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GROWTH AND AFLATOXIN PRODUCTION BY SELECTED ASPERGILLI ON CURED AND AGED MEATS

by

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Dean of Graduate College

Iowa State University
Ames, Iowa

1968
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INTRODUCTION

Since molds are frequently present on foods, the occasional contamination of human food with biologically active compounds of mold origin represents a situation that has been with us for many years. Scientific literature during the Nineteenth Century contained warnings of possible poisonings of farm animals by moldy feeds. Likewise, in the first half of the Twentieth Century, a number of toxic syndromes were associated with the consumption of mold-infested grains by animals and man. In spite of the warnings against the feeding of moldy feeds to livestock, and the reported cases of toxicoses caused by molds in man and animals, until recent years, it has been rather common practice to regard mold damage in a purely economic light; that is, from the standpoint of non-saleability of foodstuffs rather than as a potential health hazard.

Molds are used in manufacture of a variety of foods found in many parts of the world. These include many Asian foods, fermented foods, cheeses and certain cured and aged meats produced widely in Europe, and to a more limited extent, in the United States.

When one considers the many ways in which molds are associated with human food, it seems strange that the public health aspects involving the growth of these organisms on
foods have not been rigorously investigated. Indeed, prior to 1960, little more than passing attention was given to these organisms and their relationship to the human food supply. But, in 1960, a strange disease occurred in Great Britain involving over 100,000 young turkey poultts and a number of ducklings and other young animals (Blount, 1961). The cause of the malady was not readily apparent, and at first it was thought that this was a new disease, so it was called Turkey "X" disease. A toxic factor, found to be fluorescent when exposed to long-wave ultraviolet (UV) light, was found in peanut meal, a constituent of the animals' feed. The material consisted of at least two components which were chloroform-extractable and separable by chromatography; one fluoresced blue and the other green when exposed to UV light. These compounds were traced to Aspergillus flavus, a common contaminant of all toxic lots of feed. The materials were called "aflatoxin" for Aspergillus flavus toxin, and were designated aflatoxin B and aflatoxin G because of their blue and green fluorescence, respectively (Nesbitt, et al., 1962). Toxicity studies have shown that the toxins are acutely toxic and carcinogenic to a wide range of animals.

In the light of knowledge acquired since 1960, it seems desirable to re-evaluate the presence of and use of molds, especially members of the genus Aspergillus, in the production
of human food. This study was designed to determine (1) the aflatoxin-producing capabilities of molds isolated from country cured hams and European type salami, (2) the levels of aflatoxins produced by known toxinogenic strains of A. flavus on meats during storage under various conditions, (3) the levels of aflatoxins produced by known toxinogenic strains of A. flavus on salami under simulated conditions of manufacture, and, (4) the levels of aflatoxins produced by known toxinogenic strains of A. flavus on country cured hams at different stages of aging.
REVIEW OF LITERATURE

Physical and Chemical Nature of Aflatoxins

Aflatoxins are metabolites of *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare (Sargeant et al., 1961b; Austwick and Ayerst, 1963; Codner et al., 1963). The compounds are both toxic and carcinogenic to a wide range of animals (Wogan, 1966).

Aflatoxins are highly substituted coumarins containing bifuran rings and lactone configurations (Asao et al., 1963; Asao et al., 1965; Wogan, 1966). The aflatoxins have been reported to consist of at least six closely related compounds, designated as B$_1$, B$_2$, G$_1$, G$_2$, M$_1$, and M$_2$. Aflatoxins B$_1$ and G$_1$ predominate in culture extracts, with B$_2$ and G$_2$ usually being present in small to trace amounts. Aflatoxins M$_1$ and M$_2$, thought to be hydroxylated forms of B$_1$ and B$_2$, were first observed in the milk of lactating animals fed aflatoxin, hence the M designation (Asao et al., 1963; Asao et al., 1965; De Iongh et al., 1964b; Holzapfel et al., 1966). Dutton and Heathcote (1966) recently reported two new hydro-aflatoxins. These are hydroxy derivatives of aflatoxin B$_2$ and G$_2$ which are also present in culture extracts and have been designated B$_{2a}$ and G$_{2a}$. The structures of the aflatoxins are shown in Figure 1.

Aflatoxins are soluble in many slightly polar solvents.
Figure 1. Structural formulae, empirical formulae and molecular weights of the various aflatoxins
The most commonly-mentioned solvents are chloroform, methanol, ethanol, water and various mixtures of these with other solvents. A number of these solvents are suitable for use in extracting the toxins from organic or biological materials.

Wogan (1966) and Borker et al., (1966) have extensively reviewed the physical properties of aflatoxins. The compounds are fluorescent when exposed to ultraviolet light (wavelength 365 m\(\mu\)). Aflatoxins \(B_1\) and \(B_2\) emit a bluish fluorescence while \(G_1\) and \(G_2\) fluoresce greenish. Aflatoxin \(M_1\) and \(M_2\) also emit a bluish fluorescence. Ultraviolet absorption of the toxins occurs at 265 and at 363 m\(\mu\). Aflatoxins \(B_1\) and \(G_2\) absorb more intensely than do \(B_2\) or \(G_1\). Owing to the close similarities of the structures of the toxins their infrared absorption spectra are likewise very close (Table 1) (Wogan, 1966). The melting points of the aflatoxins are all quite high, and they decompose rather than actually melt (Table 1).

A number of chromatography systems were used to separate the toxins. Paper chromatography was one of the first tried but resolution was not good. Thin layer chromatography (TLC) gave much better separations. Both alumina and silica gel were used as coatings for thin layer plates; the use of silica gel resulted in superior separations so this support is now used almost exclusively. Many solvent systems have
Table 1. Summary of physical and spectral data on the four principal aflatoxins

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Melting Point</th>
<th>Ultraviolet absorption (E)</th>
<th>Infrared Absorption (CHCl₃) (cm⁻¹)</th>
<th>v max.</th>
</tr>
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<tr>
<td>Aflatoxin B₁</td>
<td>268-269° C</td>
<td>13,400</td>
<td>21,800</td>
<td>1,760</td>
</tr>
<tr>
<td>B₂</td>
<td>286-289° C</td>
<td>9,200</td>
<td>14,700</td>
<td>1,760</td>
</tr>
<tr>
<td>G₁</td>
<td>244-246° C</td>
<td>10,000</td>
<td>16,100</td>
<td>1,760</td>
</tr>
<tr>
<td>G₂</td>
<td>237-240° C</td>
<td>11,200</td>
<td>19,300</td>
<td>1,760</td>
</tr>
</tbody>
</table>

a Wogan, 1966.
b Decompose.
been employed with the thin layer chromatographic technique. The most commonly used solvent systems have consisted of combinations of chloroform and methanol; chloroform and acetone; and benzene, ethanol and water, upper phase. The \( R_F \) values of these compounds have been difficult to reproduce in most solvent systems; however, they are generally in the range of 0.3 to 0.6. Aflatoxin \( B_1 \) has the highest \( R_F \), followed in order by aflatoxins \( B_2, G_1, G_2, M_1 \) and \( M_2 \).

Catalytic hydrogenation of aflatoxin \( B_1 \) results in the uptake of 3 moles of hydrogen to yield a tetrahydrodeoxy derivative. If the hydrogenation is stopped after the uptake of one mole of hydrogen, aflatoxin \( B_2 \) is formed from \( B_1 \) in quantitative yields (Wogan, 1966; Borker et al., 1966). Aflatoxin \( G_1 \) can be hydrogenated to yield \( G_2 \) in the same manner. Aflatoxin \( B_1 \) adds a hydroxyl group when treated with a strong acid; when treated with a strong base, the lactone ring of aflatoxin \( B_1 \) is opened. Ozonolysis of aflatoxin \( B_1 \) degrades it to form levulinic, succinic, malonic and glutaric acids. While aflatoxins are not destroyed by temperatures of less than 300° C, they are somewhat unstable (Fishbach and Campbell, 1965). The toxins are subject to destruction by oxidation and are sensitive to light, especially ultraviolet light. Prolonged exposure to UV light results in the formation of many fluorescent products of decreased toxicity (Andrellos et al., 1967).
Biological and Biochemical Activity
of the Aflatoxins

Action in vivo

The effects of aflatoxins in vivo vary with the dose, duration of exposure and animal species affected. The toxins are acutely toxic; they are lethal to many animals as well as to cells in culture when administered in high doses (Wogan 1966). The oral 7 day LD$_{50}$ values for 1-day-old ducklings, based on a 50 g body weight, are 18.2 µg for $B_1$, 84.8 µg for $B_2$, 39.2 µg for $G_1$, 172.5 µg for $G_2$, 16 µg for $M_1$ and 61.4 µg for $M_2$ (Carnaghan et al., 1953; Purchase, 1967). The LD$_{50}$ values for other animals range from 0.5 to 10 mg/kg of body weight (Wogan, 1966). Young animals of any species apparently are more susceptible than are older individuals of the same species (Allcroft, 1965). In most animal species the acute toxic effects of aflatoxin consist of a general pattern of gross liver damage with necrosis and hemorrhage of the intestinal tract and peritoneal cavity (Allcroft, 1965; Wogan, 1966).

Sublethal doses result in a number of moderate to severe histo-pathological changes, again primarily in liver. There may be lesions consisting of acute necrosis and hemorrhage, chronic fibrosis, regeneration nodules, bile duct hyperplasia, veno-occlusive disease, enlarged hepatic cells or liver tumors. Bile duct hyperplasia is the most consistent lesion
observed (Wogan, 1966). Subacute toxicity can be determined semiquantitatively by the pathologist and used as a test for aflatoxin activity in the duckling based on the extent and severity of histopathological changes (Wogan, 1965).

Prolonged administration of sublethal amounts of aflatoxin results in multiple liver tumors in rats; some of these animals have been observed to develop lung metastases. This was first observed by Lancaster et al. (1961) and has since been confirmed by numerous investigators (Schoental, 1961; Le Breton, 1962; Salmon and Newberne, 1963; Barnes and Butler, 1964; Butler and Barnes, 1964; Butler, 1965; Newberne, 1965; Wogan, 1966). Continued feeding of aflatoxins is not required for tumor development; tumors have been observed up to 1 year after the toxic feed had been removed from the diet. Barnes and Butler (1964) fed a total dosage of 2.5 mg of aflatoxin to rats in an 89 day period and observed tumor development 1 year later. Newberne (1965) reported that 90% of the rats fed 1.8 ppm of aflatoxin B1 for 370 days developed tumors. Aflatoxin is now thought to be the most potent carcinogen known (Butler 1965; Kraybill and Shimkin 1964).

Many animal species are affected by aflatoxins. Cattle and pigs develop severe fibrosis and there is generalized jaundice in the pig from aflatoxin poisoning (Harding et al., 1963). The disease in both cattle and pigs is similar to
ragwort poisoning (Loosemore and Markinson, 1961; Loosemore and Harding, 1961). Lactating cows, when fed aflatoxin $B_1$, excrete toxic substances in the milk, which are now known to be aflatoxins $M_1$ and $M_2$ (Allcroft and Carnaghan, 1963a; De Iongh et al., 1964b; Allcroft et al., 1966). Ducklings, turkey poult, young pigs and trout are very susceptible to the toxin, and acute poisoning with aflatoxin results in necrosis and hemorrhage (Allcroft, 1965; Halver, 1965). Guinea pigs are more susceptible to the acute effects of aflatoxins than are rats, but rats do develop liver lesions and hepatomas with prolonged exposure to the toxins (Lancaster et al., 1961; Butler and Barnes, 1964; Butler and Barnes, 1966, Butler, 1966). Aflatoxin causes biliary cirrhosis and fatty livers in rhesus monkeys, and in these animals the effects are similar to those observed with ducklings (Tulpule et al., 1964; Madhavan et al., 1965a,b). Sheep, mice and chickens are relatively resistant to aflatoxins (Asplin and Carnaghan, 1961; Platnow, 1964; Allcroft, 1965).

Other organisms affected by aflatoxins are insects, plants and microorganisms. Matsumura and Knight (1967) found that aflatoxins reduced the number of eggs produced and the percentage of eggs hatched when the toxins were fed to the yellow fever mosquito (Aedes aegypti), house fly (Musca domestica), and fruit fly (Drosophila melanogaster). The
germination of water cress seeds was inhibited by 100 ppm of aflatoxin and 10 ppm of toxin induced chlorophyll deficiency in young cress seedlings (Shoental and White, 1965). Aflatoxins have been observed to prevent the growth of several species of microorganisms belonging to the genera Bacillus, Clostridium, Flavobacterium, Streptomyces, Nocardia, Aspergillus and Penicillium (Burmeister and Hesseltine, 1966; Ciegler et al., 1966; Arai et al., 1967, Lillehoj et al., 1967a). Usually, levels of 10-30 μg of aflatoxin/ml were required to inhibit microbial growth. In microorganisms, subinhibitory toxin levels caused aberrant cell forms (Lillehoj et al., 1967b). Aflatoxin C₁ caused abnormal growth of Tetrahymena pyriformis, but the organism degraded aflatoxin B₁ (Teunisson and Robertson, 1967). Flavobacterium aurantiacum and Nocardia asteroides removed aflatoxins from liquid media (Ciegler et al., 1966; Lillehoj, 1967b; Arai et al., 1967).

Action in vitro

Early studies by Juhasz and Greczi (1964) demonstrated the toxicity of aflatoxin to calf kidney cells; 0.1-0.5 ppm of aflatoxin destroyed the cytoplasm and nucleus of cells after 48 hours of incubation. Legator and Withrow (1964) observed that aflatoxin suppressed mitotic division of heteroploid and diploid human embryonic lung cells. The phenomenon occurred 4 hours after exposure, and reached a
maximum in 8-12 hours. They found that 0.1 ppm of aflatoxin produced 51% reduction in mitosis. In a later study, it was shown that 0.5-1.0 ppm of aflatoxin reduced cell growth of heteroploid and diploid human embryonic lung cells; growth was almost completely inhibited at a level of 5.0 ppm of aflatoxin (Legator et al., 1965). When heteroploid human embryonic lung cells were exposed to 1 ppm aflatoxin B₁ for 8-12 hours, a 92% increase over controls, in giant cell formation, was noted. There were many abnormal morphological patterns including vacuolization and accumulation of cellular debris (Legator, 1966). Vacuolization and giant cell formation were observed at aflatoxin levels as low as 0.1 ppm.

The cytotoxic effects of aflatoxins are generally thought to be related to involvement of the toxins in processes related to DNA replication, transcription of messenger RNA, or translation of messenger RNA into protein (Ciegler and Lillehoj, 1968). Wragg et al., (1967) showed that 1-5 μg/ml of aflatoxin B₁ inhibited the growth of Escherichia coli and they suggested that the inhibition of growth was due to inhibition of DNA synthesis. Clifford and Rees (1966) showed that 0.032 mM of aflatoxin B₁ prevented orotic acid incorporation into RNA, and protein synthesis in rat liver slices in vitro. Trakatellis et al., (1964) concluded that aflatoxin B₁ attached to rat liver DNA and inhibited messenger
RNA synthesis, resulting in decreased protein synthesis which they felt might be responsible for hepatic necrosis. Sporn et al. (1966) verified the report that aflatoxin binds with DNA by showing that the DNA caused a shift in the absorption maximum of $B_1$ from 362-364 m$\mu$ to 366-368 m$\mu$. Gelboin et al. (1966) showed that injection of aflatoxin $B_1$ in vivo in the rat rapidly interfered with RNA polymerase activity. Other work indicates that aflatoxin interferes with DNA metabolism; more specifically DNA synthesis and mitosis are decreased in tissue culture cells treated with aflatoxins (Gabliks et al., 1965; Legator, 1966). Black and Jirgensons (1967) found that aflatoxin $B_1$ bound to calf thymus histone as well as to DNA. They concluded that aflatoxin bound to histones with equal or greater force than it did to DNA. They postulated that, since histones have been implicated in the replication-transcription processes, conformational changes brought about in the DNA and histones by aflatoxin could be produced in the nucleic acid coding.

There is no direct evidence of aflatoxin involvement in human disease. It is highly unlikely, however, when one considers the diverse group of organisms as well as the cell systems of human origin that are affected by aflatoxin, that these compounds would not also affect man. In addition, there has been some speculation concerning possible connec-
tions between moldy foods, especially corn, in the diets of some African natives, and the high incidence of liver cancer among Africans (Borker et al., 1966). The implication is that aflatoxin or some other mycotoxin might be involved. Whether or not aflatoxins cause human disease is not known. It is generally held by governmental and regulatory agencies, however, that aflatoxins constitute a potential threat to human health and should be kept from the food supply. In another respect these compounds are valuable and powerful tools that can be used in studies of mechanisms of toxicity and chemical carcinogenesis.

Analysis and Assay of Aflatoxins

Two basic types of methods are used in the determination of aflatoxin. The first, which is most widely used, consists of physical and chemical procedures, while the other consists of biological testing which is used primarily for the verification of toxicity rather than for routine testing.

