultraviolet and infrared spectra similar to those of previously reported aflatoxins. Mass spectrophotometric data showed that aflatoxins B₂a and G₂a have molecular formulae of C₁₇H₁₄O₇ and C₁₇H₁₄O₈, respectively. NMR spectra indicated that hydroxy groups were located at the 2-position of the terminal dihydrofuran ring. Dutton and Heathcote (1968) reported that aflatoxins B₂a and G₂a are non-toxic to ducklings even at dosage levels over 60 times the amount required for aflatoxin B₁. Isolation of aflatoxin B₂a and G₂a from A. flavus cultures could be due to the culture's acidity or to extraction procedures employed by respective investigators (Pohland et al., 1968; Chipley et al., 1974).
Figure 2. Structural formulae of the aflatoxins and related compounds.
Aflatoxins $M_1$ and $M_2$

Aflatoxin $M_1$ and $M_2$ were first reported in milk of cows ingesting aflatoxins (Allcroft and Carnaghan, 1963) and were later observed in lactating rats (de Iongh et al., 1964). Holzapfel et al. (1966) isolated aflatoxin M from urine of sheep administered mixtures of aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$. Duckling assays showed the LD$_{50}$ of aflatoxins $M_1$ and $M_2$ to be 16 and 61 µg, respectively, while that of $B_1$ was 12 µg (Purchase and Steyn, 1967). Carnaghan et al. (1963) found the LD$_{50}$ of aflatoxins $M_1$ and $M_2$ to be 61.4 µg which indicates that both aflatoxins $M_1$ and $M_2$ retain their toxicities.

Incidence of Aflatoxin

A. flavus and A. parasiticus are ubiquitous and appear capable of growth and aflatoxin production on almost an endless variety of foods including peanuts. Since the initial report in 1961, the natural occurrence of aflatoxin in peanuts and peanut meal has been reported by several investigators (Borker et al., 1966; Wogan, 1966b).

Sampling of harvested crops in northern Nigeria in 1963 and 1964 showed that they contained aflatoxin (McDonald and Harkness, 1967). The authors also found that wet weather delayed aflatoxin appearance and shell damage predisposed kernels to toxin contamination. In India, during 1967-1968,
peanuts and peanut cakes were collected from various peanut oil mills; there was visible mold growth on peanuts and high levels of aflatoxin present in both peanuts and peanut cakes.

In the United States, Taber and Schroeder (1967) analyzed 78 samples of warehouse stock peanuts grown in 9 different regions and they found aflatoxin concentrations varied from 0-91 ppm. Tung and Ling (1968) recovered aflatoxin in a variety of foodstuffs including rice, peanut oil, sweet potatoes, and peanut butter. Shotwell et al. (1966) observed that rice is an excellent substrate for aflatoxin production. A. flavus-contaminated rice is usually found during post-harvesting seasons (Schroeder, 1970). Dried rice has a moisture content below 18 percent, thereby preventing fungal growth and subsequent aflatoxin production. Matsura et al. (1970) analyzed 46 samples of Japanese domestic rice, 11 samples of imported rice, 28 samples of Koji and 238 industrial strains of Koji-mold, but no aflatoxin was detected in any of the samples. No toxin was detected in rice collected from markets in Taiwan (Tung and Ling, 1968).

Allcroft and Carnaghan (1963) reported that no aflatoxin M1 was detected in milk or cow-peas, clotted blood, livers, or serum of cows or eggs from pullets fed rations containing toxic peanut meal. Peer (1968) reported that from 968 samples of foods and 96 locally brewed beers in the Muranga district of Kenya, analyses revealed that 22 of these samples
contained aflatoxins. Two samples which were positive for aflatoxin M were the only 2 samples containing milk. Campbell (1969) analyzed a wide variety of local foods in the Philippines and found that peanut butter produced locally contained more than 30 ppb aflatoxin. He also found that peanuts, corn, beans, cassava, and coconut products contained aflatoxins.

Aflatoxin was reported to be absent from fresh or raw beef because of bacterial overgrowth (Wildman et al., 1967). Frank (1968) isolated aflatoxin from a number of foods including smoked bacon. Bullerman et al. (1969a) found that aflatoxin was produced on fresh beef (on which bacterial spoilage was delayed with antibiotics), ham, and bacon. The presence of curing ingredients, especially pepper and sodium nitrite, was found to reduce the amount of aflatoxin produced (Bullerman et al., 1969b).

It appears that in areas of the world with high humidity, moderate to high temperature, and with poor harvesting, storing, and marketing facilities, the frequency of mycotoxin-contaminated foods, particularly aflatoxin, will be high.

**Metabolism of Aflatoxin**

Many investigators have studied the metabolism of aflatoxin in order to provide more information on how it exerts its toxic and carcinogenic effects on animals. Patterson and Allcroft (1970) reported that homogenates
from chick, duckling, turkey, quail, rabbit, and mouse livers metabolized aflatoxin much more rapidly than comparable preparations from the tissues of calf, goat, pig, rat, and sheep liver. With the exception of mouse liver, the tissues of the former group were found to possess a cytoplasmic enzyme capable of rapidly reducing aflatoxin $\text{B}_1$ to a product tentatively identified as aflatoxicol (Patterson and Roberts, 1971). Crude microsomal preparations from mouse liver were capable of converting aflatoxin to its hemiacetal aflatoxin $\text{B}_2\alpha$ (Peterson and Allcroft, 1970).

Studies of the metabolism of aflatoxin $\text{B}_1$ in the rhesus monkey (Dalezios and Wagon, 1972) indicated that when the toxin was administered interperitoneally, the urinary metabolites were present mainly in water-soluble form. Aflatoxin $\text{P}_1$ represented the primary urinary aflatoxin derivative. Aflatoxin $\text{M}_1$ accounted for only 2.3 percent and unmetabolized aflatoxin $\text{B}_1$ accounted for about 0.05 percent of the administered dose.

Livers of both rat and monkey exhibit a similar pattern of retention of $[^{14}\text{C}]$ aflatoxin $\text{B}_1$ for long periods of time (Dalezios and Wogan, 1972; Lijinsky et al., 1970; Wogan et al., 1967). In addition, most of the retained radioactivity is associated with the total liver protein (Dalezios and Wogan, 1972). De longh et al. (1964) and Allcroft and Carnaghan (1963) reported that cows whose ration contained toxic peanut meals excreted milk with no detectable toxin levels, either
in the bulk milk supply or in the tissues of the cow. However, the level of aflatoxin in the milk was within the limits of sensitivity of the duckling bioassay. Van der Linde et al. (1965) studied aflatoxin metabolism in cows fed rations containing 4 ppm of aflatoxin for 18 days. Through duckling bioassay and chromatographical examination, they found that aflatoxin appeared in the milk within 12-24 hours after an aflatoxin-contaminated diet was fed to the cows. Presence of aflatoxin persisted for 4 days after the toxic feed was withdrawn.

Tissue distribution and metabolism of aflatoxin B\(_1\)-\(^{14}\)C in layer chickens were reported by Mabee and Chipley (1973a). Their results indicate that layer chickens can metabolize the majority of aflatoxin B\(_1\) when administered at low levels. Chemical examination of pooled, lyophilized, radioactive excreta, blood, organs, and tissues of layers indicated that 81.2 percent of the radioactivity was observed in these substrates. Conjugates of a 4-hydroxy-aflatoxin B\(_1\)-\(^{14}\)C (aflatoxin M\(_1\)-\(^{14}\)C) metabolite was the predominant form produced. Broiler chickens administered low levels of aflatoxin B\(_1\)-\(^{14}\)C by crop intubation daily for 14 days excreted 90.64 percent of the \(^{14}\)C administered (Mabee and Chipley, 1973b). They further reported that of the \(^{14}\)C retained, 11.04, 9.83, 4.30, 12.52, 31.66, and 30.63 percent were detected in the blood, liver, heart, gizzard, breast, and leg, respectively. Further metabolism of \(^{14}\)C aflatoxin
in chicken tissues was investigated (Chipley et al., 1974). They reported that dried and frozen ethyl acetate extracts of liver, heart, gizzard, breast, leg, blood and fecal samples were obtained from either layer or broiler chickens fed subclinical levels of $^{14}\text{C}$ aflatoxin B$_1$. Treatment of these extracts with either carboxypeptidase A, leucine aminopeptidase, pepsin, or trypsin revealed that an average of 50 percent of the detected $^{14}$C in the acetate extracts was a liberated peptide (or amino acid) conjugate of $^{14}$C aflatoxin B$_2\alpha$. Kinetic studies of ring-labeled aflatoxin B$_1$ through the intestinal route revealed that radioactivity is ultimately excreted in the feces and was derived from bilinary excretion (Falk et al., 1965; Bassir and Osiyemi, 1967).

Nabney et al. (1967) pointed out that aflatoxin M$_1$ in sheep could not have arisen from aflatoxin B$_1$ by methylation, and they suggested that 90 percent of the ingested dose which could not be recovered was demethylated to a phloroglucinol-like structure which was broken down to non-fluorescent metabolites. The comparative resistance of the sheep to aflatoxin was due to its efficiency in this detoxification mechanism.

Ciegler et al. (1966b) screened a number of yeasts, molds, bacteria, actinomycetes, algae and fungal spores for their ability to degrade aflatoxin. Some molds and mold spores partially transformed aflatoxin B$_1$ into new fluorescing
compounds and *Flavobacterium aurantiacum* was capable of removing aflatoxin from the medium. Mann and Rehn (1975) in a similar experiment reported that *Corynebacterium rubrum* and *Mycobacterium phei* were able to transform aflatoxin $B_1$ into a non-toxic fluorescing compound.

