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I am submitting herewith a thesis written by Sarah Gwendolyn Carson entitled "The Influence of Fat and Lean Tissues of Country Cured Hams on Growth of Aspergillus parasiticus and Production of Aflatoxin." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology.

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A

THE INFLUENCE OF FAT AND LEAN TISSUES OF COUNTRY CURED HAMS ON GROWTH OF *ASPERGILLUS PARASITICUS* AND PRODUCTION OF AFLATOXIN

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

Experimental studies were undertaken to determine the influence of ham tissue type on growth of Aspergillus parasiticus and production of aflatoxin. There was no detectable aflatoxin produced on lean or fat tissue incubated at 10, 21, or 32 C. for 14 days. This would suggest that ham tissue will not support growth of A. parasiticus or aflatoxin production under these conditions. However, these data do not eliminate the possibility that ham tissue actively inhibits mold growth and toxin production. Therefore, experiments were performed in which ham tissue was added to a control media.

Production of aflatoxin in a control nutrient broth was influenced by the presence of ham tissue. A mean of 6.174 µg of aflatoxin was produced in 75 ml of the broth. Incorporation of 5 grams lean tissue increased aflatoxin production to a mean level of 7.862 µg, but incorporation of 5 grams adipose tissue decreased the amount of aflatoxin to 4.044 µg.

These data indicate that the lack of aflatoxin production on fat tissue may be the result of inhibition of aflatoxin biosynthesis or dilution of nutrients. The lack of growth on lean tissue is more likely the result of some other presently unknown factor.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The aflatoxins are a group of mold metabolites produced by three species of the Aspergillus flavus group, A. flavus, A. oryzae (Diener and Davis, 1969), and A. parasiticus (Austwick and Ayerst, 1963). The A. flavus group is a constituent of the microflora in soil and air and is found in dead or living plants and animals (Ainsworth, 1965). The mold has been isolated from a number of natural substrates (Feuell, 1969) and grown successfully on synthetic media under laboratory conditions (Diener and Davis, 1969).

The aflatoxins are stable compounds which are readily destroyed only by strong alkali. In dilute solution they are very sensitive to light (Dollear, 1969).

The optimum temperature range for aflatoxin production on adequately moist (A_w 0.65) substances is between 20 and 30 C., depending on A. flavus strain and substrate tested (Hesseltime, et al., 1966; Schindler, Palmer and Eisenberg, 1967). As incubation temperatures are lowered, aflatoxin production decreases. Minimal temperatures have been reported in the range of 10 to 13 C. (Schroeder and Hein, 1967; Sorenson, Hesseltime and Shotwell, 1967) for production of toxins and 6 to 8 C. (Anderson, 1954) for mycelial growth of strains of A. flavus.

I. HISTORY

Outbreaks of disease in turkey poults, ducklings, pigs and calves in England in 1960 led to the initial investigations of Aspergillus flavus and aflatoxin (Allcroft and Carnaghan, 1964). The common denominator linking these diseases was feed containing Brazilian peanut meal (Blount, 1961).

Workers at the Central Veterinary Laboratory, Weybridge, England, noted the meal had fungal hyphae invasion and isolated the toxic factor from the meal. Austwick and Ayerst (1963) were unable to detect hyphae in a sample of non-toxic meal.

Attempts at culture from some meal samples showed the hyphae from the peanut meal was dead. However, Sargeant et al. (1961) succeeded in growing fungal species isolated from a toxic Ugandan peanut meal on Czapek agar. A chloroform extract of the culture showed the identical R_f value of 0.7. Feed trials of the isolate proved toxic to ducklings and produced the characteristic symptoms - loss of appetite, lethargy, wing weakness, arched neck, drawn head and legs extended backward (Asplin and Carnaghan, 1961) - associated with turkey X disease. The toxin producing fungus was identified as Aspergillus flavus link ex Fries, and the toxin was given the name "aflatoxin" in view of its origin (Barnes, 1970).