A number of problems are connected with the determination of aflatoxins. Since the aflatoxins are usually found in agricultural products, which may be stored in large lots, the problem of sampling is encountered. Usually the amount of toxin is small, in the μg/kg range, and it is difficult to obtain a representative sample since the toxin may be unevenly distributed throughout the lot. Sometimes only a few kernels
are contaminated, but these may be quite high in aflatoxin and may contaminate the entire batch when it is ground or mixed. Cucullu et al. (1966) found that individual peanut kernels infected with A. flavus may contain as much as 4-10 mg of aflatoxins per kernel.

Since aflatoxins are found in agricultural products or some other substrate on which the mold has grown, the next problem encountered in the assay of aflatoxins is that of extracting the toxin from the substrate for assay. This is usually done by solvent extraction; the exact method is often determined by the substrate, since a solvent which is adequate for one substrate may not be for another because of the solubility of interfering materials. The presence in the extract of interfering materials, such as pigments or lipids, often necessitates a purification step prior to analysis. There has been a great deal published on the subject of methods for analysis of aflatoxins; these have been summarized in detail in several recent reviews (Borker, et al., 1966; Marth, 1967; Ciegler and Lillehoj, 1968).

**Physiochemical methods of analysis**

The majority of the methods used in the analysis of aflatoxins involve techniques which make use of the chemical or physical properties of these compounds. Most of these methods were developed for use with specific substrates and a great many of them for use with peanut products. With
most of these methods, the first step consists of defatting the peanut product, if this has not already been done. This is usually accomplished by extraction with fat solvents, such as hexane or petroleum ether, in which the aflatoxins are insoluble. The next step is extraction of the toxins. Two general methods have been used: (1) extraction in a Soxhlet extractor for 4-18 hours, or (2) rapid batch extraction in a high speed blender or on a shaker. The former method has found wide use in Europe, while the latter method has been favored in the United States (Borker et al., 1966).

Early methods for the analysis of aflatoxins in peanuts were proposed by Sargeant et al. (1961a, b) and Allcroft et al. (1961). These studies showed that toxins were extractable with methanol. They used ducklings to assay for the aflatoxins by feeding the extracts to the ducklings and then measuring the extent of liver damage. In a method developed at the Tropical Products Institute (TPI), methanol extraction was used to recover the aflatoxins from peanut products; the aqueous methanolic extract was then extracted with chloroform and the toxins were separated using paper chromatography (Anon., 1962). Paper chromatography gave inadequate separation because it did not separate $\text{B}_1$ from $\text{B}_2$ or $\text{G}_1$ from $\text{G}_2$. The procedure was later revised by use of thin layer chromatography (Anon., 1964).
European methods, the samples were either defatted in a Soxhlet extractor prior to extraction of the aflatoxins or the extracts themselves were defatted and then the aflatoxins removed (Coomes et al., 1965; De Iongh et al., 1964a). Lee (1965), proposed a method involving direct chloroform extraction of the toxins from peanut products in the presence of small amounts of water. He found that this method was very rapid, and that recovery was equal to the more lengthy, methanol-extraction methods. From the point of extraction on, the methods are essentially the same, since almost all employ analysis by thin layer chromatography and estimation of toxin levels by comparison of the fluorescence intensity of the unknown samples to aflatoxin standards.

Since the objective of most workers in the U.S. has been to develop a rapid, sensitive procedure suitable for the analysis of many samples, the American methods differed from those used in Europe (Borker et al., 1966). When rapid analytical methods were employed, high speed blenders or shakers were used in place of Soxhlet extractors. Combinations of solvents such as acetone-water-hexane or hexane-methanol-water were also used (Heusinkveld et al., 1965; Robertson et al., 1965; Nesheim et al., 1964). The basic method was as follows: the samples were extracted, and the fat and other interfering materials were removed by phase separation, or by various column chromatography treatments.
The samples were concentrated, spotted on TLC plates, the plates developed and the fluorescence of the unknowns compared to that of aflatoxin standards.

The official A.O.A.C. method for peanut products in use at the present time is as follows (Anon., 1966; Anon., 1967): peanuts, or the peanut product, are defatted and the aflatoxins extracted in a blender with hexane and methanol:water (55:45, v/v) (with peanut meal the hexane is omitted); debris is removed by centrifugation, 50 ml of the aqueous methanol layer is transferred to a beaker and 55 g of acid-washed Celite 545 is added; the mixture is transferred to a chromatographic column, packed, and washed with 500 ml of hexane; the aflatoxins are eluted with 600 ml of hexane: chloroform (1:1); the toxin solution is evaporated to near dryness and then made up to a desired volume with chloroform; the samples are spotted on silica gel G-HR TLC plates along with aflatoxin standards and developed with chloroform:methanol (93:7) in an equilibrated lined tank; the plates are examined under ultraviolet light, and the fluorescence of the unknown is matched with that of the appropriate standard spot showing the same type and amount of fluorescence; the amount of aflatoxin is calculated using the following formula:

\[ \mu g/kg = S \times Y \times V/X \times W \]
Where $S = \mu l$ of aflatoxin B$_1$ standard equal to the unknown; $Y =$ concentration of the aflatoxin standard, $\mu g/ml$; $V =$ volume in $\mu l$ of the final dilution of the sample extract; $X =$ $\mu l$ of sample extract spotted giving a fluorescent intensity equal to $S$, the aflatoxin standard; and $W =$ grams of sample applied to the Celite column. This method is essentially that published by Campbell and Funkhouser (1966) which is very similar to the procedure outlined by Nesheim (1964); it reportedly is suitable for assay of levels as low as 20 ppb of aflatoxin B$_1$.

Eppely (1966b) improved on the direct chloroform extraction method of Lee (1965) for peanut products. A purification step using a silica gel column was added; the revised method was rapid, simple, and one which permitted detection and estimation of as little as 1 $\mu g$ of aflatoxins/kg of sample. This method permitted analysis of a larger sample size, and detected lower levels of aflatoxin than did the current accepted A.O.A.C. method. Recently, a collaborative study of this method showed that it was equal in accuracy and sensitivity to the A.O.A.C. method (Eppley et al., 1968).

Engebrecht et al., (1965) developed a method for the extraction of aflatoxin from cottonseed meal. The meal was defatted with hexane and the toxins were extracted with acetone. Pigments and residual lipids were removed by cold
filtration. The acetone was evaporated, and the residue was dissolved in hot methanol. The methanol-soluble material was then taken up in chloroform and analyzed by thin layer chromatography. Other procedures for cottonseed products were developed by Chen and Friedman (1966), Pons et al. (1966a), Pons and Goldblatt (1965) and Stoloff et al. (1966). All of these methods involved extraction with either aqueous methanol or aqueous acetone. Gossypol pigments and other interfering substances were removed by precipitation with lead acetate and the extracts were then further purified by column chromatography (silica gel, celite or liquid-liquid). Sensitivities of these methods in terms of a lower detectable limit ranged from 30 to 0.02 ppb of aflatoxin $B_1$.

Many of the methods developed for peanut and cottonseed products are also suitable for other products, such as cereal grains, beans, potatoes, leafy green and root vegetables, tobacco and mixed feeds (Pons et al., 1966a). Wiley (1966), modified the purification procedure of Pons and Goldblatt (1965) by adding development on TLC plates in methyl acetate prior to quantitation by thin layer chromatography. This method gave good results on a wide variety of agricultural products, such as feeds, grains, nuts, vegetables and alfalfa.

Denault and Underkofler (1967) applied the direct
chloroform extraction method of Lee (1965) to fungal enzyme preparations and other fermentation products, with good results. Direct chloroform extraction has also been applied to various fermentation products, especially materials such as rice, oats, wheat and liquid culture used for the production of aflatoxins (Shotwell et al., 1966; Stubblefield et al., 1967; Ciegler et al., 1966; Davis et al., 1966a, b).

Purchase and Steyn (1967) tested numerous solvents for extraction of aflatoxin M from freeze-dried milk powder. They compared Soxhlet extraction to extraction on a mechanical shaker, and found that the most efficient method was extraction in a Soxhlet apparatus using an azeotropic mixture of acetone-chloroform-water as the solvent.

A number of solvent systems have been used for the development of thin layer chromatographic plates; however, three basic systems are preferred: chloroform:methanol (93:7), chloroform:acetone (9:1) and benzene:ethanol:water (46:35:19), upper phase. Chloroform:methanol (93:7) is the solvent used in the A.O.A.C. method (Anon., 1966). Engebrecht et al. (1965), first used chloroform:acetone (9:1) in an equilibrated, lined developing tank; however, Eppley (1966a) found that if this solvent system was used in an un-lined, unequilibrated developing tank, there was a marked improvement in resolution of the four aflatoxin spots. The benzene:ethanol:water solvent prevents tailing of the toxin
spots and is especially useful for the separation of aflatoxins $G_1$ and $G_2$ from interfering pigments sometimes found in peanut butter extracts (Anon., 1966). Stoloff et al. (1968) recently reported that benzene was superior to chloroform as a spotting solvent for aflatoxin on silica gel thin layer chromatography plates. Spots applied to the TLC plates were more compact and uniform when dispensed from benzene than from chloroform, and the toxins were more stable in benzene than in chloroform.

Visual determination of fluorescence and estimation of aflatoxins on TLC plates is subject to a deviation of $+17\%$ with an accuracy within $20\%$ (Ciegler and Lillehoj, 1968). Several attempts have been made to improve this accuracy by reading the aflatoxin fluorescence using fluorodensitometers (Ayres and Sinnhuber, 1966; Pons et al., 1966b; Peterson et al., 1967; Stubblefield et al., 1967). The densitometer methods are accurate to an average deviation of $+2\%$; however, the instrumentation that is required for these methods is expensive.

A spectrophotometric method suitable for determining aflatoxins was reported by Nabney and Nesbitt (1965). With this method the ultraviolet absorption at $363 \text{ m\AA}$ was used to calculate the amount of aflatoxin. This method was fairly accurate, but required a considerable amount of work since the toxins had to be separated on TLC, the separated bands
scrapped from the plates, the toxin eluted from the silica gel and then the optical density determined. In addition to being time-consuming and not suited for routine analysis, the method required a rather expensive spectrophotometer.

Andrellos and Reid (1964) developed three confirmatory tests to identify aflatoxin B₁. These consisted of treating the isolated toxin with (1) formic acid-thionyl chloride, (2) acetic acid-thionyl chloride and (3) trifluoroacetic acid. The reaction products of the treated toxin were compared to an untreated sample, a treated standard toxin and an untreated standard on TLC plates. The reaction products had altered chromatographic patterns and compared with the reaction products of the standard. Stoloff (1967) reported the results of a collaborative study of a modification of this procedure; an improved silica gel column cleanup step was used; this gave fewer false-positive readings.

**Biological methods of analysis**

Numerous biological systems have been studied as potential bioassay organisms for aflatoxin. The first organism suggested for this purpose was the duckling (Sargeant et al., 1961a, b; Carnaghan et al., 1963; Newberne et al., 1964; Armbrecht and Fitzhugh, 1964; Wogan, 1965).

In the duckling bioassay, 1 day old Peking ducklings are used. Samples, in water or propylene glycol, are given via
stomach tube or capsule over a several day period. Control ducklings are fed a known amount of aflatoxin B$_1$. The surviving birds are sacrificed 2 to 7 days after administration of the toxin has ceased and a liver section is taken for examination. The degree of hyperplasia is recorded and compared to the known controls, and from this a determination of aflatoxin level is made, since the degree of bile duct cell hyperplasia is roughly related to the amount of aflatoxin fed. The duckling is quite sensitive to low levels of aflatoxin but the response is not specific; other toxic agents gave similar results. When the results of this test are combined with TLC data, however, reasonably accurate identification of aflatoxin can be made. The main value of this test is as a confirmatory test because it is too expensive and time-consuming to be used for routine analyses. Also, highly skilled and trained technicians are required to perform the test properly. It is, however, the most conclusive and widely-accepted biological test.

Sensitivity of the chicken embryo to aflatoxins was first reported by Platt et al. (1962). They found that when aflatoxin was injected into the yolk sac of a 5 day old chicken embryo, it was much more sensitive to the toxin than the 1 day old duckling. The chicken embryo required only 1/200th of the amount of toxin as the duckling for a positive test. Verrett et al. (1964) confirmed the work of Platt
and developed a chicken embryo assay test for aflatoxins. They found that the maximum toxic effect was obtained when the toxin was injected via the air sac, prior to incubation of the embryonated egg. Verrett et al. (1964) and Gabliks (1965) found that sensitivity to the toxin decreased as the age of the embryo increased. Gabliks (1965) reported that the duck embryo was more sensitive than the chicken embryo. Verrett et al. (1964) determined the LD50 at 21 days incubation of aflatoxin B₁ to chicken embryos to be 0.048 µg/egg when administered via the yolk and 0.025 µg/egg when the toxin was applied via the air cell with eggs injected prior to incubation. Jayaraman et al. (1968) found that 9 day old chicken embryos, incubated in egg cartons in a laboratory incubator and turned manually twice daily, were more sensitive to aflatoxin than embryos incubated in a commercial incubator with an automatic egg turner. The LD50 of aflatoxin B₁ to embryos incubated in cartons was 0.01 µg/egg; the LD50 to automatically-turned eggs was 1.0 µg/egg. With the extreme sensitivity of the chicken embryo to aflatoxin, very small amounts of toxin can be assayed. This test is somewhat simpler to run and is much less expensive than the duckling test, making it more useful as a routine test. However the chicken embryo is sensitive to many other compounds so the embryonated egg method is less specific than the duckling test. But, when the chick embryo
test is combined with TLC data or other tests, it can be quite valuable in assessing toxicity.

In vitro cell cultures are quite sensitive to aflatoxins and Legator and Withrow (1964) proposed that they be used as assay systems. The use of such cell cultures, however, has not yet been developed into a routine test for aflatoxins.

Several attempts have been made to develop microbiological assays suitable for routine analysis of aflatoxins. Burmeister and Hesseltine (1966) surveyed 329 microorganisms for inhibition by aflatoxins. They found one strain of Bacillus brevis and two strains of B. megaterium to be the most sensitive to aflatoxins. But even these required aflatoxin levels as high as 10 and 15 µg/ml before inhibition occurred. Clemmens (1968) reported that a strain of B. megaterium was sensitive to aflatoxin levels of 4-6 µg using zone inhibition as a measure of toxic effect. Using the "paper disc plate" method of antibiotic assay, Jayaraman et al. (1968), reported that levels of 1-4 µg/disc of aflatoxin B₁, 4-8 µg/disc of aflatoxin G₁ and 8 µg/disc of an aflatoxin mixture could be assayed with a sensitive strain of B. megaterium. The main drawback with microbial inhibition assays is a lack of sensitivity as compared to other methods. However, in another respect, it has been observed that certain mutagenic and carcinogenic compounds are capable of inducing lysogeny in lysogenic bacteria (Heinemann and Howard,
1964; Lein et al., 1962). Legator, (1966) showed that aflatoxin concentrations as low as 0.06 µg/ml induced lysogeny in lysogenic strains of *Escherichia coli* and *Staphylococcus aureus*. To date this information has not been utilized in developing any type of assay.

A number of other biological systems have been considered as possible bioassay test organisms. Townsley and Lee (1967), found that aflatoxin B1 inhibits cell cleavage in fertilized eggs of the mollusk *Bankia setacea* without preventing fertilization or nuclear division. They proposed a bioassay which was said to be simple to run and sensitive to concentrations of aflatoxin as low as 0.05 µg/ml. When the test was observed with a low power microscope, toxin levels of 0.05 µg/ml final volume were measured when egg division was observed, or 0.005 µg/ml final volume could be measured when observing swimming larvae. The limiting features of the test are that the larvae require temperatures of 10-20° C and sea water. Also, the mollusk is not commonly available. Brown et al. (1968), studied the temperature and dose relationships of brine shrimp (*Artemia salina*) to aflatoxins. Optimum sensitivity occurred at 37.5° C; positive results could be observed with as little as 0.5 µg of aflatoxin B1/ml of artificial sea water. Mortality at this dose level was over 60%. When the dose level was increased to 1 µg/ml the mortality was greater than 90%.
Schoental and White (1965) suggested that plant albinism might be used as a bioassay system; they observed increasing degrees of albinism in the leaves of cress (Lepidum sativum) with increasing levels of aflatoxin from 1 to 10 µg/ml. Germination was completely inhibited at 25 µg/ml.

Since Halver (1965) found that trout were extremely sensitive to aflatoxins, it has been suggested that small fish such as guppies or young trout might serve as a sensitive, low-cost test animal (Ciegler and Lillehoj, 1968).