**Toxicology of Aflatoxin**

Aflatoxicosis has been well-documented in domestic and laboratory animals and recently in man. In chicks, ducklings, goslings, pheasants, and poult's fed various levels of aflatoxins, Muller et al. (1970) found the most significant pathological changes occurred in the livers. Grossly effected birds had livers that were brown to tan in color with some varying degrees of greenish discolorations. Hepatic cell degradative changes and bile ductule hyperplasia were also present in varying degrees. Boočuvil and Hamilton (1975) found increased mortality rates, enlarged livers, and decreased total blood serum in chickens fed aflatoxins. Incorporation of 20 ppm of aflatoxin into the feed of mature breeder males caused clinical aflatoxicosis as evidenced by a significant decrease in body weight, but did not produce any other significant characteristics (Briggs et al., 1974). Newberne et al. (1966a) noted that supplementation of the diet with arginine and lysine increased the sensitivity of ducklings to aflatoxicosis whereas additional methionine, cysteine, or glutathione had no effect. The duckling appears
to be the most susceptible test species and Armbrecht et al. (1964) found liver lesions in ducklings fed 1 mg of aflatoxin B₁/g of feed per day for 12 weeks in addition to the usually reported pathology. Newberne (1976) fed rats (after weaning) with diets supplemented with aflatoxin B₁ and observed the development of liver tumors. Butler and Baines (1966) observed tumors in rat stomach containing aflatoxin. Spontaneous tumors of this type were so rarely encountered that this gave rise to speculations that aflatoxin might be implicated in carcinomas other than those of the liver.

Aflatoxin has been shown to cause abortion or fetal death in pregnant dogs, but it has not yet been established that this has a direct effect on the fetus. Dogs are highly susceptible to both oral and interperitoneally-administered aflatoxin B₁ and the pathological picture produced was similar to that of "hepatitis X" (Newberne et al. 1966b). Repeated doses of aflatoxin were found to be less toxic to dogs than a single equivalently large dose.

Allcroft et al. (1966) checked the tissues and urine of dosed animals (sheep and goats) for aflatoxin or its metabolites. Aflatoxin B₁, G₁ and M were found in the urine, liver, and kidneys with M being more concentrated in the latter organ. Nabney et al. (1967) determined the excretion of aflatoxin components in milk, urine, and feces of a lactating ewe after oral administration of a single dose (1 mg mixed aflatoxin/kg of feed). Only 8.1 percent of the total dose was recovered in
an identified form. Aflatoxin $M_1$ was found primarily in the urine while $G_1$ was excreted mainly in the urine and feces with some traces in milk.

Clerk and Caurie (1968) reported that $A. \text{flavus}$ previously isolated from contaminated cassava caused various biochemical changes in the cassava tuber. The total nitrogen content was lowered, and the amino acid and soluble carbohydrate contents of the tuber were varyingly altered, both quantitatively and qualitatively by $A. \text{flavus}$. Schoental and White (1965) found that 100 ppm of aflatoxin completely inhibited the germination of cress seed and 10 ppm of the toxin induced chlorophyll deficiency in the seedlings. Uritani et al. (1970) utilized sweet potato slices as an assay system for determining the effect of aflatoxin on plant cells. A dramatic shift in metabolism was observed following injury to the root which was characterized by an increase in the levels of several enzymes related to respiration and polyphenol synthesis. The number of mitochondria also increased subsequent to injury which paralleled an enhanced cytochrome oxidase activity.

The pathogenesis and etiology of primary pulmonary adenomatosis in man was investigated by Belchetz et al. (1976). They reported that pulmonary adenomatosis was due to inhalation of aflatoxin. Epidemiological studies in tropical areas have shown that high cancer in the population may be related to the ingestion of contaminated food (Peer and
Linsell, 1973). In similar work, Foy et al. (1966) related the high incidence of primary liver cirrhosis and cancer among Africans to dietary insufficiencies, particularly pyridoxine, in conjunction with ingestion of aflatoxin. Payet et al. (1966) described some observations made in Senegal on children who inadvertently had been fed aflatoxin-contaminated meal for a prolonged period of time. Four children of one year of age were involved. The concentrations of aflatoxin-contaminated peanuts they consumed ranged from 70-140 \( \mu \text{g} \) per day for 10 months. No signs of primary carcinoma or any indication of carcinogenic degeneration was seen in their liver tissues. However, liver biopsies from 2 of the 4 children revealed lesions of fibrosis.

Tung and Ling (1968) extensively studied foodstuffs in Taiwan for aflatoxin contamination. They found aflatoxin in a variety of foodstuffs where agricultural products were dried and stored in damp warehouses. They further surveyed 3 families involving 25 of 39 members where 3 children had died. Moldy rice was incriminated as the causal agent. Analyses of the rice revealed concentrations of 23 ppm of aflatoxin. Similar results of liver cirrhosis and aflatoxin-contaminated diets were obtained among the Bantus of South Africa (Acherman, 1972). However, the conclusion still remains that only circumstantial evidence exists for the involvement of aflatoxin as the etiological agent of liver cancer in humans.
Biochemical and Intracellular Effects
of Aflatoxins

Acute poisoning with aflatoxin B₁ had been shown to produce alterations in the liver metabolism of rats and ducklings (Shank and Wogan, 1966). The liver size was reduced, but total lipid was increased. In ducklings, the incorporation of label from ^14C-glucose into lipid was reduced but that from ^14C-leucine into protein, after an initial reduction, was increased.

Ingestion of toxic meal has been shown to produce changes in the levels of oxidized pyridine nucleotides in the livers and sera of chickens. Although the liver levels returned to normal after 4 weeks, the lowered serum concentration persisted for 2 weeks, which suggested an enhanced utilization of these nucleotides (Plantonow, 1965). Brown and Abrams (1965) have published the results of an extensive study of aflatoxicosis in ducklings and chickens involving key enzymes in the glycolytic and tricarboxylic acid cycles, the respiratory chain, and oxidative respiration mechanisms. Marked decreased activity was recorded for mitochondrial dehydrogenase and oxidative phosphorylation systems of the liver. Concomitant increases in the activity of plasma enzymes were correlated with hepatic damage and were suggested as possible diagnostic indicators of aflatoxicosis. Abnormal plasma was obtained from affected birds which led the authors to suggest that the reduction in plasma albumins
could account for growth impairment, while the reduction in plasma globulins could be the cause of increased susceptibility to infection. They concluded that suppression of protein synthesis was due to a lowered ATP synthesis consequent to mitochondrial damage in the liver cells.

Aflatoxin inhibited the DNA-dependent RNA polymerase system in rat liver nuclei soon after its administration in vivo interfering with gene transcription with levels of aflatoxin that were much lower than the rat LD$_{50}$ or that required to inhibit cytidine incorporation into RNA. The inhibition of RNA polymerase explained the effect of aflatoxin in lowering the RNA-DNA ratios and could be due to the binding of aflatoxin to DNA (Lilly, 1965). Cliford and Rees (1967) have proposed that upon entering the rat liver cell, aflatoxin passes to the nucleus where it binds to DNA and the RNA polymerase system. The reduced RNA synthesis involves reduction or inhibition of the messenger RNA which leads to reduced protein synthesis.

Singh and Venkitasubramanian (1975) found that a single intraperitoneal injection (6 mg/kg body weight) of aflatoxin B$_1$ in propylene glycol resulted in a decrease in total protein and pyridine nucleotides in the liver. Levels of NADP and NADPH remained unchanged, but levels of NAD and NADH were decreased. The authors further observed that the activities of hepatic malate dehydrogenase and isocitrate dehydrogenase were not altered although isocitrate dehydrogenase showed an increase when expressed in terms of total protein concentration.
Rats fed diets marginally deficient in choline were protected against the acutely toxic action of aflatoxin B₁, but the diet did not prevent the rapid inhibition of RNA polymerase or changes in nuclear morphology following the administration of aflatoxin B₁ (Butler and Neal, 1975). Hepatocytes prepared by the simplified enzymatic technique of McIntosh et al. (1975) were found to be active in incorporation of RNA and protein precursors into acid-insoluble materials. The incorporation of RNA precursors was markedly inhibited by low levels of aflatoxin B₁ and G₁, but not by aflatoxin B₂ and G₂. Aflatoxin B₁ highly elevated sensitivity to chromosome-damaging in livers of several species (Stich and Laishes, 1975).

Ultrastructural alterations and modifications of nuclear RNA of rat liver occurred when aflatoxin was administered to thioacetamide-treated rats (Reynier et al., 1975). Synthesis of nuclear RNA not only stopped but RNA that accumulated in the nuclei by thioacetamide action disappeared. Rat livers, when exposed to a single dose of aflatoxin B₁ lethal to 50 percent of the animals (7.20 μg/kg), revealed periportal cell necrosis and biliary cell proliferation (Kalengay and Desmet, 1975). Periportal cytoplasmic glycogen and RNA depletion occurred during an early period and subsequently extended to the whole lobule. Histochemical changes reverted to normal after cessation of the necrosis.
The Assay of Aflatoxins

Aflatoxin continues to dominate mycotoxin research. An area which has attracted the most attention and interest is the analytical methods, particularly those involving the development of sensitive methods for determination of aflatoxin in certain foodstuffs.

Physiochemical Method

The physiochemical procedure is based on the fluorescence exhibited by aflatoxins in ultraviolet light. This involves extraction and purification of extracts, followed by chromatograms, either by dilution to extinction or by comparison of the observed fluorescence with that of standard aflatoxin preparations. There have been many modifications of the original procedure of Sargeant et al. (1961), such as those of Neishem et al. (1964), Trager et al. (1964), and Eppley (1966), and thin-layer chromatography on silica-gel. High pressure liquid chromatography has been effectively adapted by Leitz, (1975), Hsieh et al. (1976), Kmeiak (1976), and Pons (1976) for aflatoxin determination in most foodstuffs. The aqueous-acetone procedure of Pons and Goldblatt (1965) has also been adapted to a microtechnique suitable for determination of aflatoxins in sections of peanuts. With this method, the "heart" of an apparently sound peanut has been shown to contain 4 μg of aflatoxin/g of peanut and cottonseed as having 6 μg of aflatoxin/g (Cucullu, 1966).
Interfering pigments can be separated from lipid-free extracts by precipitation as insoluble lead or copper salts (Pons et al. 1966) or by the use of zinc acetate in extraction during purification (McKinney, 1975). Chen and Friedman (1966) have used an aqueous methanol-hexane extraction, lead acetate precipitation, and partition chromatography on celite to eliminate a blue fluorescing material occasionally encountered between aflatoxin B₁ and B₂ on chromato-plates of extracts.