Almost simultaneously with the "turkey X disease", an outbreak of trout hepatoma was discovered by inspectors at the California state border (Wolf and Jackson, 1964). Liver lesions were also noted. It was

suggested that a change to the use of dry feeds (peanuts and cotton-seed meal) in trout hatcheries could have exposed the young fish to mycotoxins (Wales and Sinnhuber, 1966). Consequently, Halver (1965) found through test feeding trials that rainbow trout hepatoma was caused by aflatoxin.

II. BIOLOGICAL EFFECTS

The effects of aflatoxin may be acute or chronic depending on the dosage, frequency of exposure, and method of exposure (Wogan and Pong, 1970). Aflatoxin has been shown to be both a toxin and a carcinogen (Legator, 1969).

Researchers have made significant advances on the structure of aflatoxins and relationships of structure to activity. Buchi and Rae, (1969) and Biollaz et al. (1970) performed extensive studies on the biosynthesis of aflatoxin and demonstrated that the carbon skeleton was derived entirely from acetic acid. The toxins have closely similar structures and form a unique group of highly oxygenated, heterocyclic compounds. Aflatoxin B_1 has been shown to be the most active of the aflatoxins (Wogan, 1965). The structure has been presented in Figure 1.

Since aflatoxin has been found to localize in the nuclei of liver cells soon after dosing (Clifford and Rees, 1966), most studies have concentrated on the interaction of aflatoxins with materials in the nucleus or with the effects of aflatoxin on the gene action system (Childs, 1971).

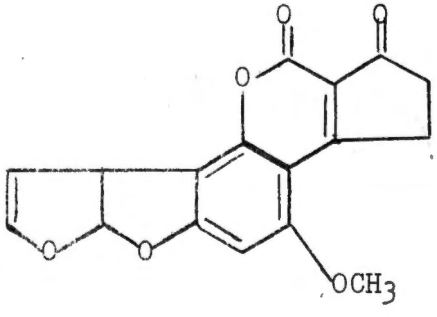

Figure 1. Structure of Aflatoxin B_1.

Dickens (1964) first postulated that the toxin's activity was due to its unsaturated γ-lactone structure. Wogan, Edwards, and Newberne (1971) demonstrated that the furofuran moiety of the aflatoxin structure was essential for its toxic and carcinogenic activity. Ayres et al. (1971) suggested that the hepatocarcinogenicity of aflatoxin B_1 depended on the bifunctional activity of the molecule with a requirement for both the dihydrofurofuran ring system and the unsaturated γ-lactone moieties. The presence of the double bond in the terminal furan ring and substituents on the lactone portion of the molecule determined potency differences among the different aflatoxions.

Like most carcinogens, aflatoxin B_1 must be activated by mammalian microsomal enzymes to give rise to toxic compounds (Clifford and Rees, 1967; Edwards and Wogan, 1970; King and Nicholson, 1967). Goze et al. (1975) noted that suggested metabolites of aflatoxins acted as inducers of E. coli K12 (λ) at a high efficiency whereas unmodified aflatoxin B_1 had no effects. Thus aflatoxin must be converted to an active metabolite in vivo before it exhibits biological activity.

In Vivo

The lethality of aflatoxin B_1 has been studied in laboratory animals including: ducklings, rats, hamsters, guinea pigs, rabbits, dogs, rainbow trout, and monkeys. Oral dosing has been established as the most potent administration method (Wogan, 1966). Metabolism studies with rats (Wogan, et al. 1967) and monkeys (Dalezios, 1971) showed that aflatoxin B_1 administered intraperitoneally disappeared rapidly with

conversion in the liver. Consequently oral LD_{50s} have been cited and appear in Table 1.