De Vogel et al. (1965) described a rapid screening test for determining potentially toxic mold strains. With this method molds were cultivated on Czapek-Dox agar enriched with an aqueous extract of raw peanuts and with ammonium chloride substituted for sodium nitrate. The agar mixture was overlaid on a thin layer of infusorial earth in a standard petri dish. The mold to be tested was streaked on one-half of the plate, and the other half was left as a control. The plate was incubated for 48-72 hours at 30°C, at which time the plate was inverted and examined under strong ultraviolet light. Aflatoxin B₁-producing strains exhibited a bright blue fluorescence in the infusorial earth layer. The authors stated that 5 µg of aflatoxin B₁/ml of medium was the lowest concentration at which a definite blue fluorescence occurred. At very low aflatoxin concentrations, the color tended to become violet due to the reflection of ultraviolet
light. The method is not specific, and the sensitivity is low; however, it is useful when screening for strongly toxigenic strains.

Organisms that Produce Aflatoxins

Shortly after their discovery, the aflatoxins were found to be metabolites of *Aspergillus flavus* Link (Sargeant et al., 1961a). At first, aflatoxins were thought to be the unique products of *A. flavus*. Later, *Aspergillus parasiticus* Speare was found to produce aflatoxins (Austwick and Ayerst, 1963; Codner et al., 1963). Now there is some question as to whether or not aflatoxin-producing cultures of *A. flavus* should not actually be classified as *A. parasiticus* (Ciegler and Lillehoj, 1968). As early as 1963, Austwick and Ayerst reported that some toxin-producing strains of *A. flavus* could be considered as identical with *A. parasiticus*.

In 1964, Hodges et al. reported that *Penicillium puberulum* Bainer produced aflatoxins. Kulik and Holaday (1966) reported that a number of fungi in addition to *A. flavus* and *A. parasiticus* produced aflatoxins. They stated that *A. niger* Van Tieghem, *A. ruber* Estienne, *A. wentii* Wehmer, *P. citrinum* Thom, *P. variable* Sopp, *P. frequentans* Westling and *P. puberulum* were all capable of producing aflatoxins. Parrish et al. (1967) screened 14 species of *Aspergillus* and
8 species of *Penicillium*. They found that all of the strains of *A. parasiticus* and 26 out of 93 *A. flavus* strains produced aflatoxins. However, no *Aspergillus* species other than *A. flavus* and *A. parasiticus*, and no *Penicillium* species could be shown to produce aflatoxins.

Scott *et al.* (1967) reported that *Aspergillus ostianus* Wehmer, produced aflatoxins. This particular strain was isolated from a Japanese dried fish product (katsuobushi).

Recently, van Walbeek *et al.* (1968) found that 16 of 128 fungi isolated from 74 food samples produced aflatoxins. Besides *A. flavus* isolates, a *Rhizopus* sp., an *A. ochroceus* strain and a strain of *P. puberulum* produced aflatoxins. The *Rhizopus* sp. and the *A. ochraceus* strain produced only small amounts of aflatoxins. It was also found that 6 strains of *A. flavus* var. *columnaris* produced only aflatoxin B$_2$; this is the first time that the exclusive production of B$_2$ has been reported in the literature. These strains of *A. flavus* were only very weakly toxinogenic.

The reports of aflatoxin production by fungi from other than the *A. flavus* group has been seriously questioned by Wilson *et al.* (1968). These workers found that of 121 fungal isolates representing 29 species (including 8 isolates already reported by other workers to produce aflatoxins) no organisms other than *A. flavus* and *A. parasiticus* produced aflatoxins.
Not all *A. flavus* and *A. parasiticus* strains produce aflatoxins. Mateles and Wogan (1967) state that about 10-30% of the strains of *A. flavus* and *A. parasiticus* examined have the potential to produce aflatoxins. Diener and Davis (1966), however, stated that about 80% of their *A. flavus* isolates from peanuts produced aflatoxins; of these 90% produced only aflatoxin B₁. Boller and Schroeder (1966) found that 94% of 284 *A. flavus-oryzae* strains isolated from rice produced aflatoxins on rice and 91% produced aflatoxins on peanuts. Taber and Schroeder (1967) reported that isolates of the *A. flavus-oryzae* group isolated from peanuts produced primarily aflatoxin B₁ with only traces of B₂ and no G₁ or G₂.

In general, toxic *A. parasiticus* strains are more strongly toxinogenic than are toxic *A. flavus* strains. Aflatoxin B₁ may predominate in culture extracts of *A. flavus* or the ratio of B₁ to G₁ is usually a 50:50 ratio. In extracts of *A. parasiticus* cultures, aflatoxin G₁ usually predominates. These ratios of toxins are also affected by the substrate and cultural conditions (Mateles and Wogan, 1967; Codner et al. 1963; Boller and Schroeder, 1966; Schroeder, 1966).
Conditions Affecting Aflatoxin Production

Production in nature

The factors influencing aflatoxin production have been studied extensively. This work has been largely concerned with toxin production in culture, so more is known about this aspect of toxin production than about production under natural conditions (Ciegler and Lillehoj, 1968). As mentioned previously, mold strains vary considerably as to the kind and amount of aflatoxins produced. Some mold strains lose their ability to produce aflatoxins, especially if they are carried on synthetic laboratory media for long periods of time. To maintain optimum toxin-producing ability it is best to carry isolates on natural or semisynthetic media which contains some organic nitrogen material. Rice, peanuts, potato plugs, soil, or media supplemented with yeast extract or corn steep liquor are satisfactory substrates for maintenance of toxic strains (Diener and Davis, 1966; Schroeder and Hein, 1967).

According to Spensley (1963), the two most important factors affecting aflatoxin production in vivo are temperature and moisture. A. flavus grows rapidly but requires more moisture than most other molds for growth. Optimum conditions for growth have been reported to be 80 to 85% relative humidity and 30°C (Spensley, 1963). This corresponds to a moisture content of 10% in peanut kernels and 16% in defatted peanut
meal. These conditions are often met under tropical conditions, (Austwick and Ayerst, 1963; Spensley, 1963). Aflatoxins are rarely found in peanuts at the time of harvest, and mold development usually does not appear until after the nuts are removed from the ground. Aflatoxins appear when kernel moisture and competitive factors favor the development of _A. flavus_. Large amounts of aflatoxin are most likely to appear when the kernel moisture is near 24%. Damaged pods and kernels favor toxin development, because of greater invasion by the mold (Ashworth et al., 1965; Schroeder and Ashworth, 1965; Spensley, 1963; Austwick and Ayerst, 1963). McDonald and Harkness (1964) showed that aflatoxin production on peanuts could be prevented if they were cured in mechanical driers.

**Production in culture**

Production of aflatoxin in culture has been studied by a number of investigators, and many factors have been found to govern the process.

Diener and Davis (1967) showed that the limiting relative humidity for aflatoxin production by _A. flavus_ on peanuts in culture was 85% ± 1% at 30°C. The limiting temperatures for toxin production in 21 days at 97-99% relative humidity was 13 ± 1°C and 41.5 ± 1.5°C. Schroeder and Hein (1967) found that the optimal temperature range for aflatoxin production on peanuts, rice and cottonseed meal by _A. flavus_
was 20 to 35°C and the optimal growth temperature was 25°C. Trace amounts of toxin were detected at 10 and at 40°C; as the temperature was increased from 25 to 30°C less time was required to obtain high yields. Aflatoxin G accumulated faster than did B at low temperatures and also disappeared faster at high temperatures. Schroeder and Hein (1967) suggested that aflatoxin production is a function of the growth rate, and that the toxins are re-metabolized faster as the fungal metabolism is accelerated by high temperature.

Schindler et al. (1967) found that maximal toxin production by *A. flavus* on wort agar occurred at 25°C in 5 days. No aflatoxins were produced below 18°C and above 35°C in 5 days; however, after 12 weeks, toxins were detected at 13°C. Schindler et al. (1967) also reported that the ratio of B₁ to G₁ varied with the temperature, and that aflatoxin production was not related to the growth rate. Maximal growth of the *A. flavus* strains occurred at 29°C and 35°C. Heavy growth occurred at 41°C but no aflatoxins were detected at this temperature. Sorenson et al. (1967) found that the optimal temperature range for aflatoxin production by *A. flavus* NRRL 2999 on rice was 28 to 32°C. Shotwell et al. (1966) reported an optimal temperature of 28°C for this organism on rice. The temperature limits for aflatoxin production by this organism on rice were found to be 8 to 37°C. Very low levels of toxin (100 ppb) were
detected in 3 weeks at 11° C, and moderate to high levels were obtained at 15° C. Again, temperature was reported to influence the ratio of toxins produced, with $B_1$ predominating at the higher temperatures. From the work of these investigators, it can be seen that temperature has a marked effect on aflatoxin production by $A. \text{flavus}$ and these effects seem to be roughly the same for several different substrates.

In addition to rice and peanuts, a number of other agricultural commodities have been studied as potential substrates for aflatoxin production. Wheat has been shown to be a good substrate for the production of $G_1$. Oats are a poorer substrate than wheat for toxin production, probably because of the rather impervious hull (Stubblefield, 1967). Armbrecht, et al. (1963) reported that they successfully produced aflatoxins on wheat, corn, oats, rye, buckwheat, rice, soybeans, peanuts and Czapek-Dox broth and agar. Hesseltine, et al. (1966) observed aflatoxin production on rice, sorghum, peanuts, corn, wheat and soybeans. Soybeans did not readily support toxin production, but high yields were obtained from rice, substantial yields from peanuts and sorghum, and lower yields from wheat and corn. When wheat and corn cultures were shaken, increased yields resulted, suggesting a positive effect from aeration.
Aeration has been shown to stimulate aflatoxin production in most cases. Increased yields of aflatoxin in shaken as opposed to unshaken rice cultures were reported by Shotwell et al. (1966). Hayes et al. (1966) observed that with high aeration rates (9000 ml air/min) aflatoxin yields were 212 mg/l and with low aeration rates (3000 ml/min) aflatoxin yields were 11 mg/l. Codner et al. (1963) showed that Czapek-Dox broth fortified with corn-steep liquor gave yields of 100-200 mg/l of aflatoxin in shake flasks, but they were unable to obtain aflatoxins in stirred, aerated fermenters. Ciegler et al. (1966) found that maximum toxin levels could be obtained in 72 hours at 25° C in well-agitated, well-aerated, 20-liter fermentors, after which aflatoxin levels declined rapidly due to non-enzymatic, non-specific degradation. This degradation could be simulated by reacting aflatoxins with peroxidized methyl esters of vegetable oils. The effect of atmospheric gases (carbon dioxide, oxygen and nitrogen) on aflatoxin production by A. flavus on peanuts was studied by Landers et al. (1967). They found that reducing the oxygen concentration reduced the amounts of aflatoxins obtained, as did increasing the carbon dioxide concentration. Increased CO₂ also decreased fungal growth, with no growth or toxin production occurring in an atmosphere of 100% CO₂.

Production of aflatoxin in semi-synthetic and synthetic
culture media has been studied extensively. A synthetic, glucose-ammonium-nitrate-salts medium developed by Brian et al. (1961) has been used to obtain satisfactory yields of aflatoxins (Raper and Fennell, 1965). Schroeder (1966) showed that the addition of corn steep liquor (up to 8%) to Czapek's broth stimulated aflatoxin production by A. parasiticus. Davis et al. (1966a) reported very high yields (630 mg/liter) of aflatoxins using a broth consisting of 20% sucrose and 2% yeast extract. Mateles and Adye (1965) used a synthetic glucose-ammonium-salts medium in submerged culture; yields of 60 to 80 mg of aflatoxins/liter of medium were obtained. Preferred carbon sources were glucose, sucrose and fructose; casamino acids were the preferred nitrogen source. Davis et al. (1967), using a chemically defined salts medium and stationary culture, found glucose, sucrose and fructose to be the most satisfactory carbon sources for aflatoxin production by A. flavus. Complex nitrogen sources, such as yeast extract and peptone, gave higher yields of aflatoxin than did individual amino acids. Davis and Diener (1968) studied the effect of carbon source on aflatoxin production by A. parasiticus. They found that glucose, ribose, xylose and glycerol were excellent carbon sources for growth and toxin production by this organism. The organism grew poorly, or not at all, and produced no aflatoxin when Krebs cycle intermediates other than fumaric
acid were used as carbon sources. The authors suggested that, for a carbon compound to support both growth and toxin production, it must be metabolized through the hexose monophosphate and glycolytic pathways. Lee et al. (1966) found that the trace metals zinc and cadmium stimulated toxin yield in a glucose-ammonium-salts medium. Iron and magnesium were also required. Barium ions reduced aflatoxin levels and manganese inhibited toxin production.

Davis and Diener (1967) observed that A. parasiticus did not produce aflatoxin when p-aminobenzoic acid (PABA), potassium sulfite or potassium fluoride was added to the medium. PABA inhibited growth and aflatoxin synthesis at all levels used. The authors postulated that PABA inhibition may be the result of competitive or feedback inhibition of the shikimic acid pathway. Potassium sulfite inhibited aflatoxin synthesis but not growth. Potassium fluoride inhibited growth and toxin production when used in high concentrations.

Concerning the biosynthesis of aflatoxins, relatively few data have been published. Although there has been much speculation. What information is available has been reviewed by Mateles and Wogan (1967).
Aflatoxins and Human Food

A number of investigators have studied the formation of aflatoxins on human food. Lie and Marth (1967) inoculated 3 month old cheddar cheese pieces with *A. flavus* and *A. parasiticus* and incubated them at room temperature for 10 and 52 days. Aflatoxins were found in the top 1.3 cm of cheese. The greatest amounts of toxin were in the top 0.64 cm of the cheese where levels of 7-10 and 15-30 mg of aflatoxin/kg of cheese were noted after incubation for 1 and for 7 weeks, respectively. The mold mycelium which was scraped off of the cheese surface contained much higher amounts of the toxins (60-100 mg/kg).

Frank (1968) studied the production and diffusion of aflatoxins in apple juice, rye and wheat breads, and Tilsit cheese. A semi-solid model substrate of "Cream of Wheat," sucrose and tap water was constructed to study diffusion of aflatoxins. Using a strain of *A. flavus* isolated from food he obtained 120 μg of aflatoxin B₁/100 ml of apple juice. With bread, levels of 61 μg of B₁ and 60 μg of G₁/g were obtained. The proportion of G₁ to B₁ was found to increase the farther away from the point of inoculation that the measurement was made. With Tilsit cheese, 0.2 μg of B₁ and 0.6 μg of G₁/g were obtained. The toxins diffused about 1 cm into the cheese. With the model system, a maximum of
248 μg of B$_1$/g was obtained in 3 days of incubation. By 5 days, the aflatoxins had diffused to all parts of a 3 cm cube. The authors stated that the depth to which aflatoxins could be found in mold-contaminated food depended on the mold strain, water content and physical properties of the food and the time and temperature of storage. van Walbeek et al. (1968), isolated a wide range of fungi from foods and determined the aflatoxin-producing ability of these molds. Organisms that produced aflatoxins on laboratory media were isolated from a meat pie, baked apple squares, dry spaghetti, orange juice, cocoa powder, various types of cheeses, fresh vegetables, rice portion of a T.V. dinner, shelled Brazil nuts, and grains used for animal feeds. Some of the isolates were from foods linked to human illnesses, others from moldy foods and the rest from routine testing. It was presumed that, had proper conditions been met, these organisms could have produced aflatoxin on the foods from which they came. The authors suggested that foods linked to human illness should be assayed for mycotoxins and toxino-genic fungi in addition to the common food poisoning bacteria and associated toxins. Wildman et al. (1967) inoculated a number of solid foods and fruit juices with A. flavus. Aflatoxins were produced on non-sterilized peanuts, grapes, bread, cocoa, oranges, potatoes, cantaloupe, peaches and cheese; on sterilized grape juice, beef infusion and beef
pieces; and on many fruit juices; apricot, peach, grape, grapefruit, orange, pineapple, apple, mixed vegetable, pear, tomato and cranberry. Owing to bacterial overgrowth no aflatoxins were produced on raw beef. Sterilized beef infusion and beef pieces supported yields of 15 and 11 μg of aflatoxin/g of meat, respectively. Most fruit juices yielded 44 μg aflatoxin/g of juice. The highest yields of aflatoxins were obtained from peanuts (82 μg/g) and grapes (60 μg/g). Cranberry juice, oranges, potatoes, cantaloupe and cheese yielded very low amounts of aflatoxins. Frank (1966) obtained aflatoxins from A. flavus grown on a large number of foods, including smoked bacon, condensed and powdered milk and egg noodles.

In addition to mold contamination of foods and subsequent aflatoxin production on these foods, there is another way that aflatoxins might enter the food supply; that is, through carry-over of aflatoxins into animal products from animals consuming aflatoxins. This is known to occur in the case of milk, where aflatoxins have been fed to lactating animals (Allcroft et al., 1961; De Iongh et al., 1964b; Allcroft and Carnaghan, 1963b; van der Linde et al., 1965). However, livers, blood serum and meat from animals, and eggs from chickens fed aflatoxins have not been shown to cause harmful effects when fed to test animals (Campbell, 1965).