Improved solvent systems such as acetone-chloroform have been developed (Eppley, 1966) and Uraih and Chipley (1976) have demonstrated that developing thin-layer chromatography plates twice in chloroform-acetone (9:1) maximizes aflatoxin separations. Due to the varying fluorescent and toxicological properties of different aflatoxins and the varying proportions of the four main compounds encountered in infested materials, the TLC method is somewhat limited for the assessment of aflatoxin toxicity. Andrellos and Reid (1964) have developed confirmatory chemical tests for aflatoxin B₁ separated on TLC plates and these have been collaboratively tested in a number of laboratories (Stoloff, 1966). Andrellos and Reid (1964) treated portions of isolated toxin with three reagents - formic acid and thionyl chloride, acetic acid, and trifluoroacetic acid and spotted the three fluorescent reaction products on TLC plates. Unreacted standards were spotted on the same plate. The $R_f$ values of various spots were compared under ultraviolet light after
development. The two main products of the reaction of acetic acid and thionyl chloride with aflatoxin B₁ were subsequently identified as the X and B-isomers of 1-acetyl-2-hydro-6-methoxydifurocoumarone (Wiley et al. 1969).

In an attempt to put the physiochemical method on a more accurate quantitative basis, Nabney and Nesbitt (1965) have developed a procedure based on the ultraviolet spectrophotometry of eluents of the aflatoxins separated on TLC plates, and Ayres and Sinnhuber (1966) have described a method based on the direct fluorodensitometric scanning of chromatoplates of the separated aflatoxins. Beckwith and Stoloff (1968) modified the densitometric procedures to eliminate inherent inaccuracies. Tested with spiked extracts of butter, the procedure gave an average 101 ± 3 percent recovery of added aflatoxin B₁ and 89 ± 8 percent recovery of aflatoxin B₂. As a result of an extensive collaborative study of aflatoxin determination (Campbell and Funkhouser, 1966), the Association of Official Agricultural Chemists have adopted a modified version of the Nesheim et al. (1964) procedures with the reservation that further improvement was considered necessary.

Biological Assay

In a biological assay, the presence of aflatoxin is judged mainly by the appearance of bile-duct hyperplasia in day-old ducklings. Newbome et al. (1964) reported that pure aflatoxins as well as extracts from molded wheat
produced a similar bile-duct hyperplasia. Due to variation in response from an assay procedure, Butler (1964) suggested that the method was not suitable for an assay procedure and that \( LD_{50} \) determinations should be used. Armbrecht and Fitzhugh (1964) showed that the sensitivity of the duckling test could be increased ten-fold by the use of repeated doses. Changes in the serum protein patterns have been reflected in the development of a polarographic method of determination of the plasma protein index reported by Datta and Gojan (1965). The index closely parallels the observed histopathological changes in the livers of ducklings.

Burmeister and Hesseltine (1966) surveyed 329 microorganisms for their sensitivity to aflatoxin and found only gram-positive sporeforming bacilli and one streptomycete to be sensitive to concentrations of 15 \( \mu g/ml \). Vangone and Vunakis (1976) demonstrated the formation of aflatoxin \( B_1 \) specific antibodies and their use in radioimmunoassay. Due to the specificities of the antisera with respect to various aflatoxins, levels as low as 1 \( \mu g \) of aflatoxin \( B_1 \) per kilogram of foodstuffs could be detected.

The chick embryo method of Platt et al. (1962) has been further developed by Verrett et al. (1964) who showed that the embryos were sensitive to pure aflatoxins as well as fungal
extracts. Crude mixtures of aflatoxins and purified preparations of aflatoxin B₁ have been shown to inhibit mitotic division in human embryo lung cells growing in tissue culture (Legator and Withrow, 1964). The ID₅₀ of aflatoxin B₁ with calf kidney cells was reported by Juhasz and Graczi (1964) as being 0.3 μg/ml. Smith (1965) has demonstrated that inhibition by aflatoxin of amino acid utilization in cell-free liver and E. coli preparations can occur and has suggested the use of this system as the basis of an in vivo assay.

Despite the wide range of assay systems, the most prevalent method is still the TLC fluorescence method with the results being confirmed by duckling bioassay.

Factors Affecting Aflatoxin Production

Medium

Effects of growth medium on aflatoxin production by A. flavus have been widely investigated. Lee et al. (1966) found that zinc was specifically required for production of aflatoxin by A. flavus. Davis et al. (1967) concluded that the influence of minerals on aflatoxin synthesis may be through their essentiality for growth or more directly on the process of toxin synthesis. Marsh (1975) reported that certain metals added as salts to a defined basal medium influenced the level of aflatoxin production by A. parasiticus. Zinc added at levels of 25 micrograms/ml of medium decreased aflatoxin production, but mycelial weight was unaffected. At
levels of 25 micrograms or less of metal per ml, salts of iron, trivalent chromium, silver, and mercury partly or completely inhibited aflatoxin production without influencing the mycelial weight.

**Maturity**

Bampton (1963) found that peanuts left in the ground 4 weeks after maturity contained aflatoxin. Toxin was also found in late planted crops in sandy soil in semi-arid regions, although plants had been in the ground for only the normal length of time. McDonald and Harkness (1964) demonstrated that both pods and kernels from one-year-old peanuts were more readily invaded by *A. flavus* than freshly dug immature and mature pods and kernels. Higher percentages of *A. flavus* invasion occurred in over-matured kernels and pods than in immatuered kernels and pods from the same plants at harvest (Deiner et al., 1965). In 1963 and 1964, peanut crops in northern Nigeria harvested at or earlier than the normal time were found to be free from aflatoxin, whereas late harvesting usually resulted in some toxicity being present (McDonald and Harkness, 1967). Increased physiological activity associated with maturity or high moisture in the soil were reported to favor invasion of palm-kernels and pods by *A. flavus* with subsequent production of aflatoxin (Deiner et al., 1965).

**Modified Atmosphere**

Modified atmospheres have shown promise in controlling *A. flavus* growth and subsequent aflatoxin production (Jay, 1971).
Further studies by Wilson and Jay (1974) demonstrated that corn stored in a modified atmosphere did not accumulate more than 15 micrograms of aflatoxin B₁ per kilogram. Corn from CO₂ treatment (61.7% CO₂, 8.7% O₂, and 29.6% N₂) was visibly molded at 4 weeks. In N₂ (99.7%) and O₂ (0.3%) and controlled atmosphere (13.5% CO₂, 0.5% O₂, and 84.8% N₂) treatments, no aflatoxin was produced but fermentation-like odors were detected.

**pH**

Maximal aflatoxin production reportedly occurred at both alkaline and acidic pH extremes (Lie and Marth, 1968). Joffe and Lisker (1969) reported that more aflatoxin accumulated in Czapek liquid medium at an initial pH of 4.0 than at 7.0. However, Reddy et al. (1971) reported that initial pH had no effect on aflatoxin production in yeast extract-sucrose medium. These results suggest that initial pH has no universal effect on production of aflatoxins by A. flavus or A. parasiticus. Apparently, the effect of pH was dependent on the composition of the medium.

**Temperature**

Rabie and Smalley (1965) found that maximum growth of A. flavus occurred at 18°C, maximum amounts of aflatoxin B₁ were produced at 24°C, and maximum amounts of aflatoxin G₁ were produced at 30°C. However, Diener and Davis (1966) showed that A. parasiticus produces the highest levels of
aflatoxin between 25 - 30°C, with a significant decrease occurring at 35°C and only slight-to-trace amounts at 40°C. Schroeder and Hein (1967) reported that the lower limit of toxin production was 10°C, but Schindlet et al. (1967) found that maximum aflatoxin was produced at 24°C whereas maximum growth occurred at either 29°C or 35°C for A. flavus. With the exception of work done by Schroeder and Hein (1968), studies on the relationship of aflatoxin production and temperature have been conducted at constant temperatures. Whereas the above studies have provided considerable and significant information, aflatoxin generation in a natural environment is likely to occur under conditions of varying temperatures. Stutz and Kruperman (1976) found that by recycling temperature, under conditions of diurnal and nocturnal time - temperature sequencing, total heat input was an important factor for aflatoxin production by A. parasiticus. No growth was detected at thermal inputs of less than 208 degree hours/day, growth was detected at thermal inputs between 208 and 270 degree hours/day, and sporulation and aflatoxin biosynthesis occurred above 270 degree hours/day.

Air

Stotzky and Goog (1965) observed that A. flavus did not grow under 100 percent nitrogen or carbon dioxide, but the mold was not killed since it was capable of growth following return to aerobic conditions. Lander et al. (1967) have
investigated the effects of various concentrations of atmospheric gases on aflatoxin production by *A. flavus* growing on peanuts. Increasing the carbon dioxide concentration from 0.03 to 20% did not reduce the mold development, but aflatoxin elaboration was progressively reduced in increasing carbon dioxide levels. A dramatic reduction in mold growth and aflatoxin production was observed when the oxygen level was reduced from 5 to 1%. Examination of aflatoxin production on solid substrates has shown that culturing in rotary shakers considerably increases yields (Hesseltine *et al.* 1966). Davis *et al.* (1966) observed the production of 60 mg of toxin/100 ml of liquid medium in static culture. Hayes *et al.* (1966) found that when the same culture was placed in a chemically defined medium, in a mechanical shaker to maximize aeration, maximum levels of 213 mg of aflatoxin in 100 ml of medium could be recorded. Mateles and Adye (1965) reported routine yields of 6.0-8.0 mg/100 ml of a synthetic medium in fluted rotary shaker flasks. Ciegler *et al.* (1966) demonstrated that proper aeration facilitated production of 20-30 mg of toxin/100 ml of growth medium. Their study indicated that adequate aeration was a critical feature for aflatoxin production.