Researchers have studied the in vivo effects of aflatoxin in the liver as well as in other organs. Rats, because of their inexpensiveness and the large body of knowledge concerning their physiology, have been the primary species studied. A summary of effects and dosages (when known) is presented in Table 2. Most species studied in vivo have shown one of three general effects: (1) interference of nucleic acid or protein synthesis (de Recondo et al., 1966, Edwards et al., 1971), (2) genetic mutations resulting in cell growth abnormalties (Goldblatt, 1969; Lancaster, Jenkins and Philp, 1961; and Garner, 1973), or (3) necrosis and/or liver lesions (Manhavan and Gopalan, 1965).

In Vitro

The in vitro toxicity of aflatoxin B_1 has been examined in cell culture systems and embryos. A variety of random observations have been made. Legator (1969) stated that aflatoxin concentrations of 0.01 to 50 µg/ml inhibited mitosis and produced chromosome abberations in human embryonic lung cells and leucocytes. Adekunle and Elegbe (1974) noted that aflatoxin B_1 binded to lysosomes of bird embryos. Several researchers have suggested in vitro binding of aflatoxins to nucleic acids and proteins (Maher and Summers, 1970). Gurtoo and Dahms (1974) demonstrated that aflatoxin B_{2a}, a metabolite of B_1 bound to cultured rat liver microsomes. Pai, Bai and Venkitasubramanian (1975) showed

TABLE 1

LITERATURE VALUES FOR LD_{50}'S OF ORALLY ADMINISTERED AFLATOXIN B_1 TO LABORATORY ANIMALS

Animal	LD_{50}	Days	Reference
Male Rats	7.2 mg/kg	3-7	Butler, 1964a
Guinea Pigs	1.4 mg/kg	1-4	Butler, 1966
Ducklings	.0335 mg/kg	6	Butler, 1964b
Dogs	1 mg/kg	7	Newberne, et al, 1966
Monkeys	2.2 mg/kg	4	Rao and Gehring, 1971
Hamsters	10.2 mg/kg	7	Wogan, 1965

TABLE 2

LITERATURE SUMMARY OF TOXIC EFFECTS OF AFLATOXIN B_1 IN THE LABORATORY RAT

Organ - Tissue	Effect	Dose	Reference
Liver	Hepatomas	0.015 ppm	Wogan and Newberne, 1967
Liver	Inhibition of parenchymal cell mitosis	3 mg/kg	Rogers and Newberne, 1967
Liver	Lesions	4-5 ppm	Butler, 1966b
Utero-Placental Junction	Hemorrhages causing fetal death or growth inhibition	7.2 mg	Le Breton, et al. 1964
Kidney	Tubular necrosis		Butler, 1966b
Adrenals	Hemorrhages, compact cell change		
Spleen	Dimunition of white pulp		
Pancreas	Changes in inlet		

that aflatoxins B_1, M_1, and G_1 (at concentration of 1×10^6 M) inhibited electron transport at the chain site between sytochrome b and c or c_1, and thus reduced energy efficiency.

All of these in vitro observations have been heavily scrutinized for a number of reasons. Two samples have been cited. Edwards et al. (1971) reported weak in vitro binding of DNA and aflatoxin B_1, but stronger binding in vivo. Childs (1971) noted that interaction of aflatoxin B_1 with histone did not alter the ability of histone to bind to negatively charged macromolecules such as RNA polymerase of DNA which occur in chromatin. He therefore doubted the disruption of normal interactions of chromatin macromolecules by the binding of DNA or histone to aflatoxin.

It has been shown that aflatoxin must be metabolized by the liver before it is toxic. All of these metabolites have not been isolated or positively identified and could not be added to culture systems. Therefore the methods used for studying aflatoxin in cell culture systems have been questionable since the toxic form was not used.

Carcinogenic Effects

Aflatoxin B_1 is the most potent liver carcinogen known for the rat (Goldblatt, 1966; Wogan, 1966). Wogan and Newberne (1967) showed that levels as low as 0.015 ppm in the diet induced a high level of hepatic carcinomas. Aflatoxin B_1 is carcinogenic per se (Wogan and Newberne, 1967), however it is suggested that metabolic activation is also a necessary prerequisite for tumor initiation (Goodall and Butler, 1966; Lijinsky, Lee and Gallagher, 1970; Wogan, Edwards and Newberne, 1971).