In addition to mold contamination of human food, a number
of fungi are used in the manufacture of a variety of food products, found in various parts of the world. A number of Oriental foods including Tempeh, Sufu, Ragi, Miso, Shoyu and Ang-kak are produced by the action of molds growing on soybeans, rice or other plant or animal material (Hesseltine, 1965). These processes use organisms of the genera Rhizopus, Actinomucor, Mucor, Aspergillus and Monascus. Aspergillus oryzae is used in the manufacture of soy sauce, an oriental food imported by the United States. Aspergillus tamarii is used in the preparation of Tamari sauce (Frazier, 1967). Fermented fish are produced in Japan and China using molds, especially members of the genus Aspergillus. The possibility that some of these molds might produce aflatoxins has been considered. Murakami et al. (1962) screened 214 cultures of Aspergillus used in Japanese industrial fermentations. No aflatoxins were found; there were spots on TLC plates which corresponded to aflatoxins, however, further study proved that these were not aflatoxins. A. oryzae is used in many oriental food fermentations, and is a close relative of A. flavus. For this reason, 53 A. oryzae cultures used in food manufacture were studied for the production of aflatoxins. None of these strains were shown to produce aflatoxins, and none of the food products produced by them contained aflatoxins (Hesseltine et al., 1966). However, this does not preclude the possibility that
aflatoxins cannot be found in these foods. Many of the Asian foods are made from cultures or starters used in home fermentations that contain many kinds of molds. It is quite possible that these sources could infrequently become contaminated with A. flavus or other organisms capable of producing aflatoxins.

A number of aspergilli and penicilli are used in fungal fermentations for the production of vitamins, amino acids and enzymes which may be used as food additives or in food processing. Denault and Underkofler (1967) surveyed fungal enzyme preparations for aflatoxin, and found none.

Other foods develop mold growth during aging. Certain cheeses are produced by fermenting milk inoculated with Penicillium camemberti and P. roqueforti and then aging for several months.

Cured and aged meats frequently develop mold growth. This growth may be from undesirable chance contamination on stored meats, such as ham, bacon or aging carcasses, or may be part of a desired characteristic flora that develops during aging and ripening of certain meats such as "country cured hams" and European type dry salami. Several patents have been issued in the U.S. for aging beef carcasses inoculated with spores of Thamnidium elegans. The inoculated beef is hung at temperatures of 7 to 16° C and at relative humidities of greater than 80%. The fungus is supposed to prevent
bacterial growth and improve flavor and tenderness (Hesseltine, 1965). These conditions, however, might also allow the development of *A. flavus*.

Country cured hams are produced in the southern U.S. and European dry salami are traditionally produced in Hungary, Italy, Spain, Greece, Yugoslavia, Romania, Czechoslovakia, and to a limited extent in the San Francisco-Oakland Bay area of the U.S. (Ayres et al., 1967).

Country cured hams are dry cured hams that may or may not be smoked, and which are aged for long periods of time. The aging may be from 6 months to 2 years, and in most cases is done in rooms or attics in which the temperature and humidity are not controlled, but allowed to fluctuate with the environmental conditions. Thus, summer temperatures may get as high as 30-35°C, allowing mold development. The hams will lose 12 to 15% of their weight in the form of moisture if they are aged 6-9 months and 25-30% if aged 1-2 years. Usually heavy mold growth occurs on the flesh side of the hams. The type of mold found is determined by the moisture content of the ham. Thus, during the early stages of aging, penicillia predominate. As the water activity of the ham decreases, aspergilli begin to grow, and eventually as the water activity decreases to a lower level (0.65), *A. ruber* and other xerophilic molds predominate (Leistner et al., 1965; Christian, 1964; Ayres et al., 1967; Lillard
and Ayres, 1968).

Dry European type salami are made from ground pork and beef combined in variable proportions, but usually 2/3 pork to 1/3 beef. Salt, spices, nitrate and nitrite are added to the meat and the mixture is kept at 2-3° C for several hours to allow the spice mix to diffuse through the meat. The meat is then stuffed into natural or artificial casings and hung overnight. Hungarian salami are then cold-smoked for 2 hours each day for 8 to 10 days. Italian salami are not smoked, but rather hung in a "greening" room for 4-5 days where excess moisture drips from the sausage, and characteristic mold begins to grow. After smoking, or after growth has started, the salami are moved to a room where they are aged for 30-60 days. During this time the temperature is maintained at 10-16° C and the relative humidity at 75%. During aging profuse mold growth may develop. The predominant molds are either penicillia, or penicillia and Scopulariopsis. Aspergilli may be present in small amounts (Leistner et al., 1965; Ayres et al., 1967).
MATERIALS AND METHODS

Except where otherwise indicated, chemicals used in all of the laboratory experiments were analytical reagent-grade.

Screening of Mold Isolates for Aflatoxin Production

Toxinogenic molds to be used in this study were selected from known aflatoxin-producing strains of *A. flavus* and *A. parasiticus* and from potentially toxinogenic strains that were isolated from cured and aged meats (Sargeant et al., 1961b; Codner et al., 1963; Ayres et al., 1967). Since over 600 isolates were available, final selections were based on the abilities of the fungi to produce aflatoxins. The organisms that were screened for aflatoxin production were selected as representative strains from the most numerous species present on the meats, and the ones most likely to produce aflatoxins based on literature reports (Sargeant et al., 1961b; Hodges et al., 1964; Kulik and Holaday, 1966). The number of strains of each species of organism studied were, as follows: *Aspergillus* species (*A. flavus-oryzae* group) (1), *A. ruber* (53), *A. wentii* (2), *A. niger* (3), *A. flavus* (1), *Penicillium frequentans* (3), *P. variable* (1), *P. puberulum* (2). The large number of *A. ruber* strains were selected because this organism was present in large numbers.
on most fully aged country cured hams; on some hams this species was almost the only one detected (Ayres et al., 1967).

The mold strains were screened first for fluorescence in culture according to the method of De Vogel et al., (1965). This method employed a screening medium consisting of: 30 g sucrose, 1 g K$_2$HPO$_4$, 0.5 g MgSO$_4$$\cdot$7H$_2$O, 0.5 g KCl, 1.6 g NH$_4$Cl, 0.01 g FeSO$_4$$\cdot$7H$_2$O, 100 ml of a hot distilled water extract of ground raw peanuts and 900 ml of distilled water. The peanut extract was made by heating 100 g of ground raw peanuts with 100 ml of distilled water at 100° C for 10 min. This slurry was added to the salts solution, and the mixture was heated 100° C for an additional 3-5 min.; the broth medium was filtered hot through a double thickness of cheesecloth. Then 20 g of agar was added to the peanut extract-salts medium and the mixture was sterilized by autoclaving for 15 min. at 121° C. The sterile medium was poured over a layer of sterile infusorial earth (Hyflo Super Cel, Johns-Manville, New York) contained in a standard glass petri dish. Conidia of the mold to be tested were streaked on the surface of one-half of a plate of screening medium; the other half remained uninoculated as a control. For purposes of comparison, A. flavus strains of varying aflatoxin-producing abilities, were streaked in the same manner. The cultures were incubated for 3 days at 25-30° C, at which time the plates were inverted and examined under long-wave
(365 μm) ultraviolet light. Strains that showed no fluorescence by 3 days were incubated an additional 4 days and examined again. Mold isolates from meats were compared with known aflatoxin-producing strains. Since the latter exhibited bright blue fluorescence in the infusorial earth layer, all cultures having this same property were considered suspect.

Following this initial selection, mold strains which showed fluorescence in culture were further screened by growing each isolate on rice, extracting the culture with chloroform, and analyzing the chloroform extracts by thin layer chromatography (TLC). The method used was similar to that of Sorenson et al. (1967). Twenty-five milliliters of tap water was added to 50 g of polished long-grain rice in a 250 ml Erlenmyer flask. The flasks were stoppered with polyurethane foam plugs, allowed to stand for 1-2 hours, and then autoclaved at 121° C for 15 min. After cooling, the rice was inoculated with 1 ml of a spore suspension of the organisms to be tested. Spore suspensions were made by adding 2 ml of a sterile 0.02% solution of agar in water to well-sporulated, 7-21 day-old, potato dextrose agar slant cultures of the organisms in question, and loosening the conidia with a flamed inoculated loop. Known aflatoxin-producing strains of A. flavus were inoculated into rice in the same manner and used as positive controls. The cultures were then incubated at 25-30° C
for 7-14 days, depending on the rate of growth of the organism. Following incubation, 100 ml of chloroform were added to each culture in its own culture flask and the mixture was allowed to stand overnight. The chloroform was then decanted, a second 100 ml portion of chloroform was added to the culture and the mixture was allowed to stand for an additional 2 hours. The second chloroform extract was combined with the first and the total 200 ml volume of extract was evaporated to dryness in vacuo at 50° C using a Buchler rotary evaporator. Uninoculated rice was also extracted with chloroform and treated in a manner similar to that used for the inoculated samples. The residue was dissolved in 5 ml of chloroform and transferred to small, 4 dram, screw cap vials. The extracts were spotted using Hamilton microliter syringes on 20 x 20 cm glass TLC plates coated with a 0.25 mm thickness of silica gel G-HR (Brinkman Instruments, Westbury, N.Y.), which had been activated by heating at 100° C for 1 hour. Aflatoxin standards (Southern Utilization Research and Development Division, U.S.D.A., New Orleans, La.) containing known amounts of aflatoxins B1, B2, G1 and G2 were also spotted on the TLC plates. The plates were developed on unlined, unequilibrated developing tanks, with chloroform:acetone (9:1) used as the developing solvent (Eppley, 1966a). The developed TLC plates were examined under UV light (365 mū) and the extracts of the mold isolates were compared to the
standards and extracts of known aflatoxin-producing molds and examined for the presence of aflatoxins. A mold strain was considered tentatively positive for aflatoxins if fluorescent spots were observed that corresponded to the aflatoxin standards and the fluorescent spots of known toxinogenic molds (Anon., 1966).

To verify that the fluorescent spots from a tentatively-positive strain were aflatoxins, each extract was subjected to several additional analyses. An extract was purified using preparative TLC, and the broad band corresponding to aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ was scraped from the plate (Kwon and Ayres, 1967). The aflatoxins were eluted from the silica gel with chloroform:methanol (97:3) using a sintered glass filter (pore size, 4-8 μ) and a small amount of suction. The purified extract was transferred to a small vial and evaporated to dryness. The residue was dissolved in 0.5 ml of chloroform and compared to aflatoxin standards and internal standards by TLC on 5 x 20 cm glass plates, each coated with a 0.25 mm thickness of silica gel G–HR (activated as above). Eight different solvent systems were used as follows: chloroform:acetone (9:1), chloroform:methanol:acetic acid (96:3:1), chloroform:methanol:acetic acid (92:7:1), benzene:methanol:acetic acid (24:2:1), benzene:methanol:acetic acid (100:10 10), benzene:acetone:acetic acid (80:9:10), benzene:ethanol:water (46:35:19), upper phase and acetone:chloroform/ethyl
ether (10:90) (chloroform/ether 30:10) (Anon., 1966; Eppley, 1966a; Scott et al., 1967). The compounds were then further purified and separated using preparative TLC. The separated bands were removed from the plates and eluted, as was done previously. Ultraviolet and infrared absorption spectra were obtained for the compounds that corresponded to aflatoxins $B_1$ and $G_1$ and these were compared to spectra for authentic aflatoxins $B_1$ and $G_1$. Biological toxicity data for the fraction corresponding to aflatoxin $B_1$ were obtained using four-day-old chicken embryos and one-day-old ducklings. The reading of the duckling tests was performed by Dr. Gideon Chassis, Department of Food Science, University of Georgia, Athens, and confirmed by Dr. D. E. Tyler, Head of Pathology and Parasitic Veterinary Medicine of the University of Georgia.

**Extraction and Assay of Aflatoxins from Meats**

The extraction and assay of aflatoxins from any substrate is hampered by the presence of chloroform-soluble interfering materials, such as pigments and lipids. With meats the primary interfering substances are lipids, especially fats.
Since this study required the analysis of a large number of samples, it was desirable to use the simplest method possible to extract aflatoxins from meats without sacrificing accuracy and precision. Lee (1965) used direct chloroform extraction of aflatoxins from defatted peanut products, in the presence of a small amount of water, and obtained essentially quantitative recovery of the toxins. Eppley (1966) verified this finding and improved on Lee's method by the addition of a silica gel column purification step similar to that used by Pons et al. (1966). In this study, aflatoxins were extracted from meats by a method modified after the procedures of Lee (1965) and Eppley (1966b).

The procedure used to extract and assay aflatoxins from meats, in all remaining work to be described hereafter, was essentially that outlined below. One-hundred gram samples of meat were used. Solid pieces of meat were broken into small pieces by placing them in a pint Mason jar and blending in an Osterizer blender at low speed, a few seconds at a time, until the sample was well-pulverized. A 100-ml portion of chloroform was added to the homogenized sample in the blender jar, and the jar was closed with an ordinary Mason lid. The mixture was mechanically shaken using a rotary platform shaker (Sensaur Model R-650 with automatic timer) at 200 rpm. for 30 min. The chloroform was decanted and filtered (gravity flow) through 1-2 g of anhydrous sodium
sulfate on Whatman No 2V fluted filter paper in an ordinary 100-cm diameter glass funnel. A second extract was made in a similar manner; the second filtrate was combined with the first.

The filtered extracts were subjected to a very brief exposure to ultraviolet light to observe the intensity of fluorescence. If the samples showed intense fluorescence they were analyzed directly by thin layer chromatography without prior concentration. If fluorescence was weak, the samples were concentrated on a steam bath and the residue was dissolved in 15 ml of chloroform. The concentrate, high in fat and oily materials, was purified using a silica gel column according to existing methods (Pons et al., 1966; Eppley, 1966; Anon., 1967). The chromatographic columns were prepared using standard Butt extraction tubes (Corning Glass No. 92195). A small amount of glass wool was placed loosely in the bottom of the tube, and ca. 5 g anhydrous sodium sulfate was placed on top of the glass wool. The column was filled about 2/3 full with chloroform and ca. 10 g silica gel (Baker, chromatography grade) was added to the column with stirring to prevent air entrapment. When the silica gel had settled, ca. 15 g of anhydrous sodium sulfate was placed in a uniform layer on top of the silica gel, and the chloroform was drawn off to the top of the column. The sample extract, in chloroform, was then added to the column.
The column was eluted at a flow rate of 10-20 ml/min with 150 ml of hexane followed by 150 ml of anhydrous diethyl ether. The aflatoxins were eluted with 150 ml of chloroform:methanol (97:3). This fraction was collected from the time that the chloroform:methanol was added until the flow stopped. The purified extract was evaporated to dryness on a steam bath, the residue was dissolved in 3-5 ml of chloroform, and the solution was transferred to a small vial. The chloroform was again evaporated by placing in a hood overnight. The residue was then dissolved in 0.5 ml of chloroform and subjected to TLC analysis.

The extracts were spotted, either directly or after partial purification, on 20 x 20 cm glass TLC plates that were prepared with a 0.25 mm coating of silica gel G-HR and activated by heating at 100° C for 1 hour. The samples were subjected to a preliminary TLC analysis in which the approximate concentration was determined. Known amounts of sample were then spotted 2.5 cm from the bottom of the TLC plates using Hamilton microliter syringes. Quantitative aflatoxin standards were spotted on the TLC plates in the same manner. The usual levels of aflatoxin B₁ and G₁ standards used were: 0.00125, 0.0025, 0.005, 0.010, 0.015, 0.025, 0.035, 0.045 and 0.055 µg. The plates were developed in benzene:ethanol:water (46:35:19) upper phase, using a solvent path length of 14 cm. Since aflatoxins B₁ and G₁
always predominated in culture extracts, whereas $B_2$ and $G_2$ were present in very small to trace amounts, and sometimes not at all, only the amounts of $B_1$ and $G_1$ were determined. The intensities of the $B_1$ and $G_1$ spots of the sample were compared with those of the standards. The aflatoxin levels were then calculated in a manner similar to the A.O.A.C. method (Anon., 1966), taking into account the dilution or concentration of the sample. Each sample was subjected to two TLC determinations, and each determination was compared to aflatoxin standards on two different occasions. Since two replicates were always employed and averaged, the final aflatoxin level was reported as the average of eight separate visual estimations of the amount of aflatoxins present. This was done to control, as much as possible, the error connected with the visual method of estimating toxin levels.

To determine if aflatoxins could be extracted and recovered from meats using the above described methods, several levels of aflatoxins were added to different meats, the meats were held for 1 or 24 hours to permit distribution of the aflatoxins through the product, and then attempts were made to recover the added aflatoxins.