Growth and sporulation of *A. flavus* appear to be somewhat more fastidious in moisture requirement than many molds (Ayerst and Budd, 1960). Dickens and Pattee (1966) found aflatoxin in two days at a moisture content between 15 and 30% at 32 C and four days at a moisture content between
20 and 31% at 21 C in freshly dug peanuts. Unpolished rice with a moisture content of 24-26 percent developed relatively large amounts of aflatoxin (305-750 μg/kg) in 7-21 days during warm weather storage. High concentrations of aflatoxin also occurred under conditions of high initial moisture (22-26 percent) and low aeration rates (0.5-1 cfm) (Calderwood and Schroeder, 1968). Maximum aflatoxin production developed within 2-3 weeks in corn naturally infested with toxigenic strains of A. flavus at a moisture content of 19-20 percent at 25 C (Van Warmelo et al., 1968).

Control of Aflatoxin Production

The possible presence of toxic metabolites of certain molds has been recognized only in the past few years. Laws specifically prohibiting the presence of mold as contaminants of food are of recent origin and are based specifically on esthetic and sanitary considerations. Steps are being taken to eliminate the smallest traces of mycotoxin under the United States Federal Food, Drug, and Cosmetic Act relative to the presence of microorganisms and their toxins in foods.

Although there are laws prohibiting the sale of aflatoxin-contaminated food, the most effective method of aflatoxin control or regulation is by inhibiting its production. Applegate and Chipley (1973) found that growth and toxin production by toxigenic strains of A. flavus were greatly reduced in both wheat and synthetic media by exposure of spores to 300 krad
of gamma irradiation and complete inhibition occurred from exposure to 400 or 600 krad. Schroeder et al. (1974), Rao and Harein (1972), Yao and Hsien (1974) reported that dichlorvos (dimethyl 2, 2-chlorovinyl phosphate) at 10 μg/ml reduced aflatoxin production in A. flavus. Sodium chloride at concentrations equivalent or greater than 12g/100 ml, sodium acetate (4 g/100 ml), malonic acid (50 mM), benzoic acid or sodium benzoate (100 mg/25 ml) were found to effectively inhibit growth and aflatoxin production by A. flavus (Uraih and Chipley, 1976). Davis and Diener (1967) reported that aflatoxin production by A. parasiticus was inhibited by 500 mg para-amino benzoic acid/25 ml medium. Bullerman (1974) noted that raisin bread containing cinnamon did not support extensive mold growth. He further reported that 2 percent cinnamon could be used to control growth and aflatoxin production by A. parasiticus.

Two-Mercaptoethanol inhibits growth and aflatoxin production in A. parasiticus when added to liquid media by inhibiting [1-^{14}C] acetate incorporation into both aflatoxin and neutral lipids, thereby showing that it acts at an early stage of aflatoxin biosynthesis (Gupta et al., 1976). With the levels of smoke obtained in smokehouse treatment of foods, Arseculeratne (1976) found a total suppression of fungal growth on the majority of palm kernels and in the few instances where growth occurred, it was marked by a lack of toxin production.
Detoxification of Aflatoxin

Ceigler et al. (1966a) intensively screened yeasts, molds, bacteria, actinomycetes, algae, and fungal spores for their ability to degrade aflatoxin. None of the yeasts, actinomycetes, or algae examined appeared to degrade aflatoxin. Pseudomonas species appeared to degrade aflatoxin between pH 6-9 but at a constant pH of 6.5, aflatoxin degradation was not observed. Penicillium raiskrickii gave partial conversion of aflatoxin B$_1$ to a compound similar to aflatoxin B$_2$. Only Flavobacterium aurantiacium removed aflatoxin from the solution. Both resting and growing cells of F. aurantiacum irreversibly detoxified aflatoxin, and duckling bioassays confirmed that no new toxic product was formed. Mann and Rehn (1975) found that Cornyebacterium rubrum degraded more than 99 percent of the added aflatoxin B$_1$ (1.48 mg) in liquid culture after 4 days. Mycobacterium phlei also degraded aflatoxin B$_1$ quickly. Anascosporogenous yeasts (Candida sp.) degraded 80 percent of aflatoxin B$_1$ in 20 days. Among molds, Cunningamella echinulata, Stachybotrys labulate, Penicillium islandicum, Aspergillus niger, and A. ochraceus were found to degrade aflatoxin B$_1$.

Aflatoxin extracts treated for a few seconds with 5 percent sodium hypochlorite (NaOCl) lost their fluorescence and toxicity, and contaminated peanut meal exposed overnight to 10 percent chlorine gas became nontoxic to chick embryos (Fishbach and Campbell, 1965). Yanq (1972) found that both cultures of A. flavus and aflatoxins were destroyed by
commercial bleach (chlorox; active ingredient, NaOCl) or an analytical reagent grade of NaOCl (7.0 x 10^{-3} M) in 5 days. Animals injected with NaOCl- or chlorox-destroyed aflatoxin extract survived and showed no obvious liver or kidney damage. Natarajan et al. (1975) showed that both NaOCl concentration and reaction pH, and not the temperature, were important factors in reducing aflatoxin in protein isolates to non-detectable levels. Limited reduction of aflatoxin B_{2a} with sodium borohyride (NaBH_4) at a neutral or slightly alkaline pH resulted in a new reduced derivative which was non-toxic to chicken embryos at levels 100 times the LD_{50} of aflatoxin B_1.

The use of polar solvents such as aqueous acetone and hexane-acetone-water mixtures have been reported (L. A. Goldblatt, Abstr. Meeting Am. Chem. Soc., 150th, p. 5a, 1965). However, the use of solvents on whole cottonseed or peanut kernels was considered ineffective by Goldblatt since aflatoxin has been found deep in the interior of the kernel. Treatment of cottonseed meal with ammonia and methylamine has been found practical for aflatoxin inactivation (Mann et al., 1971).

Attempts to eliminate or reduce the level of aflatoxin in toxic foods or feedstuffs by heating, cooking, or autoclaving have been largely unsuccessful (Blout, 1961; Feuell, 1966). Chu et al. (1975) found that in brewing, aflatoxin B_1 was stable to heat and insensitive to cooker mash treatment. Roasting peanuts at 160°C reduced aflatoxin B_1 by 80 percent and B_2 by 60 percent (Cuccullu et al., 1966). Gamma radiation
at a dosage level of 2.5 Mrads in foodstuffs was ineffective for the destruction of aflatoxin. The radiated sample still retained aflatoxin to which ducklings were sensitive (Feull, 1966).
MATERIALS AND METHODS

Organism

Aspergillus flavus strain 3145, used throughout this investigation, was obtained from the culture collection of the Northern Regional Research Laboratory, Agricultural Research Service, Peoria, Illinois. Stock cultures were grown initially on potato dextrose agar slants for 24 hr at 25 °C. Reference stock cultures were stored at room temperature under sterile mineral oil by the method of Werham (1946). Working stock cultures were obtained by subculturing mycelia from mineral oil-covered slants to screw-cap vials (16 x 125 mm), each vial containing a 15-ml potato dextrose agar slant. Cultures used for spore development and subsequent substrate inoculation were obtained from working stock cultures after 14 days of incubation. All active cultures were maintained in loosely fitted screw-cap vials at room temperature. Spore suspensions were prepared by adding sterile distilled water to 14-day-old sporulated cultures and spores collected by membrane ultra-filtration. Spores were then washed twice using 50 ml of distilled water and refiltered.

Growth Media

The basal medium (Table I) used was that of Adyes and Mateles (1964) supplemented either with sodium chloride,
### TABLE 1

**Synthetic medium for A. flavus**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>50.0 gm</td>
</tr>
<tr>
<td>((\text{NH}_4\text{)}_2\text{SO}_4)</td>
<td>3.0 gm</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>(\text{MgSO}_4 \cdot 7\text{H}_2\text{O})</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>(\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O})</td>
<td>0.7 mg</td>
</tr>
<tr>
<td>((\text{NH}_4\text{)}_6\text{Mo}<em>7\text{O}</em>{24} \cdot 4\text{H}_2\text{O})</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>(\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O})</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>(\text{CuSO}_4 \cdot 5\text{H}_2\text{O})</td>
<td>0.3 mg</td>
</tr>
<tr>
<td>(\text{MnSO}_4 \cdot \text{H}_2\text{O})</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>(\text{ZnSO}_4 \cdot 7\text{H}_2\text{O})</td>
<td>17.6 mg</td>
</tr>
<tr>
<td>(\text{H}_2\text{O})</td>
<td>1.0 liter</td>
</tr>
</tbody>
</table>
sodium acetate, benzoic or benzoic acid derivatives and malonic acid, or their sodium salts. Unless otherwise specified, the initial pH of the medium was 4.5. The medium was filter-sterilized using a filter pore size of 0.45 µm and 25 ml dispensed into each of several sterile 125-ml Erlenmeyer flasks. One ml of spore suspension containing $10^6$ spores/ml was inoculated into each flask and cultures were incubated for 8 days at $27^\circ$C in a mechanical shaker (100 rev/min). Growth in each flask was determined by mycelial dry weight.

**Aflatoxin Analysis**

Mycelial growth was separated from the liquid phase by filtering through 900s laboratory tissue (Kimberly - Clark) into a 250-ml separatory funnel. Chloroform (50 ml) was added to the filtrate, the mixture vigorously shaken for 60 seconds, and the procedure repeated. The chloroform layers were drawn off into 500-ml roundbottom flasks and brought to a concentration of approximately 10 ml in a rotary flash-evaporator (Buchler Instruments, Inc.). The residues were transferred to 13 x 100 glass culture tubes with two 2-ml chloroform washes and dried under nitrogen gas by a micro-N-evaporator (Organomotion Assoc.).