The three primary hypotheses concerning the manner in which tumor cells are initiated are as follows: (1) mature cells are activated by carcinogenic stimuli and undergo dedifferentiation (Cohnheim, 1889), (2) deoxyribonucleic acid (DNA) of the cell is altered by mutation or addition of viral DNA (Robbins, 1968), and (3) the carcinogen acts at the stem cell level and there is an abortive attempt at differentiation which results in anomalous growth patterns that may result in the formation of cancerous cells and tissues (Pierce, 1970).

The carcinogenic action of aflatoxin was first demonstrated by Lancaster, Jenkins and Philp (1961) who reported a high incidence of liver tumors in rats fed toxic meal. These observations have been extended to other animals by using pure aflatoxin B_1.

Species differences have been shown in degree of carcinogenicity. Gablik et al. (1965) induced hepatic tumors within 14 months in ducks fed dietary levels of 0.035 ppm aflatoxin. Halver et al. (1966) noted a 96 percent incidence level in rainbow trout fed dietary levels of 20 ppm for 20 months. Barnes (1967) demonstrated aflatoxin induced hepatomas in the guinea pig. Goplan et al. (1972) induced a hepatocellular carcinoma in a male Rhesus monkey two years after being fed a dietary level of 200 µg/day of aflatoxin B_1 in 20 percent toxic meal.

Other organs are also affected by the carcinogenicity of aflatoxin. The kidney was a target organ in the guinea pig (Madhaven and Rao, 1967), in hamsters (Herrold, 1971) and the rat (Butler, Greenblatt and Lijensky, 1969). The colon was demonstrated as a cancerous area for rats deprived

of vitamin A (Newberne and Rogers, 1972). Wieder et al. (1968) showed local tumors in mice administered aflatoxin intraperitoneally.

From these observations, it is clear that aflatoxins are potent carcinogens. Barnes (1970) suggested the species response difference was due to the production of different molecules (metabolites) after metabolism of aflatoxins.

Occurrence in Products

Goldblatt (1966) suggested that the presence of mycotoxins was potentially the most serious quality problem faced by the food industry. The significance of these toxins as possible health hazards has necessitated the examination of certain feeds and foods for the presence of the toxinogenic mold. While occurrence of the mold on a produce does not necessarily mean that mycotoxins are present, it has been an important indicator of potential health hazards (Campbell and Stoloff, 1974). Therefore assay methods have been of extreme importance in the examination of consumption products.

Soybeans

In a survey by Howell (1966), A. flavus could not be isolated from more than 3100 samples of soybean seed. Shotwell et al. (1969) reported only two of 866 samples of soybeans contained aflatoxin. This, and other data had led researchers to suggest that under normal conditions, aflatoxins were not a serious problem on soybeans. However, during an unusually wet growing season, Bean, Schillinger and Klarman (1972) reported 47 percent of samples isolated contained A. flavus and

25 percent of these were possible aflatoxin producers.

Under controlled environmental conditions, A. flavus has been found to grow extensively on soybean seed, however only weak aflatoxin production was reported (Borker et al. 1966). Hesseltine (et al. 1966) demonstrated that aflatoxin production of 109 µg/g soybeans was 50 to 90 percent of the amount produced on corn, wheat, rice, peanuts and sorghum. Conversely, Eldridge (1964) obtained similar aflatoxin production of 300 to 375 µg/g on both soybeans and peanuts.

Laboratory and field data, have suggested that a potential does exist for production of aflatoxin from A. flavus present in soybeans. No conclusive work has been published for ideal growth conditions of A. flavus or its production of aflatoxin on soybean substrate.

Corn

Trenk and Hartman (1970) demonstrated that A. flavus produced aflatoxin in corn at moisture levels above 17.5 percent when the temperature was 24 C or higher.