Aflatoxins used for addition to the meats were produced by \textit{Aspergillus parasiticus} Speare C.M.I. 15957 (Commonwealth Mycological Institute Culture Collection, Surrey, England) grown on polished rice at 25-30\textdegree C. The toxins were ex-
tracted from the culture with chloroform and the extract quantitated by comparison with aflatoxin standards on TLC plates as previously described. Quantitation was also achieved by calculation using the optical density reading at 363 μm and the extinction coefficient for this wavelength according to the method of Nabney and Nesbitt (1965). These results were comparable, and were averaged to determine the concentration.

The meats used in the study were ham, bacon, smoked salami and beef. One hundred-gram portions of the meats were placed in pint mason jars and the toxins, in 350 μl of chloroform, were added. Four levels of aflatoxin B₁, 0.25, 2.5, 25 and 250 μg, were studied; these levels were equivalent to 2.5, 25, 250 and 2500 ppb, respectively. The corresponding amounts of G₁ were 4.0, 40, 400 and 4000 ppb. The chloroform was removed by gentle warming. Duplicate samples were prepared; one sample was extracted within 1 hour after addition of the aflatoxins, and the other was stored for 24 hours prior to extraction. The extraction was then performed as previously described. The experiment was replicated twice.
Production of Aflatoxin on Cured Meats in Storage

This study was designed to determine if known aflatoxin-producing molds could produce aflatoxin on meats under storage conditions. The effects of type of meat, mold strain, storage temperature, time of storage, number of mold spores present, and light were investigated.

Three types of meat were studied, beef, smoked ham and smoked bacon. The use of beef was intended to simulate beef undergoing aging. To keep bacterial contamination at a minimum, the beef was obtained as one large piece (beef round, ca. 4.5 kg) from a local Ames store and then ground with the coarse cutting blade of a sterilized grinder. Antibiotics were added to the meat at the time of grinding to further prevent bacterial overgrowth. The antibiotics and their concentrations used were as follows: chlorotetacycline, 15 μg/g of meat; polymyxin B, 20 units/g of meat; penicillin G, 0.5 units/g meat. The meat-antibiotics mixture was allowed to equilibrate overnight at 2-3°C and then was dispensed in 100-g portions into sterile pint Mason jars. The jars were closed with ordinary Mason lid assemblies except that two sheets of Whatman No. 1 filter paper (diameter 7.0 cm) were used in place of the regular flat portion of the Mason lid. The modified Mason lid assembly was sterilized on the jar by autoclaving at 121°C for 15 min
prior to the addition of the meat.

The ham used in this study was freshly produced Hormel "Cure 81" boneless smoked ham obtained directly from the Geo. A. Hormel Company, Fort Dodge, Iowa. The ham was cut into small pieces (ca. 1-2 cm cubes) using a sterilized knife dipped into alcohol and flamed to reduce bacterial contamination as much as possible. Other measures that were taken to keep contamination at a minimum were the wearing of sterilized disposable examination gloves when handling the meat, and use of sterilized aluminum foil to lay the meat on while cutting. No antibiotics were added to the ham because the curing salts used in the production of the ham provided enough bacteriostatic action, under these conditions, to allow mold growth. One hundred-gram portions of diced ham were dispensed into sterile pint Mason jars, and the jars closed as described above.

The bacon used in this study was Hormel thick-sliced bacon obtained from a local Ames store. The bacon was cut across the slice to make slices of about 6 cm lengths. Enough of these slices were used to make a sample size of 100 g. The samples were placed in sterile pint Mason jars in the same manner as with ham, using the same precautions to prevent contamination.

The toxic mold strains used were *A. parasiticus* Speare C.M.I. 15597; *A. flavus* Link NRRL 2999 (Northern Regional
and A. flavus M 262 (Iowa State University, Department of Dairy and Food Industry, Culture Collection number), isolated from an American-produced Italian type salame. The latter organism was deposited in the Northern Regional Research Laboratory Culture Collection under the accession number NRRL A16,100. The NRRL number will be used in all remaining references to this organism.

Four storage temperatures were employed, 10, 15, 20 and 30°C. The time of storage was determined by the storage temperature. Two storage times were used for each temperature except 30°C where one time was used. The times used were as follows:

- 10°C: 28 and 56 days
- 15°C: 14 and 28 days
- 20°C: 7 and 14 days
- 30°C: 7 days

Conidia used for inocula were produced by growing the individual molds at 25-30°C for 7-10 days on thin layers of potato dextrose agar in Roux bottles. Aspergillus flavus NRRL A16,100 did not sporulate readily in the Roux bottles; sporulation was increased by continuous flushing of the culture with sterile moistened air. The conidia were harvested by adding 150 ml of a sterile 0.05% Tween 80 (Mann Research Laboratories, Inc., New York, N.Y.) solution to each of the Roux bottle cultures. The cultures were
allowed to stand a few minutes to permit wetting of the spores. The wetted spores were loosened by "teasing" with a sterile bent glass rod, and sterile glass beads. The spore suspension was collected, concentrated by centrifugation and washed with additional sterile Tween 80 solution. The spores were then suspended in 100 ml of sterile 0.05% Tween 80 solution and the number of spores per milliliter determined by direct microscopic count, using a Petroff-Hauser bacterial counting chamber. This determination was made by counting the spores contained within 100 small squares of the chamber grid using the 45X objective of a light microscope and applying the following formula:

\[
\text{Spores/ml} = \frac{\text{Total Number of Spores counted} \times 20,000,000}{\text{Number of squares counted}}
\]

Portions of each spore suspension were then diluted with sterile 0.005% Tween 80 solution to obtain levels of \(10^2\) and \(10^6\) conidia/ml, respectively; the meat samples were inoculated using 1 ml of inoculum to obtain spore levels of \(10^2\) and \(10^6\) conidia/100 g of meat. The experiment was constructed so that each meat sample received all of the treatments; there were two replicates of each treatment.

The organisms used on meats were also inoculated into flasks of sterile rice, in the manner described above, using \(10^2\) and \(10^6\) spores per flask respectively. The flasks were incubated at each of the temperatures and longest times used with
meats.

Because of color changes of the cured meat pigments when exposed to light, cured ham is frequently protected from light during storage. Since aflatoxin might also be protected by the exclusion of light, especially the ultraviolet region, the effect of light on the production of aflatoxin on ham was investigated. The ham was prepared as previously described, and inoculated with $10^6$ spores/100 g of meat. Each organism was tested at storage temperatures of 10, 15 and 20° C. Storage times used for each temperature were, as follows: 10° C, 56 days; 15° C, 28 days; 20° C, 14 days. The samples were stored in the presence of fluorescent light and in closed boxes in the absence of light. The samples were done in duplicate, and the experiment was replicated twice.

After the proper storage time had elapsed, all samples were placed in a freezer at -18° C until they could be analyzed. Mold growth was estimated visually using the following scale:

- no visible growth
+ scant growth (0-25% covered)
++ moderate growth (25-50% covered)
+++ abundant growth (50-75% covered)
++++ profuse growth (75-100% covered)

Since the growth of *Aspergillus* species was readily distinguishable by characteristic color and appearance, distinction was made between the growth of the aspergilli and that of
other fungi. The samples were extracted and aflatoxin levels determined in the manner described in the section "Extraction and Assay of Aflatoxins from Meats".

Production of Aflatoxin on Aged Salami

In this study the factors affecting aflatoxin production on European type salami undergoing aging were studied. Since 

A. flavus NRRL A16,100 was originally isolated from an Italian type salame, this organism was studied for its aflatoxin-producing potential on aging salami.

Two basic types of salami, with three different treatments were studied. The types of salami studied were:

Italian type, not smoked; Hungarian type, inoculated before smoking; and Hungarian type, inoculated after smoking. The salami were made according to Ayres et al., (1967). The same formula was used for both Italian and Hungarian type salami: 8 kg lean pork, 2 kg fresh side pork, 5 kg lean beef, 350 g sodium chloride, 75 g ground white pepper, 22 g garlic powder, 140 g sucrose, 28 g sodium nitrate, 3 g sodium nitrite and 10 g monosodium glutamate. The meat was ground with the fine cutting blade of a sterilized grinder. The salts and spices were evenly mixed with the meat and the mixture was kept at 2-3° C overnight before stuffing into 4 cm diameter natural beef casings. The stuffed casing was
tied off into ca. 8 cm lengths to give miniature salami weighing about 100 g each.

The inoculum levels used were $10^2$ or $10^6$ spores per salame. The salami were inoculated by dipping an individual salame for 30 seconds in a spore suspension containing the desired number of spores/ml. The salame was removed from the inoculum and allowed to drain until no more liquid dripped from its surface. The liquid that drained from the salame was returned to the inoculum. When the salami were inoculated in the above fashion, it was found that the spore suspensions used for inoculum lost about 1 g of weight per salami dipped. The spore inocula ($10^2$ or $10^6$ spores/ml) was based on the assumption that each dipped and drained salame had absorbed an average of 1 ml of inoculum, thus depositing the approximate desired number of spores on the salame. One-half of the Hungarian type salami were inoculated before smoking and the other half after smoking. In both cases the salami were smoked for 1 hour a day for 8 days at a temperature that never exceeded 32° C.

Temperature and humidity were controlled during aging of the salami. The temperatures used were 10, 15 and 20° C. Humidities were controlled at 65-70, 75-80 and 85-90%. Each inoculated salame was hung in a sterile quart Mason jar equipped with a Mason lid. The Mason lid was modified by cutting, off center, a 1.5 cm hole to accommodate a No. 1, one-
hole rubber stopper fitted with a 5 cm length of glass tubing, plugged with cotton. A small wire hook was attached to the center of the lid, and extended into the jar on which the salame was hung.

A 150-200 ml portion of a saturated salt solution was placed in the bottom of each jar. The type of salt solution used was selected to give the desired humidity at the temperatures used (Spencer, 1926, O'Brien, 1948). The humidities obtained from various saturated salt solutions at the temperature used are shown in Table 2. As the salami aged and lost moisture, the salt solutions became less saturated; this was compensated for by adding excess salt to the solution at the beginning of the experiment to maintain saturation. The excess salt was maintained by adding additional salt, while the experiment was in progress if it appeared that saturation was diminishing. The cotton-plugged glass tubing allowed sufficient gas exchange so the atmosphere within the jar was aerobic. It was assumed that this arrangement would not permit such a large exchange of air that the humidity of the environment within each jar would be altered excessively. At the time that the experiment was done, atmospheric humidity was very low, and it was thought that this would help prevent large humidity fluctuations in the jars.
Table 2. Salt solutions used to obtain humidities within the desired humidity range for each temperature at which salami were aged

<table>
<thead>
<tr>
<th>Desired Humidity Range (%)</th>
<th>Actual Humidity (%)</th>
<th>Temperature (°C)</th>
<th>Saturated Salt Solution Used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>65-70</td>
<td>68.0</td>
<td>10°</td>
<td>NH₄NO₃</td>
<td>Spencer, 1926</td>
</tr>
<tr>
<td></td>
<td>68.0</td>
<td>15°</td>
<td>NH₄NO₃</td>
<td>Spencer, 1926</td>
</tr>
<tr>
<td></td>
<td>67.0</td>
<td>20°</td>
<td>NH₄NO₃</td>
<td>O'Brien, 1948</td>
</tr>
<tr>
<td>75-80</td>
<td>76.3</td>
<td>10°</td>
<td>NaCl</td>
<td>O'Brien, 1948</td>
</tr>
<tr>
<td></td>
<td>77.2</td>
<td>15°</td>
<td>NaNO₃</td>
<td>O'Brien, 1948</td>
</tr>
<tr>
<td></td>
<td>75.8</td>
<td>20°</td>
<td>NaCl</td>
<td>O'Brien, 1948</td>
</tr>
<tr>
<td>85-90</td>
<td>86.0</td>
<td>10°</td>
<td>KBr</td>
<td>O'Brien, 1948</td>
</tr>
<tr>
<td></td>
<td>86.7</td>
<td>15°</td>
<td>KCl</td>
<td>O'Brien, 1948</td>
</tr>
<tr>
<td></td>
<td>86.3</td>
<td>20°</td>
<td>KCl</td>
<td>O'Brien, 1948</td>
</tr>
</tbody>
</table>

The salami were aged for 8 weeks, at which time they were stored in a freezer at -18° C until they could be analyzed for aflatoxins. Another group of salami inoculated with 10⁶ spores/salami and aged at 75-80% humidity at each temperature, were frozen each week for the first 4 weeks until aflatoxin analyses could be performed. This was done to determine if aflatoxin levels were higher in the early stages of aging, at a humidity considered to be normal for a commercially produced product, than at the end of the aging period.

The amount of growth of the *Aspergillus* spp. and other fungi were estimated visually as previously described. Random loopfuls of *Aspergillus*-like growth were streaked on
potato dextrose agar slants. These cultures were grown and examined for (1) characteristics typical of *A. flavus* NRRL A16,100 and (2) ability to produce aflatoxins on rice. The salami were extracted and assayed for aflatoxins using the method described in the section, "Extraction and Assay of Aflatoxins from Meats". The results of these experiments are expressed as the average of two replicates.

The effects of the salami ingredients on growth and aflatoxin production by *A. flavus* NRRL A16,100 were evaluated. Glucose-ammonium-nitrate (GAN) broth described by Brian et al. (1961), a chemically defined medium which supports aflatoxin production, was used to study the effects of the curing salts and spices on growth and toxin production.

GAN broth was prepared by dissolving the following ingredients in 100 ml of distilled water: 50 g glucose; 2.4 g NH$_4$NO$_3$; 10 g KH$_2$PO$_4$; 2 g MgSO$_4$·7H$_2$O; 1.3 ml of a solution consisting of 20 g ZnSO$_4$·7H$_2$O, 2 g CuSO$_4$·5H$_2$O and 1 g Co(NO$_3$)$_2$·6H$_2$O per liter; 1.3 ml of a solution of 50 g CaCl$_2$/liter. The GAN broth was dispensed in 50-ml quantities into 250 ml Erlenmeyer flasks and autoclaved for 10 min at 121° C. Individual curing salts and spices were added to the hot broth in the same percentages as used in making the salami. Other flasks contained GAN broth only and GAN broth plus all of the salts and spices in the same percentages as used individually. The flasks used in this study contained GAN broth plus the
following percentages of spices: none; 2.2% NaCl; 0.48% ground white pepper; 0.14% garlic powder; 0.9% sucrose; 0.17% NaNO₃ 0.02% NaNO₂; 0.07% monosodium glutamate; and all of the above ingredients in the percentages given. A mixture of 2/3 pork to 1/3 beef, with and without the combined curing ingredients, and casings alone were also studied. The samples were inoculated with loopfuls of spores and sclerotia of A. flavus NRRL A16,100 and were incubated at 25° C. for 7 days. The meats were extracted as previously described. The GAN broth was filtered through Whatman No 2v filter paper, by gravity filtration; and the mycelial mats were collected, dried and weighed. The filtered GAN broth for each culture was extracted with two 50-ml portions of chloroform in a separatory funnel, and the extracts were combined. The extracts were concentrated and analyzed by TLC as described above. The results are reported as the average of two replicates.

Production of Aflatoxin on Country Cured Hams During Aging

Portions of country-cured hams which had been experimentally produced, and aged to 3, 6, 9 and 12 months were available (Lillard and Ayres, 1968). In the study by Lillard and Ayres the hams were experimentally prepared as follows: thirty-two fresh hams, weighing about 9 kg each, were treated
with 2.3 kg of sterile sodium chloride per 45.4 kg of meat. The salt was rubbed into the hams. One group of 8 hams was uninoculated; this was the control group. A second group of 8 hams was inoculated with strains of *P. expansum*, *P. chrysogenum* and *P. veridicatum*. A third group of 8 hams was inoculated with strains of *A. ruber* and *A. repens*. A fourth group of 8 hams was inoculated with all of the above organisms. All of the hams were wrapped in white butcher paper and placed in sterile knit bags. The 32 hams were kept at 0°C for one night and then removed to a humidity room, hung shank down and aged under controlled temperature and humidity conditions for various periods of time (3, 6, 9 and 12 months). Two hams from each group were removed for various analyses at three month intervals during the curing time of one year. The hams were sampled for these analyses such that a shank and a butt portion, each weighing about 2-4 kg, was available from each ham. This resulted in the availability of 64 pieces of country cured ham that had been aged for different periods of time. Each inoculation treatment was represented at each age level. The temperature was varied with each time of aging so that from 0-3 months the hams were kept at 10°C, from 3-6 months they were kept at 20°C and from 6-9 and 9-12 months they were kept at 30°C. These temperatures were selected to simulate the environmental temperature fluctuations which occur during commercial aging.
Four pieces of ham were available from each inoculation treatment within each aging time. The 4 pieces of ham representing these treatments were inoculated with toxigenic mold strains. Three pieces of ham were inoculated with $10^6$ spores of either A. flavus NRRL 2999, A. flavus NRRL A16,100, or A. parasiticus CMI 15957. The fourth piece of ham in each group was kept as an uninoculated control. The ham pieces were wrapped in white butcher paper and the aging resumed at the temperatures and times indicated below:

- 3 month hams at 10° C for 6 weeks
- 6 month hams at 20° C for 4 weeks
- 9 and 12 month hams at 30° C for 2 weeks

After the proper time had elapsed the hams were frozen and stored at -18° C until analyzed for aflatoxin content.