Approximately 30g (wet weight) of mycelial pellets separated from the liquid phase were washed twice with distilled water (50 ml) and the suspensions refiltered. The pellets
were mixed with 15 g of glass powder and ground in a mortar for about 5 min. The paste was transferred to a 250 ml separatory funnel containing 60 ml of chloroform and extracted according to procedures outlined above.

Aflatoxins were assayed by thin-layer chromatography using plates coated to a thickness of 250 μm with MN-Kieselgel G-HR silica gel (Brinkmann Instruments) and developed twice with chloroform-acetone (4:1). Aflatoxins were later isolated and quantitated using a DU-2 spectrophotometer (Beckman Instruments, Inc.) and the sum of each fraction of aflatoxins B$_1$, G$_1$, B$_2$, and G$_2$ reported are based on 85%, 82%, 78%, and 76% of aflatoxins recovered, respectively. Commercial standards were co-chromatographed and calibration curves (Figure 3) were also prepared for spectrophotometric quantitation of each toxin (Applegate and Chipley, 1974).

Analysis of Metabolites

A yellow compound accumulated in all flasks treated with benzoic acid and sodium benzoate. To obtain the compound in sufficient quantity for identification, 25 flasks of inoculated media were combined after incubation and extracted with 150 ml of acetone. The yellow compound was separated chromatographically from the acetone extract with an activity grade 1 aluminum oxide Woelm acid 8 cm x 40 cm column (ICN Pharmaceuticals, Inc.). The column was packed as a slurry with chloroform. Aflatoxins were eluted with 100 ml of chloroform and were assayed as previously outlined. The
yellow compound which remained in the column after aflatoxin elution was then eluted with acetone-chloroform (25:75) and further purified by thin-layer chromatography (MN-Kieselgel G-IR). Plates were developed in acetone-chloroform (25:75). The yellow compound was quantitated by measuring its absorbance in methanol at 312 nm (ε17,000; Hseih, Yao and Reece, Abstr. Pacific Conf. on Chemistry and Spectroscopy, 1973). Chemical and physical identification of the isolated yellow compound was then undertaken.

**Physical and Chemical Properties of Compound**

Infrared (IR) spectra were obtained with a Perkin-Elmer 237B Grating Infrared Spectrophotometer. The compound was dissolved in 0.2 ml of chloroform and the solution placed in matched sealed liquid IR cells (NaCl). Chloroform was used as a reference solvent.

Nuclear Magnetic Resonance (NMR) spectra were carried out using a Varian NMR spectrometer (Bruker HA 100). Samples of the compound were analyzed in deuterochloroform.

Mass spectra of the yellow compound were obtained using a low-resolution mass spectrometer (Dupont 21-490). All samples were introduced into a gas chromatograph (Perkin-Elmer, model 990) linked to the mass spectrometer, and programmed at 140 C for 8 min, then 4 C/min to 240 C and held for 64 min. Spectra were recorded oscillographically.
Preparation of Cell-Free Extract

*A. flavus* mycelia grown in synthetic media for 72 hr were collected in cheese cloth and washed twice with 30 ml of 0.05 M phosphate buffer, pH 7.5. Thirty g (wet weight) of mycelial pellets were mixed with 10 grams of precooled glass powder (25 μm diameter, Ultrasonics, Inc.) in a chilled mortar placed in an ice bath and ground until a smooth creamy paste was obtained. The paste was then mixed with 15 ml of phosphate buffer (pH 7.4) and the mixture centrifuged for 30 min at 5,000 x g at 0 C. The supernate was further concentrated with an Amicon diafiltration cell using a membrane with a molecular weight cut-off of 10,000 to an average protein value of 11 mg/ml.

Protein Determination

Protein was determined by the Lowry-Ciocalteau modification of the Folin protein method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard. A standard solution containing 1 mg of BSA/ml was prepared and 0.1, 0.2, 0.3, 0.4 and 0.5 ml quantities were added to separate tubes and brought to 1 ml with distilled water. The sample fractions were analyzed by placing 1-ml aliquots into separate tubes.

The following reagents were then prepared:

- Reagent A: 2% Na$_2$CO$_3$ in 0.1N NaOH
- Reagent B: 0.5% CuSO$_4$.5H$_2$O in 1% Na or K tartarate
- Reagent C: 50 ml of reagent A mixed with 1 ml of reagent B
Reagent D: 1N Folin phenol reagent
(commercial preparation)

To each of the sample and standard tubes, 5 ml of reagent C was added, mixed, and allowed to sit for 10 min at room temperature. After 10 min, 0.5 ml of reagent D was added with mixing. The color was allowed to develop a minimum of 50 min before reading at 540 nm in a Beckman DU-2 spectrophotometer. A blank was prepared by adding 1 ml of distilled water plus the reagents to a separate tube. Since Triton X-100 causes a precipitate to be formed during the Lowry protein determination, the tubes had to be centrifuged at 1500 rpm for 5 min in an International Model PR-6 Centrifuge (International Equipment Co., Needham Heights, Mass.) before reading at 540 nm. Standard curves carried out in the presence of Triton gave the same readings as shown in Figure 4.

Enzyme Assay

Study of the conversion of the yellow compound into aflatoxin B₁ was conducted in duplicate experiments at 25 C for 2 hr based on the preliminary experiment (Table 11). The reaction mixtures contained 100 mM phosphate buffer (pH 7.4), 0.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 10% glycerol, cell-free extract with a total protein value of 4 mg/ml of incubating mixture and approximately 20 mg of purified yellow compound. Incubations were carried out in a total volume of 15 ml in 50-ml Erlenmeyer flasks in a
mechanical shaker (100 rev/min) at 25 C for 2 hr. Reactions were stopped by vigorously shaking the reaction mixture with 20 ml of chloroform. Aflatoxin assays were then repeated as previously outlined.

**Protein Isolation**

Fifty ml of the cell-free extracts prepared as outlined previously were mixed with 20 ml of 60% ammonium sulphate solution and centrifuged at 20,000 xg for 30 min at 0 C. The supernate was decanted and the protein precipitate was further dialyzed with 20 ml of 0.05 M phosphate buffer solution, pH 7.4. The protein value was analyzed by the Lowry - Ciocalteau method.

**Disc - Gel Electrophoresis**

The isolated proteins were separated by the polyacrylamide disc-gel electrophoresis technique with a constant current supply. The disc-gel consisted of 2.5% large-pore upper gel, pH 6.7, and 7% small-pore gel at pH 8.3. The disc-gels were electrophoresced with 2.5 M tris (hydroxymethyl) amino methane (Tris) glycine buffer, pH 8.3, in the upper and lower reservoir. Bromophenol blue was added to the upper reservoir to serve as the tracking dye. About 10 ul of protein were added to each gel. After the entire disc-gel apparatus was loaded, it was left at room temperature, and a current of 3.5 ma was applied until the tracking dye was
about 3 mm from the bottom of the gel. After the elec­
trophoresis, the gels were removed from the gel columns with
a syringe of cold water and stained with 2% amido schwartz
(aniline blue-black) for a minimum of 24 hr. The gels were
destained with 7% acetic acid with a current flow of 12.5 ma
per gel, and scanned with a Joyce-Loebel chromoscanner for
absorption peaks.

**Marking of Column**

The Bio-Gel A-1.5 column was marked by chromatographing
15-ml standards of varying molecular weights. These
standards were prepared by dissolving 50 mg of the protein
in 10 ml of 0.05 M pyrophosphate buffer containing 1% (v/v)
Triton X-100. The void volume was determined by using
dextran blue (molecular weight 2,000,000) while acid
phosphatase (molecular weight 55,000), and horse heart
myoglobin (molecular weight 17,500) were used to determine
the separating characteristics of the column. The calibration
curve is shown in Figure 5.
RESULTS AND DISCUSSION

Effects of Various Acids and Salts on Growth and Aflatoxin Production

The present investigation showed that growth and production and release of aflatoxins could be seriously inhibited by hydroxylated benzoic acid and several benzoic acid derivatives. At concentrations of 10 mg/100 ml of medium, 2-nitrobenzoate (Table 2) stimulated growth (10%) and reduced (25%) release of aflatoxins. Para-aminobenzoic acid (Table 3) stimulated growth (30%) of A. flavus but release of aflatoxins was reduced (8%). Ethyl aminobenzoate (Table 4) at similar concentrations, did reduce growth (31%) but stimulated (5%) the release of aflatoxins. Para-aminobenzoic acid is an essential component of folic acid, and thus serves as an essential metabolite for microorganisms for folic acid synthesis (Young, 1955). At a concentration of 20 mg/100 ml of medium, 2-nitrobenzoate, para-aminobenzoic acid, and ethyl aminobenzoate reduced mycelial growth by 10%, 15% and 38%, and the release of aflatoxins by 5%, 4% and 6%, respectively with complete inhibition at 400 mg, 900 mg, and 80 mg/100 ml of medium, respectively. Differences in the results obtained from these various amino substituted benzoic acids suggest that the position of the amino groups on the aromatic ring, and the concentrations of the substituted benzoate in the medium, determined their effectiveness on both mycelial growth, production and release of aflatoxins in A. flavus (Rothe, 1976).
<table>
<thead>
<tr>
<th>NBA (mg/100 ml)</th>
<th>MYCELIAL DRY WEIGHT (grams)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (ug)</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B1</td>
<td>G1</td>
</tr>
<tr>
<td>0.0</td>
<td>1.0</td>
<td>±.050</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>1.1</td>
<td>±.010</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>20</td>
<td>0.9</td>
<td>±.007</td>
<td>4.4</td>
<td>4.2</td>
</tr>
<tr>
<td>40</td>
<td>0.6</td>
<td>±.010</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>80</td>
<td>0.45</td>
<td>±.040</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>100</td>
<td>0.20</td>
<td>±.004</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>200</td>
<td>0.16</td>
<td>±.022</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>400</td>
<td>--a</td>
<td></td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>800</td>
<td>--</td>
<td></td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

a -- no growth  
b -- none detected
### TABLE 3

**EFFECTS OF PARA-AMINOBENZOIC ACID (PABA) ON GROWTH AND PRODUCTION OF AFLATOXINS BY ASPERGILLUS FLAVUS**

<table>
<thead>
<tr>
<th>PABA (mg/100 ml)</th>
<th>MYCELIAL DRY WEIGHT (grams)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (µg)</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B₁</td>
<td>G₁</td>
</tr>
<tr>
<td>00</td>
<td>1.0</td>
<td>+0.050</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>1.3</td>
<td>+0.050</td>
<td>6.2</td>
<td>6.1</td>
</tr>
<tr>
<td>20</td>
<td>0.85</td>
<td>+0.040</td>
<td>5.7</td>
<td>5.0</td>
</tr>
<tr>
<td>40</td>
<td>0.60</td>
<td>+0.008</td>
<td>4.8</td>
<td>4.3</td>
</tr>
<tr>
<td>80</td>
<td>0.40</td>
<td>+0.005</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>100</td>
<td>0.31</td>
<td>+0.002</td>
<td>2.9</td>
<td>2.5</td>
</tr>
<tr>
<td>200</td>
<td>0.28</td>
<td>+0.001</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>400</td>
<td>0.23</td>
<td>+0.002</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>800</td>
<td>0.10</td>
<td>+0.001</td>
<td>--b</td>
<td>--</td>
</tr>
<tr>
<td>900</td>
<td>--a</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