Studies have found that aflatoxin invasion of corn in the Corn Belt has been low (Rambo, Tuite and Caldwell, 1974; Trenk and Hartman, 1970). Rambo et al. (1974) found only 1.6 percent A. flavus in Indiana corn samples. Trenk and Hartman (1970) reported only two of 14 Iowa samples contaminated with A. flavus produced aflatoxin. Shotwell, Goulden and Hesseltine (1974) observed only two of 186 samples of corn from the Midwest infected with A. flavus. Conversely, much higher levels

of A. flavus were found in corn samples from the Southern states (Shotwell, Hesseltine and Goulden, 1973).

Detroy, Lillehoj and Ciegler, et al. (1971) produced large quantities of aflatoxin on autoclaved corn. Trenk and Hartman (1970) demonstrated the potential of aflatoxin production on corn under poor storage conditions. His study emphasized the difference in aflatoxin production on freshly harvested and rewet corn stored under similar environmental conditions. At 24 C maximum aflatoxin production was 50 ng/g in freshly harvested corn, but reached 500 ng/g in rewet corn.

Rambo et al. (1974) detected a 6 percent aflatoxin incidence in damaged corn and 0 percent in undamaged corn. Conversely, Shotwell et al. (1974) found the toxin present in all fractions, from damaged and discolored kernals (19 percent) to apparently sound kernals (75 percent). Both Fernell et al. (1973) and Shotwell et al. (1974) reported high invasion in the germ region.

From this data, it was unlikely that aflatoxin contaminated kernels could be removed by physical separation or through grading standards. Other researchers have suggested proper storage conditions as a means of control of aflatoxin production in corn. Wilson and Jay (1975) introduced modified atmospheres of high CO_2 and low O_2 levels to deter production on stored corn. Since none of their systems tested allowed significant aflatoxin contamination, a method might prove successful if a residue - free, economical system could be adopted.

Pecans

Aflatoxin existence in pecans was first noted during an investigation of a bakery mold problem. A. flavus was found in 2 to 21 percent of bakery pecans and 0 to 85 percent of market pecans. Lillard et al. (1970) noted 71 percent of these mold isolates screened on sucrose supplemented yeast extract broth produced toxin.

Reports of A. flavus in pecans from the field has been high. Chipley and Heaton (1971) reported A. flavus present in 4 percent of pecan nutmeats examined at harvest. Escher, Koehler and Ayres (1974) noted 9 percent of sound pecan kernels contaminated with fungal mycelium, and 2 percent of those plated yielded A. flavus or A. parasiticus.

Blanchard and Hanlin (1973) studied the effectiveness of propylene oxide (PO) treatment for microflora control in pecans. Under controlled conditions, PO gave 80 to 90 percent reduction of surface microflora and at least 61 percent reduction of internal flora. However, even with high doses, PO could not eliminate the fungi completely and thus proved useless commercially.

Therefore, because of the high incidence of mold present in pecans at harvest, data has suggested the possibility of extensive mold damage during storage (if fungal growth was not arrested) followed by aflatoxin production and thus a potential health problem (Escher et al. 1974). The high consumption levels of pecans by humans emphasizes the danger and should spur further research.

Peanuts

Since the aflatoxin problem was first associated with peanuts and A. flavus, researchers do not doubt the potential health hazards associated with the product. Peanuts have been described as more like green vegetables than oil or cereal crops in the care required in harvest, handling and storage to prevent mold damage (Goldblatt, 1965).

At harvest, peanuts have a 45 percent moisture content which must be reduced to 8 percent within four or five days. Delay of the drying cycle by rain or humid weather often results in mold development with toxin production (Austwick and Ayerst, 1963).

Cucullu et al. (1966) demonstrated that most samples of sound, mature peanuts do not produce aflatoxins. Five percent of moldy samples isolated showed a high incidence of A. flavus but an average toxin production of 50 ppb. Conversely, 0.24 percent of the samples showed poor or no growth of A. flavus but an average toxin production of 1,100,000 ppb.