The outer surface of the hams was sampled by removing the outer 0.5-1 cm layer of 100 g of the lean portion of each ham piece. This was the portion which most readily supported mold growth. These portions of meat were cut into small (ca. 1 cm cube) pieces and extracted and analyzed for aflatoxins in the manner previously described.
RESULTS AND DISCUSSION

Screening of Mold Isolates for Aflatoxin Production

Of the mold strains isolated from meats, only *A. flavus* M 262 (NRRL A16,100) was shown to produce chloroform-extractable, fluorescent compounds which coincided with aflatoxin standards on TLC plates (Table 3). This organism was isolated, in our laboratory, by Dr. John C. Ayres from an Italian type salame. It was identified by Dr. Lothar Leistner (present address: Institut für Bakteriologie und Histologie, Bundesanstalt für Fleischforschung, Kulmbach, Germany); the identification was confirmed by Dr. Lois Tiffany, Department of Botany, Iowa State University, Ames. The organism does not sporulate readily under ordinary laboratory conditions, but rather forms numerous macroscopic black sclerotia. It was observed that sporulation was more profuse at the tops of agar slants and at the edges rather

Table 3. Molds isolated from cured and aged meats which were examined for the production of aflatoxin

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number of Strains Screened</th>
<th>Number Fluorescing in Culture</th>
<th>Number that Produced Aflatoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. ruber</em></td>
<td>53</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td><em>A. wentii</em></td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aspergillus species</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P. frequentans</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>P. variable</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P. puberulum</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
than near the center of petri plates; when slants or plates were continuously flushed with sterile, moistened air, sporulation was improved.

The fluorescent, aflatoxin-like compounds in chloroform extracts of *A. flavus* NRRL A16,100 were compared to aflatoxin standards using TLC and 8 different developing solvents. The $R_F$ values for these compounds corresponded to the $R_F$ values of the aflatoxin standards (Table 4). Photographs of TLC plates containing the suspect and standard aflatoxins, developed in the 8 different solvents, are shown in Figure 2.

The ultraviolet and infrared absorption spectra of the sample aflatoxins $B_1$ and $G_1$ were the same as those of authentic aflatoxins $B_1$ and $G_1$ (Figures 3, 4 and 5). Both ultraviolet and infrared absorption maxima of the compounds were the same as reported in the literature for aflatoxins (Asao et al., 1963; Asao et al., 1965).

Biological toxicity data obtained from four-day-old chicken embryos indicated that the fraction corresponding to $B_1$ killed the embryos at the highest dilution (0.02 µg per embryo) within three days after administration via the air sac. This value is very close to the $LD_{50}$ value of 0.025 µg/egg, for aflatoxin $B_1$ reported by Verrett et al. (1964). When the sample aflatoxin $B_1$ was administered to one-day-old ducklings via stomach tube, histopathological examination of bile duct cells of treated animals, sacrificed 7 days after
administration of the toxin, revealed bile duct cell proliferation characteristic of authentic aflatoxin B₁ (Wogan, 1965).

These data show that this strain of *A. flavus* does indeed produce aflatoxins, and by virtue of the fact that it was found growing on a meat substrate suggests that a potential health hazard exists. On the other hand, it was not possible to demonstrate the production of aflatoxin by selected strains of *A. ruber*, *A. niger*, *A. wentii*, *P. frequentans*, *P. variable* and *P. puberulum*. While it has been suggested that these organisms might produce aflatoxins (Kulik and Holaday, 1966 and Hodges et al., 1964) this work has not yet been verified. Recently, Wilson et al. (1968), studied 121 fungal isolates representing 29 species, including 8 isolates already reported by other investigators to produce aflatoxins, and found that only *A. flavus* and *A. parasiticus* strains produced aflatoxins. Likewise, Parrish et al. (1966), found that no aflatoxins were produced by 45 strains of *Aspergillus* (which were not members of the *A. flavus* group) and 10 strains of *Penicillium*. The findings presented here support those of Wilson et al. (1968) and Parrish et al. (1966) that fungi from other than the *A. flavus* group are not likely to produce aflatoxins. However, owing to the extreme toxicity of the aflatoxins and the very versatile metabolic nature of the fungi, it may be regarded as still questionable
Table 4. Comparison of Rp values of aflatoxin standards with the Rp values of aflatoxin-like compounds isolated from rice cultures of A. flavus NRRL A16,100 using 8 different solvent systems

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Rp Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aflatoxin standards</td>
</tr>
<tr>
<td></td>
<td>B₁  B₂ G₁ G₂</td>
</tr>
<tr>
<td>Benzene:ethanol:water (46:35:19) upper phase</td>
<td>.47 .41 .35 .28</td>
</tr>
<tr>
<td>Chloroform:methanol:acetic acid (96:3:1)</td>
<td>.46 .42 .36 N.S.ᵃ</td>
</tr>
<tr>
<td>Chloroform:methanol:acetic acid (92:7:1)</td>
<td>.71 N.S. .60 N.S.</td>
</tr>
<tr>
<td>Benzene:methanol:acetic acid (100:10:10)</td>
<td>.54 .48 .42 .37</td>
</tr>
<tr>
<td>Benzene:acetone:acetic acid (80:9:10)</td>
<td>.16 .12 .11 .06</td>
</tr>
</tbody>
</table>

ᵃNot separated.
Figure 2. Comparison of aflatoxin-like compounds isolated from *A. flavus* NRRL A16,100 to aflatoxin standards using 8 different TLC solvent systems

Solvent system key:

1. Chloroform:acetone (9:1)
2. Benzene:methanol:acetic acid (24:2:1)
4. Chloroform:methanol:acetic acid (96:3:1)
5. Chloroform:methanol:acetic acid (92:7:1)
6. Benzene:methanol:acetic acid (100:10:10)
7. Benzene:acetone:acetic acid (80:9:10)
8. Acetone:chloroform/ethyl ether (10:90)
   (chloroform/ethyl ether 30:10)

Standard and sample arrangement, from left to right, was the same on each plate, and was as follows: aflatoxin standard, aflatoxin extract from A16,100, and an internal standard (aflatoxin standard superimposed on the extract from A16,100)
Figure 3. Ultraviolet absorption spectra of aflatoxins B₁ and G₁ isolated from A. flavus NRRL A16,100 compared to ultraviolet absorption spectra of standard aflatoxins B₁ and G₁.
Aflatoxins from \textit{A. flavus} NRRL A16,100

Standard aflatoxins

Absorbance

Wavelength (m\textmu)
Figure 4. Infrared absorption spectra of aflatoxin B₁ from *A. flavus* NRRL A16,100 (A) and standard aflatoxin B₁ (B)
Figure 5. Infrared absorption spectra of aflatoxins $G_1$ from *A. flavus* NRRL A16,100 (A) and standard aflatoxin $G_1$ (B)
whether some strains of these organisms (other than *A. flavus* and *A. parasiticus*) might have the potential to produce aflatoxins, and that further study of additional isolates is needed.

**Extraction and Assay of Aflatoxins from Meats**

The chloroform extracts of meats were cloudy prior to filtration. After filtration, however, a clear extract was obtained. Chloroform recovery was about 75-85%. Intense fluorescence was observed in the extracts of the samples treated with either 25 or 250 µg of aflatoxin B₁, and it was found that extracts from meats containing as little as 25 µg/100 g could be analyzed directly on TLC plates without prior concentration. Lipids and other interfering materials were not a problem in the unconcentrated extracts.

Aflatoxins were recovered from all of the treated samples. In general, the recoveries were consistent with the levels added to the meats. The actual recovery data are shown in Table 5. In most cases, variations in recovery were within the limits of error of the visual TLC estimation method, which, as already mentioned, may be as high as 20%. Much of the error may be due to the visual method of estimation of the toxin concentration rather than to errors in the recovery process. Aflatoxins were recovered equally well
Table 5. Micrograms of aflatoxins recovered from 100 gram samples of meats which were treated with four levels of aflatoxins

<table>
<thead>
<tr>
<th>Time of treatment</th>
<th>ug of aflatoxin added/100 g meat</th>
<th>ug aflatoxin recovered/100 g meat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B₁</td>
<td>G₁</td>
</tr>
<tr>
<td>Extracted</td>
<td>250</td>
<td>400</td>
</tr>
<tr>
<td>1 hr after</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>addition</td>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>of toxins</td>
<td>0.25</td>
<td>0.4</td>
</tr>
<tr>
<td>24 hr after</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>addition</td>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>of toxins</td>
<td>0.25</td>
<td>0.4</td>
</tr>
</tbody>
</table>
from samples extracted 1 hour after the addition of the toxins and after the toxins were in contact with the meat for 24 hours, suggesting that the aflatoxins were not irreversibly bound to protein or other materials in the meats. Recoveries were equally satisfactory from each of the meats tested.

This method was found to be a simple and rapid procedure for the extraction and analysis of aflatoxins from meats. It worked equally well for samples containing high levels of aflatoxins (2500 ppb) and samples containing very small amounts of aflatoxin (2.5 ppb). While it may be possible to detect levels of aflatoxins lower than 2.5 ppb with this method it appears that this level is a practical lower limit for the detection and quantitation of aflatoxin B₁ in meats. There did not appear to be any danger of misjudging a low or intermediate toxin level with a higher level using this method. No problems were encountered with interfering fluorescent materials; however, lipids, mainly fats and oils, were a problem when samples had to be concentrated. These were effectively removed with the silica gel column, to yield relatively clean extracts suitable for thin layer chromatographic analysis. Chloroform extracts of meat samples which were not treated with aflatoxins did not yield fluorescent spots on thin layer chromatograms which could be confused with the aflatoxins.
Production of Aflatoxin on Cured Meats in Storage

Of all of the factors studied in relation to the production of aflatoxins on meats, temperature had the most pronounced effect on both toxin production and growth of the organisms. Greatest levels of aflatoxin were obtained at 20°C with meats stored for 14 days. The largest amount of aflatoxin (630 μg/g) was produced at this temperature on beef by A. parasiticus CMI 15957. The lowest amount of aflatoxin detected (0.1 μg/kg) was produced at 15°C on bacon by A. flavus NRRL 2999. The 630 μg/g level of aflatoxin consisted of mainly G1, whereas with the lower level, B1 and G1 were present in equal amounts.

No aflatoxins were detected in any of the meat samples stored at 10°C for either of the storage periods of 28 or 56 days. However when sterilized rice inoculated with the organisms used in this study was incubated at each temperature and longest time used to study aflatoxin production on meats, 6 μg/g each of aflatoxins B1 and G1 were detected in cultures of A. flavus NRRL 2999 incubated at 10°C for 56 days (Table 6). Schroeder and Hein (1967) detected small amounts of aflatoxin in rice cultures of A. flavus incubated at 10°C for 24 to 48 hours. However, they stated that during their entire experiment the levels never reached practical significance. The small amounts of
Table 6. Effect of temperature on aflatoxin production (μg/g) on rice by *A. flavus* NRRL 2,999, *A. flavus* NRRL A16,100 and *A. parasiticus* CMI 15,957 at two levels of inocula and varying incubation times

<table>
<thead>
<tr>
<th>Mold strain</th>
<th>Inoculum per flask (conidia/50 g)</th>
<th>Temperature and time of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^6$ C (56 days) $15^\circ$ C (28 days) $20^\circ$ C (14 days) $30^\circ$ C (7 days)</td>
</tr>
<tr>
<td>2,999</td>
<td>$10^2$</td>
<td>B$_1$ G$_1$ B$_1$ G$_1$ B$_1$ G$_1$</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>N.D. 175 300 125 300 400 250</td>
</tr>
<tr>
<td>A16,100</td>
<td>$10^2$</td>
<td>N.D. N.D. 800 500 400 600 50 50</td>
</tr>
<tr>
<td>15,957</td>
<td>$10^2$</td>
<td>N.D. N.D. 12 185 50 180 70 70</td>
</tr>
<tr>
<td>2,999</td>
<td>$10^6$</td>
<td>6 6 125 300 150 300 500 150</td>
</tr>
<tr>
<td>A16,100</td>
<td>$10^6$</td>
<td>N.D. N.D. 800 600 400 600 6 6</td>
</tr>
<tr>
<td>15,957</td>
<td>$10^6$</td>
<td>N.D. N.D. 12 250 17 70 12 4</td>
</tr>
</tbody>
</table>

* N.D. - none detected.
toxin detected could well have been due to toxin carry-over in the inoculum, since large amounts of concentrated spore suspensions were used for inocula. Sorenson et al., (1967), detected levels of aflatoxin B₁ of less than 0.01 μg/g in rice cultures of A. flavus NRRL 2999 incubated at 8 and 11°C for 21 and 14 days, respectively. When the cultures were incubated at 11°C for 21 days, 0.1 μg/g of aflatoxin B₁ was detected by these workers. Here again the very low levels of aflatoxin could have been the result of aflatoxin carry-over in the inoculum. Another very important consideration is the fact that in both of the above cases the work was done in pure culture, as was the work with rice cultures used in this study, whereas the meats used in this study were not sterile. While an attempt was made to keep other microbial competition as low as possible, with the long incubations required for the growth of A. flavus and A. parasiticus at 10°C, considerable competitive bacterial and yeast growth was observed on all of the meats studied. While A. flavus and A. parasiticus may have been able to grow at 10°C in pure cultures incubated for long periods of time they were at a considerable disadvantage when incubated in mixed culture at this temperature. In fact, even when precautions were taken (such as use of large inocula and addition of antibiotics and curing salts) to retard microbial competition as long as possible, and give A. flavus and A. parasiticus
the competitive advantage, bacteria and yeasts tended to over-grow the toxic molds. Also, with mixed cultures, the possibility of microbial detoxification of any small amounts of aflatoxins that might have been produced at this temperature cannot be discounted. These data indicate that meats stored at 10° C, or less, would become spoiled by the growth of bacteria and yeasts before they would become toxic as the result of A. flavus or A. parasiticus growth.

Aflatoxins were produced at 15° C on all of the meats studied (Table 7). The highest levels of total aflatoxins detected (41 µg/g) at 15° C were found in beef inoculated with $10^6$ spores of A. parasiticus and stored for 28 days. Low levels of aflatoxins (0.1-0.38 µg/kg) were produced by all three organisms on ham and bacon stored for 14 days and inoculated with either $10^2$ or $10^6$ conidia/100 grams of meat. No aflatoxins were detected in beef inoculated with $10^2$ spores of A. flavus and incubated for 14 days, whereas under these same conditions A. parasiticus produced small amounts (0.05 µg/g) of both aflatoxins $B_1$ and $G_1$, even though there was no visible growth of the organism. When the beef samples were inoculated with $10^2$ spores and stored for 28 days, neither A. flavus or A. parasiticus produced detectable amounts of aflatoxins.

The high levels of aflatoxin produced by A. parasiticus consisted primarily of aflatoxin $G_1$ (Table 7). Temperature
Table 7. Production of aflatoxins (µg/g) on meats stored at 15° C for 14 and 28 days by *A. flavus* NRRL 2,999, *A. flavus* NRRL A16,100 and *A. parasiticus* CMI 15,957 at two inoculation levels (10^2 and 10^6 conidia/100 g of meat)

<table>
<thead>
<tr>
<th>Type of meat</th>
<th>Days of meat storage</th>
<th><em>A. flavus</em> NRRL 2,999</th>
<th><em>A. flavus</em> NRRL A16,100</th>
<th><em>A. parasiticus</em> CMI 15,957</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
<td>B1         G1   B1    G1</td>
<td>B1         G1   B1    G1</td>
<td>B1         G1   B1    G1</td>
</tr>
<tr>
<td>Beef</td>
<td></td>
<td>N.D.       N.D.  0.6  3.4</td>
<td>N.D.       N.D.  1.2  3.6</td>
<td>0.05        0.05  3.1  25</td>
</tr>
<tr>
<td>Ham</td>
<td>0.05</td>
<td>0.05       1.4   2.8  0.06  0.2</td>
<td>2.0         2.4   0.13  0.15</td>
<td>4.5  11</td>
</tr>
<tr>
<td>Bacon</td>
<td>0.06</td>
<td>0.06       0.08  0.14</td>
<td>0.12  0.24  0.3  0.2</td>
<td>0.18  0.12  0.8  1.0</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>N.D.       N.D.  1.8  9.0</td>
<td>N.D.       N.D.  0.8  3.8</td>
<td>N.D.       N.D.  3.8  38</td>
</tr>
<tr>
<td>Ham</td>
<td>2.0</td>
<td>4.5        6.7   11   9   12</td>
<td>19          20   3.2   12</td>
<td>9.0  21</td>
</tr>
<tr>
<td>Bacon</td>
<td>0.06</td>
<td>0.06       0.4   0.6  0.7   0.2</td>
<td>13          4    1.0  2.2</td>
<td>1.8  5.4</td>
</tr>
</tbody>
</table>

^a N.D. - none detected.