---

a — no growth  

b — none detected
**TABLE 4**

**EFFECTS OF BENZOCAINE (ETHYL AMINO-BENZOATE) ON GROWTH AND PRODUCTION OF AFLATOXIN BY ASPERGILLUS FLAVUS**

<table>
<thead>
<tr>
<th>BENZOCAINE (mg/100 ml)</th>
<th>MYCELIAL DRY WEIGHT (grams)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (μg)</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>00</td>
<td>1.0</td>
<td>±.050</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>0.69</td>
<td>±.032</td>
<td>7.1</td>
<td>7.0</td>
</tr>
<tr>
<td>20</td>
<td>0.62</td>
<td>±.047</td>
<td>4.3</td>
<td>4.0</td>
</tr>
<tr>
<td>40</td>
<td>0.45</td>
<td>±.024</td>
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<td>--</td>
</tr>
<tr>
<td>80</td>
<td>--a</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup> -- no growth

<sup>b</sup> -- none detected
Ethyl benzoate (Table 5) reduced growth (60%) and the release of aflatoxin by 68% at a concentration of 10 mg/100 ml and, at a concentration of 20 mg/100 ml, the release of aflatoxin was completely inhibited while growth was reduced by 78%. Methyl benzoate (Table 6) at a concentration of 10 mg/100 ml of medium reduced growth (68%) and completely inhibited the release of aflatoxins. Benzoic acid (Table 7) reduced mycelial growth (73%) release and aflatoxin production by 88% at a concentration of 50 mg/25 ml and complete inhibition occurred at a concentration of 100 mg/25 ml. Sodium benzoate at a concentration of 50 mg/25 ml of medium reduced growth (75%) and completely inhibited growth as well as production and release of aflatoxin at a concentration of 100 mg/25 ml of medium. Sodium benzoate and benzoic acid have an initial pH of 5.0 and 3.9, respectively, at a concentration of 100 mg/25 ml of synthetic medium. Cruess and Richert (1929) reported that between pH 2.3 to 2.4, only 0.02 to 0.03% sodium benzoate was required to prevent growth of most fermentative organisms examined. Salicylic acid (Table 8) at a concentration of 10 mg/100 ml reduced growth and the release of aflatoxins with complete inhibition of mycelial growth and the release of aflatoxin achieved at concentrations of 80 mg and 20 mg/25 ml of medium, respectively. Various concentrations of benzoic acid and its derivatives which inhibited mycelial growth and production or release of aflatoxins suggest that undissociated benzoic acid or substituted benzoate molecules might be the active antimicrobial agent affecting mycelial growth and production or release of aflatoxins by *A. flavus*. 
### TABLE 5

**EFFECTS OF ETHYL BENZOATE ON GROWTH AND PRODUCTION OF AFLATOXIN BY ASPERGILLUS FLAVUS**

<table>
<thead>
<tr>
<th>ETHYL BENZOATE (mg/100 ml)</th>
<th>MYCELIAL DRY WEIGHT (grams)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (μg)</th>
<th>STANDARD DEVIATION</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B₁</td>
<td>G₁</td>
</tr>
<tr>
<td>0.0</td>
<td>1.0</td>
<td>±.050</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>0.40</td>
<td>±.030</td>
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<td>0.22</td>
<td>±.021</td>
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</tbody>
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---

*a* -- no growth  

*b* -- none detected
TABLE 6

EFFECTS OF METHYL BENZOATE ON GROWTH
AND AFLATOXIN PRODUCTION BY ASPERGILLUS FLAVUS

<table>
<thead>
<tr>
<th>METHYL BENZOATE (mg/100 ml)</th>
<th>MYCELIAL DRY WEIGHT (grams)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (μg)</th>
<th>STANDARD DEVIATION</th>
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<td>1.0</td>
<td>+ .050</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>0.10</td>
<td>0.39</td>
<td>+ .009</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.20</td>
<td>0.30</td>
<td>+ .027</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.40</td>
<td>0.10</td>
<td>+ .018</td>
<td>--</td>
<td>--</td>
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</table>

\( a \) -- none detected
<table>
<thead>
<tr>
<th>CONCENTRATION (mg/25 ml)</th>
<th>MYCELIUM (mg/25 ml)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (MEDIUM) (ug/25 ml)</th>
<th>AFLATOXINS (MYCELIUM) (ug)</th>
<th>STANDARD DEVIATION (medium)</th>
<th>STANDARD DEVIATION (mycelium)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B$_1$ G$_1$ B$_2$ G$_2$</td>
<td>B$_1$ G$_1$ B$_2$ G$_2$ B$_1$ G$_1$ B$_2$ G$_2$ B$_1$ G$_1$ B$_2$ G$_2$ B$_1$ G$_1$ B$_2$ G$_2$</td>
<td>B$_1$ G$_1$ B$_2$ G$_2$ B$_1$ G$_1$ B$_2$ G$_2$ B$_1$ G$_1$ B$_2$ G$_2$ B$_1$ G$_1$ B$_2$ G$_2$ B$_1$ G$_1$ B$_2$ G$_2$</td>
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</tr>
<tr>
<td>00 (Control)</td>
<td>420</td>
<td>±10</td>
<td>2.0 1.6 1.0 0.8</td>
<td>1.1 0.8 0.5 0.3</td>
<td>+.03 +.018 +.004 +.001</td>
<td>+.012 +.0025 +.009 +.0022</td>
</tr>
<tr>
<td>50 (Benzoic Acid)</td>
<td>114</td>
<td>±8</td>
<td>0.8 0.4 b</td>
<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
</tr>
<tr>
<td>100 (Benzoic Acid)</td>
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<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
</tr>
<tr>
<td>50 (Sodium Benzoate)</td>
<td>105</td>
<td>±3</td>
<td>0.6 0.2 b</td>
<td>- - - -</td>
<td>+.001 +.002</td>
<td>- - - -</td>
</tr>
<tr>
<td>100 (Sodium Benzoate)</td>
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<td>-</td>
<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
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</table>

*a* no growth  
*b* none detected
<table>
<thead>
<tr>
<th>SALICYLIC ACID (mg/100 ml)</th>
<th>MYCELIAL DRY WEIGHT (grams)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (μg)</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B1</td>
<td>G1</td>
</tr>
<tr>
<td>00</td>
<td>1.0</td>
<td>±.050</td>
<td>7.0</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>0.52</td>
<td>±.037</td>
<td>5.8</td>
<td>5.5</td>
</tr>
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<td>0.47</td>
<td>±.028</td>
<td>--b</td>
<td>--</td>
</tr>
<tr>
<td>40</td>
<td>0.22</td>
<td>±.009</td>
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<td>--</td>
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<tr>
<td>80</td>
<td>--a</td>
<td></td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

a — no growth

b — none detected
Both mycelial growth and production of aflatoxins by *A. flavus* were reduced by 40% and 33%, respectively, when the medium was supplemented with NaCl at a concentration of 10 g/100 ml (Table 9). Complete inhibition occurred at a concentration of 12 g or more/100 ml. At low levels (1 g or less/100 ml), NaCl appeared to have stimulated both mycelial growth and aflatoxin production. The greatest stimulation of growth compared to unsupplemented medium occurred at a sodium chloride concentration of 0.2 g/100 ml. However, the greatest stimulation of toxin production occurred at a concentration of 1 g/100 ml. Even at sodium chloride concentrations of 2 g/100 ml to 8 g/100 ml, significant increases in toxin production were noted. These results are in agreement with the report of Bullerman *et al.* (1969b) that high salt concentrations were required to prevent growth and aflatoxin production by *A. parasiticus* during aging in country-cured hams. Kulik and Hanlin (1968) also stated that 15% NaCl was needed to inhibit growth of *A. parasiticus*. Buchanan and Ayres (1976) supplemented sodium chloride (2 g/100 ml) in modified synthetic medium and observed no inhibitory effect on aflatoxin production by *A. parasiticus*; on the contrary, toxin production was stimulated. These results suggest that high NaCl concentrations might have adversely affected the water activity required for growth and subsequent toxin production by *A. flavus*.