Austwick and Ayerst (1963) observed good growth of toxin producing A. flavus at 85 percent relative humidity at 25 to 28 C. Diener and Davis (1967) reported optimum time of seven to 15 days and temperature of 25 to 30 C for aflatoxin production on sterilized peanut kernels. Under these conditions, 1.5×10^6 spores of A. flavus produced from 0 to 39,700 µg aflatoxin per kg subtrate.

Two methods of control for the toxin have been suggested. Sanders, Davis and Diener (1968) demonstrated that A. flavus growth and aflatoxin

formation was inhibited at 86 percent relative humidity by 60 and 40 percent CO_2 at 25 C. Consequently, they advocated control by modified atmospheres. Natarajan (et al., 1975) showed that 88 percent of the aflatoxin present in peanuts remains in the solid protein fraction during wet milling, and suggested the possibility of electrolysis of the toxin for control.

Experience and laboratory data have shown that aflatoxin production on peanuts is possible. The increased utilization of peanut products in animal feeds and as protein extenders has emphasized the importance of detection of mold or toxin and thus potential health hazards.

Country Hams

Molds often grow on meats, especially cured meats during storage or aging. Some of these molds have toxinogenic potential. Bullerman and Ayres (1968) first isolated a strain of A. flavus that produced aflatoxin from cured meat. Five strains of A. flavus, four of which produced aflatoxin, were isolated from a single country cured ham by Strzelecki, Lillard and Ayres (1969).

Sutic, Ayres and Koehler (1972) found the type of mold present was dependent on the age of the hams and the moisture level in the storage room. All Aspergilli were more abundant on the surface of hams aged for 12 months or longer than on one to three month old hams. However, the younger hams were capable of supporting mold growth and toxin production. Under dry storage conditions, more Aspergilli were isolated from one to three month hams, whereas under moist conditions these

hams yielded more Penicillia. Of 366 ham molds examined, 121 were Aspergilli and only two of these yielded strains of A. flavus with toxin producing ability.

Bullerman, Hartman and Ayres (1969c) studied aflatoxin production in ham samples innoculated with 1.6 x 10^6 spores of A. flavus or A. parasiticus and stored at 10, 20 and 30 C. Inconsistent aflatoxin levels of 0.01 µg/g to 27 µg/g were produced on slices taken from six to nine month hams during aging when the temperature approached 30 C.

These data suggest that A. flavus and A. parasiticus could grow and produce aflatoxins on country cured hams during the aging process. Bullerman et al. (1969b) demonstrated that low humidities (65-70 percent) and low temperature (10 C) during aging, as well as high salt concentration on the hams could prevent growth and aflatoxin production by A. flavus and A. parasiticus.

The risk of contamination of foods or food ingredients with aflatoxins is largely determined by the extent to which mold growth is prevented at all stages of food production and utilization. Country cured hams are no exception to this generality. However, whereas most mold growth on products is caused by carelessness, mold growth on country hams develops simultaneously with flavor precursor chemical constituents, and have been traditionally accepted (Satterlee and Lillard, 1967; and Lillard and Ayres, 1969). Thus it is of utmost importance to evaluate these molds for production of aflatoxin and make an assessment of possible potential health hazards.

Summarizing, the literature shows:

1. A. flavus (A. parasiticus) is a mold which can produce aflatoxin.
2. A. flavus has been isolated from grains, nuts, oilseeds and meats.
3. Aflatoxins may be the most important hepatocarcinogen known.
4. Aflatoxins are toxic at extremely small levels.
5. There is a possibility of aflatoxin formation by A. flavus (A. parasiticus) on country cured hams.

These observations have emphasized the potential dangers and health hazards associated with aflatoxin formation. However, it is necessary to consider the economic aspects involved. If the Food and Drug Administration enforces pulling of mold infested hams by the United States Department of Agriculture, the ham industry will suffer tremendous losses. Therefore, the present study was conducted to investigate the following questions:

1. Does country ham tissue support the growth of A. flavus (A. parasiticus) or aflatoxin production?
2. Does presence of country ham tissue in a normal nutrient broth influence aflatoxin production?
3. Are there possible inhibitors in country ham tissue for toxin formation?