^b µg/kg.
affects the ratio of $B_1$ to $G_1$ produced; it can be seen that
the *A. flavus* strains, in almost all instances, also produced
more aflatoxin $G_1$ than $B_1$ at 15° C (Table 7). That the
accumulation of aflatoxin $G_1$ over $B_1$ is favored by lower
incubation temperatures has been reported by several in­
vestigators (Schindler *et al.*, 1967; Schroeder and Hein,
1967; Sorenson *et al.*, 1967), and the data presented here
are in agreement with these reports. Also, it can be seen
from these data that the accumulation of $G_1$ over $B_1$ was
greater on the beef substrate than on either ham or bacon.

Although the highest level of aflatoxin production at
15° C was by *A. parasiticus* on beef, the two *A. flavus* strains
produced their highest levels of toxins on ham. *Aspergillus
flavus* NRRL 2,999 produced its lowest level of aflatoxin
on bacon while *A. flavus* NRRL A16,100 produced its lowest
level of aflatoxin on beef. *Aspergillus parasiticus* produced
equal amounts of aflatoxins on ham and bacon. These effects
can be seen in Figure 6. This figure also gives some idea
of the relative amounts of aflatoxins produced at 15° C
with respect to those amounts produced at higher temperatures
(later figures), since the same scale is used on Figures 6, 8
and 9. The differences between strains and meats at the 15° C
storage temperature are more apparent in Figure 7, which con­
tains the same data as Figure 6.

Growth of the toxic strains at 15° C, as measured visually
Production of aflatoxins by *A. flavus* NRRL 2,999, *A. flavus* NRRL A16,100 and *A. parasiticus* CMI 15,957 on beef, ham and bacon inoculated with 10^6 conidia/100 g of meat and stored for 28 days at 15°C.
Figure 7. Production of aflatoxins by A. flavus NRRL 2,999, A. flavus NRRL A16,100 and A. parasiticus CMI 15,957 on beef, ham and bacon inoculated with $10^6$ conidial/100 g of meat and stored for 28 days at 15° C. This figure shows the same data as Figure 6, but the Y axis has been expanded.
and recorded using a + system, was greatest on ham inoculated with $10^6$ conidia/100 grams of meat and stored for 28 days (Table 8). Bacon supported the least mold growth with the growth being confined to the lean portion. Members of the penicillia were the most prevalent competing organisms on ham and bacon. A species of Rhizopus grew profusely on the beef and covered much of the meat surface. Colonies of Aspergillus spp. could be seen growing, intermixed, with the black mold. Samples stored for 28 days at 15° C supported greater mold growth than did samples stored for 14 days. This was also generally true for aflatoxin production, with the highest levels occurring with the longer storage time. The toxic mold strains were able to compete with the other organisms better when an inoculum of $10^6$ conidia/100 g of meat were used; as a result, toxin levels were higher with the larger inoculum.

All meats stored at 20° C contained aflatoxins (Table 9). Aspergillus parasiticus produced more aflatoxins than the A. flavus strains on every type of meat studied at this temperature. The maximum amounts of aflatoxins produced by A. parasiticus were 630 µg/g of beef, 340 µg/g of bacon and 190 µg/g of ham, when samples were inoculated with $10^6$ conidia/100 g and stored for 14 days. Aspergillus parasiticus CMI 15,957 and A. flavus NRRL A16,100 produced more aflatoxins
Table 8. Growth of *A. flavus* NRRL 2,999, *A. flavus* NRRL A16,100 and *A. parasiticus* CMI 15,957 on beef, ham and bacon stored at 15° C for 14 and 28 days at two inoculum levels (10^2 and 10^6 conidia/100 g of meat).^a^  

<table>
<thead>
<tr>
<th>Mold strain</th>
<th>Inoculum level</th>
<th>14 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beef</td>
<td>Ham</td>
<td>Bacon</td>
</tr>
<tr>
<td>2,999 10^2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A16,100 10^2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15,957 10^2</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2,999 10^6</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>A16,100 10^6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15,957 10^6</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

^a^Growth scale: - no visible growth; + scant growth (0-25% covered); ++ moderate growth (25-50% covered); +++ abundant growth (50-75% covered); ++++ profuse growth (75-100% covered).

on beef and bacon than on ham; *A. flavus* NRRL 2,999 produced more aflatoxins on beef than on ham and bacon (Figure 8).

*Aspergillus parasiticus* produced more aflatoxin *G₁* than *B₁* in every instance. In the majority of the samples, the two *A. flavus* strains also produced more *G₁* than *B₁*. These data are shown in Table 9.

With all of the organisms, and all of the meats the longer
Table 9. Production of aflatoxins (µg/g) by A. flavus NRRL 2,999, A. flavus NRRL A16,100 and A. parasiticus CMI 15,957 on meats stored at 20°C for 7 and 14 days at two inoculum levels (10^2 and 10^6 conidia/100 g of meat)

<table>
<thead>
<tr>
<th>Type of meat</th>
<th>Days of Storage</th>
<th>A. flavus NRRL 2,999</th>
<th>A. flavus NRRL A16,100</th>
<th>A. parasiticus CMI 15,957</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10^2</td>
<td>10^6</td>
<td>10^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B_1  G_1</td>
<td>B_1  G_1</td>
<td>B_1  G_1</td>
</tr>
<tr>
<td>Beef</td>
<td></td>
<td>5.1</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td>Ham</td>
<td></td>
<td>14</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td>Bacon</td>
<td></td>
<td>2.0</td>
<td>1.0</td>
<td>7.4</td>
</tr>
<tr>
<td>Beef</td>
<td></td>
<td>15</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>Ham</td>
<td></td>
<td>32</td>
<td>64</td>
<td>44</td>
</tr>
<tr>
<td>Bacon</td>
<td></td>
<td>11</td>
<td>32</td>
<td>18</td>
</tr>
</tbody>
</table>
Figure 8. Production of aflatoxins by *A. flavus* NRRL 2,999, *A. flavus* NRRL A16,100 and *A. parasiticus* CMI 15,957 on beef, ham and bacon inoculated with $10^6$ conidia/100 g of meat and stored for 14 days at 20°C.
storage time (14 days) resulted in the accumulation of higher levels of aflatoxins than did the shorter storage time (7 days). However, there did not appear to be significantly greater growth at the longer incubation time than at the shorter incubation time (Table 10). Likewise, the larger inoculum usually resulted in higher levels of aflatoxins on all of the meats, than did the smaller inoculum. Also, the $10^6$ conidia resulted in more growth than did the $10^2$ conidia (Table 10).

**Table 10.** Growth of *A. flavus* NRRL 2,999, *A. flavus* NRRL A16,100 and *A. parasiticus* CMI 15,957 on beef, ham and bacon inoculated with $10^2$ and $10^6$ conidia/100 g of meat and stored at 20°C for 7 and 14 days.

<table>
<thead>
<tr>
<th>Mold strain</th>
<th>Inoculum level</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Beef</td>
<td>Ham</td>
</tr>
<tr>
<td>2,999</td>
<td>$10^2$</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>A16,100</td>
<td>$10^2$</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>15,957</td>
<td>$10^2$</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>2,999</td>
<td>$10^6$</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>A16,100</td>
<td>$10^6$</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>15,957</td>
<td>$10^6$</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Growth measuring scale: - no visible growth; + scant growth (0-25% covered); ++ moderate growth (25-50% covered); +++ abundant growth (50-75% covered); ++++ profuse growth (75-100% covered).
At 30° C, abundant to profuse growth of the toxic mold strains occurred in most of the meat samples (Table 11). The growth was equal to or greater than that observed at 20° C, but toxin production was lower than that found at 20° C (Table 12). There were no extremely high amounts of aflatoxins detected at this temperature such as occurred at 20° C with A. parasiticus on beef. However, A. parasiticus

Table 11. Growth of A. flavus NRRL 2,999, A. flavus NRRL A16,100 and A. parasiticus CMI 15,957 on beef, ham and bacon inoculated with 10^2 and 10^6 conidia/100 g of meat and stored at 30° C for 7 days^a

<table>
<thead>
<tr>
<th>Mold Strain</th>
<th>Inoculum Level</th>
<th>Growth at 7 days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Beef</td>
</tr>
<tr>
<td>2,999</td>
<td>10^2</td>
<td>+++</td>
</tr>
<tr>
<td>A16,100</td>
<td>10^2</td>
<td>++</td>
</tr>
<tr>
<td>15,957</td>
<td>10^2</td>
<td>+++</td>
</tr>
<tr>
<td>2,999</td>
<td>10^6</td>
<td>++++</td>
</tr>
<tr>
<td>A16,100</td>
<td>10^6</td>
<td>++++</td>
</tr>
<tr>
<td>15,957</td>
<td>10^6</td>
<td>++++</td>
</tr>
</tbody>
</table>

^aGrowth measuring scale: - no visible growth; + scant growth (0-25% covered); ++ moderate growth (25-50% covered); +++ abundant growth (50-75% covered); ++++ profuse growth (75-100% covered).

produced consistently higher amounts of aflatoxin than either of the A. flavus strains on all of the meats. On bacon, A. parasiticus produced 135 μg/g, which was the largest
Table 12. Production of aflatoxin (µg/g) by A. flavus NRRL 2,999, A. flavus NRRL A16,100 and A. parasiticus CMI 15,957 on meats inoculated with either 10^2 or 10^6 conidia/100 g of meat and stored at 30° C for 7 days

<table>
<thead>
<tr>
<th>Type of Meat</th>
<th>A. flavus NRRL 2,999</th>
<th>A. flavus NRRL A16,100</th>
<th>A. parasiticus CMI 15,957</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B_1</td>
<td>G_1</td>
<td>B_1</td>
</tr>
<tr>
<td>Beef</td>
<td>51</td>
<td>47</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>92</td>
<td>24</td>
</tr>
<tr>
<td>Ham</td>
<td>9.5</td>
<td>10.2</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>Bacon</td>
<td>14</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>34</td>
<td>35</td>
</tr>
</tbody>
</table>
amount of aflatoxin produced on any of the substrates at 30° C, but bacon supported the least amount of toxic mold growth, of the 3 meats studied. *A. flavus* NRRL 2,999 produced more aflatoxins on ham than on any other substrate and *A. flavus* NRRL A16,100 produced the most aflatoxin on beef. This organism (A16,100) grew less, and produced less aflatoxins over all, than did either of the other two strains (Figure 9). Apparently *A. flavus* NRRL Al6,100 is not as well adapted to growth at high temperatures as are the other two aspergilli studied.

The ratio of aflatoxin $B_1$ to $G_1$ was quite different at 30° C than at the lower temperatures. With the *A. flavus* strains, the relative amounts of the two toxins were approximately equal to one another, although with strain Al6,100, $G_1$ tended to predominate slightly on beef and bacon. With strain 2,999 in some cases $B_1$ slightly predominated; in other cases $G_1$ predominated. With *A. parasiticus*, $G_1$ predominated on all meat samples tested, however the degree of predominance of $G_1$ appeared to be less at this temperature than at lower temperatures. These data clearly show the effects of temperature on the ratio of $B_1$ to $G_1$; although less total toxin was detected at 30° C, meat samples stored at this temperature may be more toxic than meat samples stored at lower temperatures because of a higher proportion of aflatoxin $B_1$ in these meats.
Figure 9. Production of aflatoxin by A. flavus NRRL 2,999, A. flavus NRRL A16,100 and A. parasiticus CMI 15,957 on beef, ham and bacon inoculated with $10^6$ conidia/100 g of meat and stored for 7 days at 30°C.
In general, the $10^6$ inoculum level resulted in more mold growth and more aflatoxin than did the $10^2$ inoculum level. When the $10^6$ inoculum level was used, no fungal growth other than the *Aspergillus* spp. could be detected at $30^\circ$ C. When the $10^2$ inoculum level was used, the presence of other fungi was observed although the *Aspergillus* spp. still predominated. The temperature of $30^\circ$ C apparently favored development of the *Aspergillus* strains because even with the $10^2$ level of spores the aspergilli were able to predominate.

The overall effects of temperature on aflatoxin production by the organisms studied in meats can be seen in Figure 10. Of the four temperatures studied, the optimum temperature for all three organisms on all of the meats was $20^\circ$ C. Other workers have found optimum temperatures for aflatoxin production in the range $20-30^\circ$ C depending on cultural conditions (Schroeder and Hein, 1967; Schindler *et al.*, 1967; Sorenson, *et al.*, 1967).

The presence or absence of light had an inconclusive effect on the production of aflatoxin (Table 13). Some differences were noted in most cases, but these were not completely consistent and appear to be due, in part, to experimental error. However, there does appear to be some indication that aflatoxin production was slightly stimulated by light, but the absence of light did not prevent aflatoxin production. The presence of light did not cause reduction of
Figure 10. The effect of temperature on aflatoxin production by *A. flavus* NRRL 2,999, *A. flavus* NRRL A16,100 and *A. parasiticus* CMI 15,957 on beef, ham and bacon at the longest storage period for each temperature. The inoculum level was $10^6$ conidia/100 g of meat.

Symbols:

- X  Beef
- O  Ham
- Δ  Bacon
the toxins to levels lower than were obtained in the absence of light.

Table 13. Production of aflatoxin (µg B₁+G₁/g) by A. flavus NRRL 2,999 A. flavus NRRL A16,100 and A. parasiticus CMI 15,957 on ham stored at 15 and 20°C in the presence and absence of light. The inoculum level was 10⁶ conidia/100 g of meat.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>A. flavus NRRL 2,999</th>
<th>A. flavus NRRL A16,100</th>
<th>A. parasiticus CMI 15,957</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>20°C</td>
<td>6.2</td>
<td>6.0</td>
<td>24</td>
</tr>
<tr>
<td>15°C</td>
<td>2.7</td>
<td>0.2</td>
<td>24</td>
</tr>
</tbody>
</table>

Production of Aflatoxin on Aged Salami

No aflatoxins were detected on any salami aged at 10°C. Trace amounts of aflatoxins were found in Italian type salami aged at 15°C. Somewhat higher amounts of aflatoxins were detected in Italian type salami aged at 20°C (Table 14).

While temperature had a pronounced effect on aflatoxin production in aged salami, the factors of humidity and type of salami produced equally striking effects. Also, there definitely appeared to be interactions between these three factors and their effects on aflatoxin production.

That aflatoxins can be produced at 15°C has already been demonstrated; however, when the humidity was controlled between 65-70% no aflatoxins were produced on salami at 15°C.
Table 14. Production of aflatoxins (μg B₆ + G₆ /g) by A. flavus NRRL A16,100 on salami inoculated with either 10² or 10⁶ conidia/salami and aged for 8 weeks at 15 or 20° C at either of three humidities (65-70, 75-80 or 85-90%)

<table>
<thead>
<tr>
<th>Type of Salami</th>
<th>15° C</th>
<th>20° C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65-70%</td>
<td>75-80%</td>
</tr>
<tr>
<td>Italian</td>
<td>10²</td>
<td>10⁶</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hungarian I</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Hungarian II</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

aN.D. - None detected.

bInoculated after smoking.

cInoculated before smoking. Smoking was done for 1 hr. each day for 8 days. While smoking the temperature was kept at 32° C. Between each smoking period the temperature decreased to 20-25° C.
At 20° C, lower levels of aflatoxins were detected at 65-70% humidity than at 85-90% humidity. The effect of smoking on aflatoxin production was equally striking. No aflatoxins were produced on Hungarian type salami at any temperature or humidity level, when the salami were inoculated before smoking. When salami were inoculated after smoking, only those salami aged at 20° C and at a relative humidity of 85-90% supported aflatoxin production. For all practical purposes, if the humidity is kept at 75% or below, the smoking of Hungarian type salami will prevent A. flavus growth and aflatoxin production during aging. Because of the pronounced effects of temperature, humidity and smoking, the effect of the level of inoculum was somewhat minimized. However at 15° C, only salami inoculated with $10^6$ conidia developed aflatoxin, and at 20° C and 65-70% humidity the amount of aflatoxin produced on Italian type salami was much less when $10^2$ conidia/salame were used for inocula than when $10^6$ conidia/salame were used.