Sodium acetate (2 g or less/100 ml) reduced growth but increased aflatoxin production (Table 10). Complete inhibition occurred at 4 g/100 ml. Hoffman *et al.* (1939) reported that acetic acid inhibited mold growth at an initial pH of 5.0
TABLE 9 - EFFECTS OF SODIUM CHLORIDE ON GROWTH AND PRODUCTION OF AFLATOXINS BY ASPERGILLUS FLAVUS

<table>
<thead>
<tr>
<th>SODIUM CHLORIDE (g/100 ml)</th>
<th>MYCELIUM (mg/25 ml)</th>
<th>STANDARD DEVIATION</th>
<th>AFLATOXINS (MEDIUM) (ug/25 ml)</th>
<th>AFLATOXINS (MYCELIUM) (ug)</th>
<th>STANDARD DEVIATION (medium)</th>
<th>STANDARD DEVIATION (mycelium)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B₁ G₁ B₂ G₂</td>
<td>B₁ G₁ B₂ G₂</td>
<td>B₁ G₁ B₂ G₂</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>420</td>
<td>+10</td>
<td>2.0 1.6 1.0 0.8</td>
<td>1.1 0.8 0.5 0.3</td>
<td>+.03</td>
<td>+.018 +.004 +.001 +.012 +.0025 +.009 +.0022</td>
</tr>
<tr>
<td>0.2</td>
<td>650</td>
<td>+12</td>
<td>4.8 3.0 2.0 1.4</td>
<td>2.5 1.0 0.7 0.4</td>
<td>+.041 +.009 +.016 +.007 +.02 +.031 +.004 +.0035</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>500</td>
<td>+13</td>
<td>5.5 3.4 2.6 2.0</td>
<td>3.2 1.8 1.3 0.8</td>
<td>+.05 +.05 +.03 +.022 +.041 +.032 +.006 +.014</td>
<td></td>
</tr>
<tr>
<td>0.8</td>
<td>465</td>
<td>+9</td>
<td>6.0 4.6 3.0 2.5</td>
<td>3.8 2.2 1.7 1.2</td>
<td>+.045 +.008 +.04 +.001 +.055 +.001 +.021 +.007</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>450</td>
<td>+12</td>
<td>6.5 5.2 4.5 3.0</td>
<td>4.4 3.0 2.0 1.7</td>
<td>+.05 +.013 +.004 +.017 +.034 +.018 +.033 +.013</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>410</td>
<td>+9</td>
<td>5.0 4.6 4.0 2.0</td>
<td>3.1 2.5 2.0 1.4</td>
<td>+.02 +.006 +.002 +.016 +.04 +.009 +.007 +.0076</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>380</td>
<td>+14</td>
<td>4.4 3.3 3.0 1.5</td>
<td>2.5 1.7 1.3 0.8</td>
<td>+.021 +.031 +.01 +.002 +.012 +.018 +.043 +.008</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>300</td>
<td>+11.2</td>
<td>4.0 2.5 1.8 1.2</td>
<td>1.0 0.6 0.8 0.5</td>
<td>+.007 +.004 +.005 +.01 +.005 +.0061 +.001 +.0032</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>250</td>
<td>+8</td>
<td>1.4 1.0 0.8 0.5</td>
<td>0.6 0.3 0.2 0.3</td>
<td>+.016 +.0015 +.006 +.007 +.003 +.002 +.007 +.0054</td>
<td></td>
</tr>
<tr>
<td>12.0</td>
<td>---</td>
<td>+b</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

a) no growth
b) none detected
<table>
<thead>
<tr>
<th>SODIUM ACETATE (g/100 ml)</th>
<th>MYCELIUM (mg/25 ml)</th>
<th>STANDARD DEVIATION (μg/25 ml)</th>
<th>AFLATOXINS (MEDIUM) (μg)</th>
<th>STANDARD DEVIATION (μg)</th>
<th>AFLATOXINS (MYCELIUM) (μg)</th>
<th>STANDARD DEVIATION (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>420</td>
<td>+10</td>
<td>2.0</td>
<td>1.6</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>0.2</td>
<td>500</td>
<td>+12</td>
<td>14.0</td>
<td>10.0</td>
<td>6.0</td>
<td>7.0</td>
</tr>
<tr>
<td>0.4</td>
<td>400</td>
<td>+14</td>
<td>10.0</td>
<td>6.0</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.8</td>
<td>250</td>
<td>+8</td>
<td>6.0</td>
<td>5.0</td>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>1.0</td>
<td>200</td>
<td>+8</td>
<td>5.0</td>
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<td>2.5</td>
<td>3.0</td>
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<tr>
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<td>180</td>
<td>+6</td>
<td>3.0</td>
<td>2.0</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>4.0</td>
<td>--</td>
<td>b</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

^a no growth

^b none detected
and the amount of acetic acid needed to inhibit growth was a function of initial pH. In the present investigation, maximum growth and release of aflatoxin production as compared to the control occurred at pH 5.4 (0.2 g of sodium acetate/100 ml) and complete inhibition was achieved at pH 5.0 (4 g of sodium acetate/100 ml). Buchanan and Ayres (1976) demonstrated that sodium acetate was inhibitory at an acid pH and concentrations of 1 and 2 g/100 ml completely inhibited growth and aflatoxin production at an initial pH of 4.5 and 5.0, respectively. Lack of complete agreement between results of the present study and those of previous studies may be due to differences in medium used and in species of fungi.

Malonic acid and 2, 4-dinitrophenol have been reported to stimulate succinate dehydrogenase at low concentrations (Kearney, 1957). Malonic acid is also a specific inhibitor of succinate dehydrogenase (Webb, 1946). In the present study, sodium malonate at 10, 20, 40, and 50 mM stimulated growth and aflatoxin production, with maximum increase (20%) of toxin production as compared to the control occurring at 50 mM (pH 5.9) (Figure 6). However, malonic acid (free acid) at 10, 20, 40, and 50 mM sharply reduced growth and aflatoxin production with complete inhibition at 60 mM (pH 2.4). Effects of malonic acid on growth and aflatoxin production by A. flavus may simply be a function of the pH and the chemical composition of the medium. Malonic acid may also affect the utilization of endogenously formed
Figure 6. The Effects of Sodium Malonate and Malonic Acid on Aflatoxin Production by *Aspergillus flavus*. Results are shown as the amount of aflatoxin produced in 25 ml of medium.

- □ - B₁ production in the presence of sodium malonate
- ○ - G₁ production in the presence of sodium malonate
- ■ - B₁ production in the presence of malonic acid
- ● - G₁ production in the presence of malonic acid
Figure 6
acetyl CoA from acetate, a precursor of aflatoxin biosynthesis (Gupta et al., 1975).

Hendricks (1964) reported that organic carboxylic acids such as acetic, succinic, and malonic were taken up by plant cells under acid conditions where the undissociated acid was predominant. Usually, when these compounds were to serve as either substrates or inhibitors of cell metabolism, they were supplied in acid solution, that is, nonionized, to enter the cells. This appears to be the case for results observed in the present study.

Growth and stimulation of aflatoxin production by low levels of sodium malonate, sodium chloride, and sodium acetate may be a function of sodium ions. Presumably, the A. flavus membrane is permeable to sodium ions, thereby creating an electrochemical gradient potential. However, a high potassium, low sodium regime within the cytoplasm is favorable for efficient working of various enzyme systems (Atkinson and Polya, 1967). Therefore, the active removal of sodium ions, together with some anions, becomes necessary. Other cell functions must provide energy for this. Mitchell (1966) in his hypothesis indicated how an ATPase could be associated with ion transport in the membrane. The ATPase is within a membrane structure and coupled with the oxidation-reduction chain. In bladders from fresh-water turtles, Klair and Bricker (1965) found a correlation between glycolysis (measured by lactate formation) and net sodium transport. They concluded that the level of glycolysis depended on the
amount of sodium present in Ringer's solution. In both plant and animal tissues, Hendricks (1964) found that ATP hydrolysis was important for Na⁺ - K⁺ exchange across the membrane and Na⁺, K⁺, and Mg⁺⁺ promoted ATPase activity in such membranes. In erythrocyte membranes, however, activation of ATPase was more effective with Na⁺ on the inside and K⁺ on the outside of the membrane (Whittam, 1962).

In the present study, the addition of sodium ions (as salts) did not appear to result in an increase in the permeability of cell membrane. Preliminary experiments have indicated no significant differences in the amounts of 260- and 280-nm absorbing material released from cultures in the presence or absence of sodium ions.

Reduction in the production of aflatoxins by benzoic acid and sodium benzoate was accompanied by the appearance of a yellow pigment (Figure 7). Yao and Hsieh (1974) have observed that dichlorvos (dimethyl 2,2- dichlorovinyl phosphate) inhibited biosynthesis of aflatoxin by A. parasiticus and those cultures treated with dichlorvos excreted an orange pigment. By direct visual comparison of thin-layer chromatography plates, the increased concentration of yellow pigment observed in the present study appeared to be directly proportional to the reduction of aflatoxins and to the increase in concentration of supplemented benzoic acid and sodium benzoate (Figure 8). On the other hand, increases in aflatoxin production were accompanied by blue and green fluorescent spots similar to the fluorescence of aflatoxins.
Figure 7. Effects of Benzoic Acid or Sodium Benzoate on the Production of Yellow Compound. Results are expressed as μg of yellow compound/25 ml of synthetic medium.

- △ - 1 mg. of benzoic acid sodium benzoate/ml of medium
- ○ - 2 mg of benzoic acid sodium benzoate/ml of medium
- □ - 4 mg of benzoic acid sodium benzoate/ml of medium
Figure 8. Effects of Benzoic Acid or Sodium Benzoate on Aflatoxin B₁ Production. Results are expressed as μg of aflatoxin B₁/25 ml of synthetic medium.

- benzoic acid
- sodium benzoate
Figure 8

AFLATOXIN (µG) vs. CONCENTRATION (MG/ML)
B₁, G₁, B₂ and G₂ standards but with lower R_f values. Treatment of aflatoxin B₁ with cold, dilute mineral acid in darkness at 5°C for 16 hr produces aflatoxin B₂a (Chipley et al., 1974). Preliminary experiments have confirmed that the blue fluorescent spots mentioned above are similar to aflatoxin B₂a described by Chipley et al. (1974). Examination of the pH of the medium showed a two-unit decrease in the initial pH of 4.5 to a pH of 2.5 within 48 hr. This could explain the presence of the blue and green fluorescent spots with lower R_f values observed in this investigation.

Characterization of Yellow Compound

The yellow compound was found to have a melting point of 267-269 °C and absorbance maxima in methanol of 312 nm. The IR spectrum (Figure 9) showed peaks at 1620 cm⁻¹ and 1675 cm⁻¹, indicating the presence of chelated and nonchelated anthraquinone carbonyl groups while the peak at 1725 cm⁻¹ indicates an additional carbonyl group (or groups). The NMR spectrum showed a five-proton signal positioned at δ 1.9 and a broad triplet centered at δ 4.0. There were two one-proton chemical shifts for aromatic protons at δ 7.4 and δ 5.85. The mass spectrum showed principal high mass ions m/e of 338, 320, 313, and 297. Ions of significance were noted at 352 and 325. Low mass ions of some significance were also observed at 41, 43, and 60, indicating the presence of an acetyl group. Based on physical and chemical characteristics and mode of production
Figure 9. Infrared Spectrum of Yellow Compound.
of the yellow compound in the present study, there is good reason to believe that it is closely related to the compound isolated by Schroeder et al. (1974), and by Yao and Hsieh (1974). The possibility exists that the pigment described by these authors could be a dihydro derivative of the pigment reported in the present study.

**Conversion of Yellow Compound to Aflatoxin**

The reaction of yellow compound (Table 11) with cell-free extract resulted in the production of aflatoxin $B_1$. In similar experiments using boiled cell-free extracts, aflatoxin $B_1$ was not produced. These results indicated that the yellow compound was an intermediate in aflatoxin biosynthesis and that enzymes were involved in the reaction (Biollaz et al., 1970; Yao and Hsieh, 1974).

**Factors Affecting the Synthesis of Aflatoxin in Cell-Free System**

The effect of protein concentration in the cell-free extract was determined (Table 11). The results suggest that the conversion of yellow compound to aflatoxin $B_1$ is related to the concentration of protein in the cell-free system. The highest amount of conversion of yellow compound to aflatoxin $B_1$ was achieved with 60 mg of total protein in the cell-free extract.
**TABLE 11**

**EFFECTS OF TIME AND PROTEIN CONCENTRATION (CELL-FREE SYSTEM) ON THE CONVERSION OF YELLOW COMPOUND TO AFLATOXIN B₁³⁶**

<table>
<thead>
<tr>
<th>PROTEIN CONCENTRATION IN CELL-FREE SYSTEM (mg)</th>
<th>AFLATOXIN B₁ (µg)</th>
<th>EFFECT OF TIME ON AFLATOXIN B₁ PRODUCTION³⁶</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.4</td>
<td>0.5</td>
<td>± .026</td>
</tr>
<tr>
<td>20</td>
<td>3.8</td>
<td>1.0</td>
<td>± .041</td>
</tr>
<tr>
<td>40</td>
<td>5.3</td>
<td>1.5</td>
<td>± .037</td>
</tr>
<tr>
<td>50</td>
<td>6.0</td>
<td>2.0</td>
<td>± .032</td>
</tr>
<tr>
<td>60</td>
<td>6.4</td>
<td>4.0</td>
<td>± .060</td>
</tr>
</tbody>
</table>

³⁶Each flask contained 2 mg of yellow compound per 15 ml medium.

³³Each flask contained 60 mg protein.
In a similar experiment, benzoic acid at a concentration of 2 g/15 ml of cell-free system, inhibited the conversion of yellow compound to aflatoxin (Table 12). Similarly, barium in the form of barium chloride (0.2 mg) (Table 13) inhibited the conversion of yellow compound to aflatoxin.

The inhibition of aflatoxin biosynthesis by benzoic acid and barium chloride, respectively, in this investigation might be explained on the basis of either competitive or noncompetitive inhibition of one of the enzyme(s) involved in aflatoxin biosynthesis (Davis and Diener, 1967; Gunter, 1976).
TABLE 12

EFFECT OF BENZOIC ACID IN CELL-FREE SYSTEM\textsuperscript{a}

<table>
<thead>
<tr>
<th>CONCENTRATION (Benzoic acid) (mg)</th>
<th>AFLATOXIN (B\textsubscript{1}) (\mu g)</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>6.5</td>
<td>±.031</td>
</tr>
<tr>
<td>1</td>
<td>1.7</td>
<td>±.004</td>
</tr>
<tr>
<td>2</td>
<td>--\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Each flask contained 60 mg protein and 2 mg yellow compound and 15 ml of cell-free extract.

\textsuperscript{b} -- none detected.
TABLE 13

EFFECTS OF BARIUM CHLORIDE ON CELL-FREE SYSTEM\textsuperscript{a}

<table>
<thead>
<tr>
<th>CONCENTRATION OF BARIUM CHLORIDE (mg)</th>
<th>AFLATOXIN B\textsubscript{1} (g)</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>6.4</td>
<td>±.031</td>
</tr>
<tr>
<td>0.1</td>
<td>1.1</td>
<td>±.004</td>
</tr>
<tr>
<td>0.2</td>
<td>-- b</td>
<td>--</td>
</tr>
<tr>
<td>0.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.8</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1.0</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Each flask contained 60 mg of protein, 2 mg yellow compound, and 15 ml of cell-free extract.

\textsuperscript{b} -- none detected.
Characterization of A. flavus Protein

No electrophoretic distinction could be made between proteins of aflatoxin-producing and non-aflatoxin producing strains of A. flavus (Sorenson et al., 1971; Schmidt et al., 1977). No correlation could be made of the protein isolated in this investigation with aflatoxin production. Similarly, Schmidt et al. (1977) were unable to detect the protein fraction responsible for aflatoxin production. They suggested that the absence of a specific enzyme band responsible for aflatoxin synthesis is based on the possibility that these proteins may be present in quantities too small in concentration for detection. In contrast, Hsieh (unpublished report) explained the lack of correlation of isolated protein and aflatoxin production on an "unknown dissociation factor" of the isolated proteins. This could explain the absence of correlation between the isolated protein and aflatoxin production in this investigation.

The results of the Bio-Gel A 1.5 column (exclusion limit 1,500,000) demonstrated that the majority of the protein appeared in the void volume. This suggests that the protein has a large molecular weight of at least 1,500,000. This was confirmed by the sodium dodecyl sulfate electrophoretic method. During staining in electrophoretic studies, the protein absorbed stain poorly. This is likely due to the hydrophobic nature of the protein. This fact was further reinforced by poor sedimentation in the ultracentrifuge due to a possible lipid-protein complex.
SUMMARY

The effects of sodium chloride, sodium acetate, benzoic acid, benzoic acid derivatives, malonic acid, and sodium malonate on growth and production and release of aflatoxins by *Aspergillus flavus* were investigated in synthetic media. Sodium chloride at concentrations equivalent to or greater than 12g/100 ml inhibited growth and aflatoxin production, while at 8g or less/100 ml, growth and aflatoxin production were stimulated. At 2 g or less/100 ml, sodium acetate also stimulated growth and aflatoxin production, but reduction occurred with 4 g or more/100 ml. Malonic acid at 10, 20, 40, and 50 mM reduced growth and aflatoxin production (over 50%) while sodium malonate at similar concentrations but different pH values had the opposite effect. Benzoic acid (pH 3.9) and sodium benzoate (pH 5.0) at 0.4 g/100 ml completely inhibited growth and aflatoxin production. Examination of the effect of initial pH indicated that the extent of inhibitory action of malonic acid and sodium acetate was a function of initial pH. The inhibitory action of benzoic acid and its derivatives appeared to be a function of their undissociated molecules. Aflatoxin reduction was usually accompanied by the appearance of a yellow pigment. Spectral analyses partially identified this compound as an acetyl derivative of a versiconal-type compound. A cell-free extract prepared from *A. flavus* grown in synthetic media was active.
in converting this yellow compound into aflatoxin B<sub>1</sub> in the presence of reduced nicotinamide adenine dinucleotide phosphate at 25°C (pH 7.4). In the presence of benzoic acid and its salt or boiled cell-free extract, conversion of yellow compound to aflatoxin B<sub>1</sub> was prevented. These results suggest that the yellow compound is an intermediate in the secondary metabolic cycle involved in aflatoxin B<sub>1</sub> production. Benzoic acid (2 mg) and barium chloride (0.2 mg) or boiling the cell-free extract appear to have respectively blocked or denatured an enzymatic step late in the biosynthetic pathway of aflatoxin B<sub>1</sub>. The conversion of yellow compound into aflatoxin B<sub>1</sub> is a function of protein concentration. No correlation could be made between the protein isolated in this investigation and the production of aflatoxin. Results of Bio-Gel A 1.5 column chromatography demonstrated that the majority of the protein isolated in this investigation appeared in the void volume which suggests a high molecular weight of at least 1,500,000. This was further verified by sodium dodecyl sulfate electrophoresis. Results from ultracentrifugal analyses revealed that the protein sedimented poorly which imply the possibility of a lipid-protein complex.
Figure 3. Calibration of Aflatoxin $B_1$, $G_1$, $B_2$, $G_2$ Standards
Figure 3  
AFLATOXINS (μg/ml)  

OD (350nm)
Figure 4. Standard Curve For Lowry Protein Determination
Figure 4

PROTEIN (μg/ml)

O.D. (540 nm)
Figure 5. Calibration Curve of Bio-Gel A 1.5 Column Eluted With 1% Triton - 0.05M Pyrophosphate Buffer. A 2.5 x 90 cm Column was Prepared and 4 - ml Fractions were Eluted.
**Figure 5**

Logarithm of Molecular Weight vs. Fraction Number

- **Blue Dextran**
- **Acid Phosphatase**
- **Horse Heart Myoglobin**
LITERATURE CITED


Hoffman, C., Schwietzer, T. R. and Dalby, G. 1939. Fungistatic properties of fatty acids and possible biochemical significance. Food Res. 4: 539-545.