When the effects of aging time were studied, it was found that no aflatoxins were produced during the first 4 weeks of aging on Hungarian type salami inoculated before or after smoking, when aged at any of the temperatures and 75-80% humidity. Also, no aflatoxins were produced during the first 4 weeks of aging on Italian type salami aged at 10 and 15° C. By contrast, with Italian type salami aged at 20° C, the
levels of aflatoxin increased during the first 4 weeks of aging and then declined by 8 weeks of aging (Table 15). These data suggest that while aflatoxins may be present during early stages of aging, the toxin levels will be much lower in the finished product when long aging periods are used than when short aging periods were used. The low levels of aflatoxins in the finished product could be due to degradation of the toxins by A. flavus itself, or other microorganisms or by chemical oxidation.

Table 15. Production of aflatoxins by A. flavus NRRL A16,100 on Italian type salami inoculated with 10^6 conidia/salami and aged for several time periods at 20° C and 75-80% humidity

<table>
<thead>
<tr>
<th>Age of Salami (weeks)</th>
<th>Aflatoxin (B&lt;sub&gt;1&lt;/sub&gt; + G&lt;sub&gt;1&lt;/sub&gt;) µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>2.8</td>
</tr>
<tr>
<td>4</td>
<td>2.4</td>
</tr>
<tr>
<td>8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

While it is significant that aflatoxins were not produced on many of the aged salami under the conditions just described, it is equally significant that the amounts that were formed were very low as compared to the levels previously obtained on the meats. When A. flavus was re-isolated from salami and grown on rice, levels of 330-480 µg of aflatoxin/g of rice were obtained. These are levels normally produced by this
strain on rice; thus, atoxinogenic mutants were not being selected during growth on the salami. It was obvious that the substrate, though permitting growth, reduced aflatoxin production. When the effects of individual salami ingredients on growth and toxin production by *A. flavus* were studied using a defined medium, it was found that NO$_2^-$ and pepper significantly reduce growth and aflatoxin production over the control medium and other ingredients (Table 16). The levels of aflatoxins found in media containing NO$_2^-$ or pepper were of the same order of magnitude as the final levels of aflatoxins found on the aged salami. The complete spice mix containing both NO$_2^-$ and pepper reduced growth and toxin production in the same manner as did the two ingredients alone. The reduced amount of aflatoxin produced in the presence of pepper is in agreement with the results of Frank (1966) who found that *A. flavus* would grow on peppercorns, but that no aflatoxins were produced. Casings did not support growth or toxin production, and the pork and beef mixture alone did not support mold growth because of bacterial over-growth. The salts, NaCl and NaNO$_3$, caused only slight reductions in toxin levels over the control. Garlic powder and sucrose were found to stimulate growth and toxin production, and monosodium glutamate had no effect on toxin production but did seem to cause a slight increase in growth.
Table 16. Effect of salami ingredients on growth and aflatoxin production by *A. flavus* NRRL A16,100

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Mycelial weight (g) or growth (+)</th>
<th>Aflatoxin[^a] (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control[^b]</td>
<td>0.74</td>
<td>38</td>
</tr>
<tr>
<td>Casings</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>Pork + Beef</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Pork + beef + spices</td>
<td>+++</td>
<td>28</td>
</tr>
<tr>
<td>Garlic powder</td>
<td>1.10</td>
<td>87</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.95</td>
<td>55</td>
</tr>
<tr>
<td>Monosodium Glutamate</td>
<td>0.98</td>
<td>32</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.72</td>
<td>20</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0.79</td>
<td>21</td>
</tr>
<tr>
<td>White pepper</td>
<td>0.35</td>
<td>3</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>0.16</td>
<td>0.19</td>
</tr>
<tr>
<td>Complete salt + Spice mix</td>
<td>0.18</td>
<td>0.55</td>
</tr>
</tbody>
</table>

[^a]: Represents B₁+G₁ and consisted of approximately 50% B₁ and 50% G₁.

[^b]: GAN broth only, no salami ingredients.

From these data it would appear that low temperatures, low humidities and smoking prevented growth and aflatoxin production by *A. flavus* on aged salami. However, when growth and aflatoxin production occurred, the amounts of toxins produced were considerably lower than those obtained on other meats at the same temperatures. Reduced growth and toxin production was probably due, in part, to the effects of NO₂⁻ and pepper in the curing mixture. Low toxin levels were also
related to the long aging time employed.

**Production of Aflatoxin on Country Cured Hams During Aging**

Aflatoxin production on country cured hams during aging was inconsistent. No aflatoxins were produced on the 3-month hams aged at 10° C. With the 6-month hams aged at 20° C, only *A. flavus* NRRL 2,999 produced aflatoxins. The levels produced were very low, with an average of 0.01 µg/g.

With 9-month hams incubated at 30° C all three molds produced aflatoxins; *A. parasiticus* produced the greatest amount (Table 17). With 12-month hams aged at 30° C, no aflatoxins were detected.

**Table 17. Production of aflatoxins by A. flavus NRRL 2,999, A. flavus NRRL Al6,100 and A. parasiticus CMI 15,957 on country cured hams inoculated with 10 conidia/ham and aged for various periods of time and various temperatures**

<table>
<thead>
<tr>
<th>Ham age (months)</th>
<th>Temperature (°C)</th>
<th>NRRL 2,999</th>
<th>NRRL Al6,100</th>
<th>CMI 15,957</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>6.3</td>
<td>0.5</td>
<td>27</td>
</tr>
<tr>
<td>12</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

On the basis of previous work one would not expect to obtain aflatoxin production on the 3-month hams aged at 10° C. However, the 6-month hams aged at 20° C would be expected to yield relatively high amounts of the toxins,
especially since toxins were obtained at 30° C with the 9-month hams. One explanation might be that the hams had been frozen for some time prior to this study and unknown changes in composition may have prevented mold growth. Also, other molds already growing on the hams may have had the advantage such that *A. flavus* and *A. parasiticus* could not compete at 20° C, since their optimum growth temperature is somewhat higher (28-30° C). The 12-month hams were fully aged, very dry hams. Even though the temperatures of 30° C may have favored growth of *A. flavus* and *A. parasiticus*, the water activity of the 12 month hams may have been sufficiently low that these organisms were not able to grow.

These data suggest that *A. flavus* and *A. parasiticus* could grow and produce aflatoxins on country cured hams during the aging process. The most likely time for this to occur would be when the aging temperature increased to 28 to 30° C but before the water activity of the hams became too low to permit growth of the toxic strains.

The data presented in this study suggest that aflatoxins could develop on meats in storage, if the storage temperature was not kept to about 10° C or below. The fact that aflatoxins were produced at 15° C on meats with mixed cultures is significant since this approaches refrigeration temperatures. Because cured meats such as ham and bacon are less susceptible to bacterial spoilage than fresh meats, they are
more apt to be mishandled in terms of poor control of storage temperatures. If the temperature of a display case or storage chamber containing cured meats were allowed to rise slightly, or if cured meats were stored in the home in a cool but unrefrigerated place, the growth of *A. flavus* or *A. parasiticus* and subsequent production of aflatoxins could result.

The fact that high levels of aflatoxins were detected on beef treated with antibiotics and stored at 15, 20 and 30° C is especially significant. Wildman et al. (1967) were unable to obtain aflatoxin production on fresh beef not treated with antibiotics, because of bacterial over-growth. The data presented here show that if toxic mold strains, especially *A. parasiticus*, have the advantage, very high levels of aflatoxins can be produced on fresh beef; these conditions could be met with certain types of beef aging processes. It has been suggested that aging temperatures of 32-43° C, in conjunction with the use of bacteriostatic agents such as antibiotics, he used to accelerate tenderization of beef carcasses (American Meat Institute Foundation, 1960).

Based on the data presented in this study, such an aging process could favor the growth of *A. flavus* and *A. parasiticus*, if spores of these organisms were present, and could possibly result in the production of aflatoxins on aging beef carcasses. The process of beef aging using
*Thamnidium elegans* uses a temperature of 15° C (Hesseltine, 1965). While the *Thamnidium* may control surface growth of bacteria in this process, if large numbers of *A. parasiticus* or *A. flavus* spores were present on the meat, it is possible that they could compete and aflatoxins could be produced. While aflatoxin production at 15° C requires a somewhat longer time than at higher temperatures, trace amounts of aflatoxins could be produced in shorter times. In view of the extremely potent carcinogenic nature of these compounds even trace amounts would constitute potential hazards to human health. However with the traditional methods of aging beef carcasses, employing temperatures of 2-3° C for 7-14 days, the development of aflatoxins would not seem to be likely.

Molds have long been associated with the spoilage of foods. Since spoilage molds are aerobic for the most part, mold growth on foods is in the form of surface growth. With foods such as cheeses, jellies and syrups, the common practice has been to remove the molds and use the remainder of the food. This is also true of mold growth on other foods such as cured and aged meats, where molds may be simply washed off of bacon or ham and trimmed from beef carcasses prior to the use of these products for human food. In the light of recent knowledge on aflatoxins showing diffusion of the toxins in foodstuffs (Lie and Marth, 1967; Frank, 1968) and the results presented here showing
toxin production at low temperatures on meats, these practices seem to be questionable and should be re-examined.

Aspergillus flavus and A. parasiticus are ubiquitous organisms found in many parts of the world. They are not as common in the air as some other molds but are very common in soil, and soil organisms are often contaminants of foods, especially animal carcasses and cut meats.

Commercial European type salami are aged at 12-15°C and 75% humidity for 30-60 days (Ayres et al., 1967). Under these conditions aflatoxins could be produced; however, based on the data obtained in this study, the amount of toxin produced would be expected to be low. Salami aged for 30 days may contain more aflatoxins under these conditions than salami aged for 60 days. Yet, even trace amounts of aflatoxins would be unacceptable, owing to their carcinogenic nature. There would appear to be less danger of aflatoxin development with the Hungarian type salami, since the added factor of smoking prevents growth of A. flavus at the temperature and humidity of commercial aging. Inoculating the Italian type salami with large numbers of conidia of known non-toxinogenic species of Penicillium or Scopulariopsis might prevent growth of A. flavus contaminants as well as aid in aging and flavor development. Using different amounts and combinations of curing ingredients, especially nitrites, might also prevent growth and toxin
production by *A. flavus* contaminants on European type salami. Country cured hams will support aflatoxin production during aging if proper conditions of temperature and water activity are met. Low temperatures (10°C) and low humidities (65-70%) during aging as well as high salt concentrations on the hams would prevent growth of *A. flavus* and *A. parasiticus*, and likewise tend to prevent the development of aflatoxins.

This study shows that when molds are used in the manufacture of foods, depending on the conditions employed, aflatoxin development can be a possibility. In the light of this study, and others already mentioned, it would be desirable that the traditional processes of food production employing molds be scientifically studied and evaluated to determine the potential of aflatoxin development.
SUMMARY AND CONCLUSIONS

Summary

A strain of *Aspergillus flavus* (NRRL A16,100), isolated as a contaminant from an Italian type salami manufactured in the United States, was found to produce aflatoxins. Other molds isolated from cured and aged meats, including strains of *A. ruber*, *A. wentii*, *A. niger*, *Aspergillus* species (*A. flavus-oryzae* group), *Penicillium frequentans*, *P. variable* and *P. puberulum*, could not be shown to produce aflatoxins.

A direct chloroform extraction method was developed for the extraction and analysis of aflatoxins from meats; using this procedure ham, bacon, salami and fresh beef were treated with four levels of aflatoxins and the toxins recovered. A silica gel column was used to partially purify the chloroform extract, when necessary, and the aflatoxin levels were quantitated by visual comparison to aflatoxin standards on thin layer chromatography plates. Concentrations of aflatoxin B₁ as low as 2.5 ppb in meat were detected using this method.

The method was used to study aflatoxin production on cured and aged meats in storage and during aging. Beef, bacon and ham were inoculated with $10^2$ or $10^6$ conidia/100 grams of meat. Three test organisms were used:
A. flavus NRRL 2,999, A. flavus NRRL A16,100 and A. parasiticus CMI 15,957. The meats were stored for varying lengths of time at 10, 15, 20 or 30° C, and then examined for mold growth and aflatoxin production. No growth of any of the organisms used was observed at 10° C; likewise, no aflatoxins were found at this temperature. At 15° C mold growth was scant to moderate, and aflatoxins were detected at this temperature. At 20° C abundant mold growth was observed on all of the meats, and aflatoxins were produced in relatively high amounts. Of all the temperatures studied, storage of meat at 20° C resulted in the production of the highest amounts of aflatoxins by each of the organisms on every type of meat. At 30° C abundant to profuse mold growth occurred on all of the meats studied. Mold growth at 30° C was as abundant or greater than mold growth at 20° C, but the production of aflatoxin was less at 30° C than at 20° C.

Of the meats studied, fresh beef containing antibiotics to reduce competition by bacteria, supported higher yields of aflatoxins at almost every temperature than did the other meats. One sample of beef supported the production of 630 μg of aflatoxins/g of meat. Bacon supported less growth of the molds, but in some cases supported very high yields of the toxins. A. parasiticus grew and produced aflatoxins especially well on beef and bacon. This organism also produced the highest yields of total aflatoxin in almost all
cases on all meats.

Aflatoxin $G_1$ always predominated in culture extracts of *A. parasiticus*. With the *A. flavus* cultures, aflatoxin $G_1$ predominated at 15 and 20° C, but at 30° C the relative amounts of $B_1$ to $G_1$ were approximately equal. In general, longer incubation times resulted in higher levels of aflatoxin, as did the use of larger ($10^6$) inoculum level. Light did not have a pronounced effect on the production of aflatoxins on ham, although in some cases, slightly higher levels of aflatoxin were found in samples incubated in the presence of light.

European type salami were inoculated with *A. flavus* NRRL A16,100 and aged at various temperatures and humidities. No aflatoxins were observed in any of the salami aged at any of the humidities at 10° C. At 15° C Italian type salami aged at 75-80% and 85-90% humidity contained aflatoxins. At 20° C all Italian type salami supported aflatoxin production, as did Hungarian type salami inoculated after smoking and aged at 85-90% humidity. Smoking inhibited growth and aflatoxin production by *A. flavus* in almost all cases. The level of inoculum had little effect on the level of aflatoxin produced. Aflatoxin levels were found to be higher during the first 4 weeks of aging than at the 8th week of aging. Salami ingredients were found to affect growth and
aflatoxin production by *A. flavus*. Pepper, nitrite and the complete spice mix reduced growth and aflatoxin production by *A. flavus* growing in glucose, ammonium-nitrate broth.

Aflatoxins were produced at 30° C on country cured hams that were 9 months of age. Trace amounts of toxins were produced by *A. flavus* NRRL 2,999 on 6-month-old hams at 20° C. No aflatoxins were produced on 3-month-old hams aged at 10° C or on 12-month-old hams aged at 30° C.

Conclusions

From this study the following conclusions can be made:

1. The production of aflatoxins by fungi other than those of the *A. flavus-oryzae* group is questionable. Certain molds that do not belong to this group have been suggested as having the potential to produce aflatoxins; this needs more study before it can be stated conclusively.

2. Extraction of aflatoxins from meats can be accomplished with direct chloroform extraction. Chloroform-soluble lipids can be removed from extracts using a silica gel column.

3. Aflatoxins were produced on fresh beef, ham and bacon at temperatures as low as 15° C. Therefore the practice of removing mold growth from foods and using the remainder of the food should be discouraged.
4. The most favorable temperature for the production of aflatoxins on meats in terms of highest toxin yields was in the range 20-30° C. Longer times were required for production of aflatoxins on meats at 15° C than at 20 or 30° C. No aflatoxins were detected in meats stored at 10° C for 56 days, but considerable bacterial and yeast growth was evident. Therefore meats stored at ordinary refrigeration temperatures below 10° C probably would be rendered unfit for human consumption by bacterial and yeast growth before becoming toxic as a result of \textit{A. flavus} or \textit{A. parasiticus} growth.

5. Fresh beef will support high yields of aflatoxins if conditions favoring the growth of toxinogenic molds are met. Therefore aging of beef at elevated temperatures (32-43° C) in conjunction with the use of bacteriostatic agents should be considered as potentially capable of allowing development of aflatoxins.

6. The use of larger numbers of conidia for inocula resulted in the toxic molds being better able to compete with other microorganisms found on meats and therefore usually resulted in higher levels of aflatoxins at any given temperature. Proper sanitation directed toward reducing the number of mold spores on processed meats would tend to retard growth and toxin production on these meats by toxinogenic molds.
7. European type salami that are not smoked and are aged for 4 weeks or less at temperatures of 15° C or above and at humidities of 75% or greater can support growth and toxin production by *A. flavus*. To prevent aflatoxin production by *A. flavus* on these meats, the products should be aged for longer periods of time, up to 8 weeks, at temperatures below 15° C and at humidities of less than 75%. Where practical, smoking can also be used to retard mold growth.

8. Country cured hams will support aflatoxin production by *A. flavus* and *A. parasiticus* if proper conditions of temperature and available water are met. Controlling the aging temperature at 15° C or less and the relative humidity at 65-70% would help prevent the growth of *A. flavus* and *A. parasiticus* on country cured hams.
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