CHAPTER II

MATERIALS AND METHODS

I. EXPERIMENTAL DESIGN

The objective of this study was to evaluate aflatoxin production by A. parasiticus influenced by adipose and muscle tissues of Tennessee country cured hams. Two major experiments were conducted. One used suspended ham portions in controlled microenvironments and the other comminuted ham tissue in sucrose supplemented yeast extract broth. The experimental systems are defined in Tables 3 and 4.

Three different country cured hams were used throughout the study. They were purchased locally and thought to be typical of other country cured hams produced commercially in Tennessee. The hams had been aged for five months which is the average aging time for commercial hams in the state. (Brook Haven Farms, Inc., Route 4, Seymour, Tennessee 37865.)

Country cured hams are produced over a range of environmental conditions. Therefore varying humidities and temperatures have not been uncommon. The incubation temperatures of 10, 21, and 32 C used in the experiments were representative of those encountered by hams during the aging period (Bullerman et al. 1969b).

Aspergillus parasiticus PC 5228 EAC was employed in both experiments. The strain was grown successively on two different media

TABLE 3

EXPERIMENTAL DESIGN FOR STUDIES CONCERNED WITH GROWTH OF A. FLAVUS AND AFLATOXIN PRODUCTION ON SUSPENDED HAM TISSUE SLICES

System	Tissue Type	Temperature	Ham No.	Replication
A	Fat	10	1,2,3	I,II,III
B	Lean	10	1,2,3	I,II,III
C	Fat	21	1,2,3	I,II,III
D	Lean	21	1,2,3,	I,II,III
E	Fat	32	1,2,3	I,II,III
F	Lean	32	1,2,3	I,II,III

TABLE 4

EXPERIMENTAL DESIGN FOR STUDIES CONCERNED WITH EFFECTS OF COMMINUTED HAM TISSUE ON AFLATOXIN PRODUCTION IN YEAST EXTRACT BROTH SUPPLEMENTED WITH 20 PERCENT SUCROSE

System	Tissue Incorporated	Ham No.	Replication	Temperature
A	Lean	1,2,3	I,II,III	32
B	Fat	1,2,3	I,II,III	32
C	----	-----	I,II,III	32

to assure its virality and purity. The spores were transferred by sterilized needle from an initial stock to Czapek dox agar (Sutic et al. 1972) prepared according to Difco. The culture was incubated at 32 C for five days and subsequently transferred by the previous method to malt agar (Leistner, 1968), prepared according to manufacturers Becton, Dickinson and Company. Inocula was produced by incubation for five days at 32 C.

Twenty (20) ml of a sterile solution of 0.05 percent tween 60 (Mann Research Laboratories, Inc., New York, N. Y.) and three glass beads were poured into an Erlenmeyer flask containing the mold and the flask shaken. The conidia were thus suspended in the solution for inoculation. Spore counts using a hemocytometer indicated one drop of solution from a 0.10 ml pipet contained 2.5×10^6 A. parasiticus spores. The 0.10 ml pipet was then used in both experiments to deliver one drop of inoculate or 2.5×10^6 spores to the samples.

The two experiments were completely different in design and are discussed as such in the following section. Conversely, the methods and materials employed in extraction, cleanup and quantitation were basically the same. The differences, if any, in these procedures are mentioned within the individual experimental section.

Experiment I

The first experiment constituted a closed-system, artificial microenvironment. Whole portion (5.0 g $\pm$ 0.2 g) samples of fat and lean tissue were randomly cut from each ham and sterilized with ethylene

oxide for 15 hours. Samples were suspended on hooks attached to Mason lids. The tissues were surface inoculated with a 0.10 ml sterile pipet in the manner previously described and enclosed in sterile one-pint Mason jars. Samples were incubated for two weeks at 10, 21 or 32 C.

After the appropriate incubation time, samples were removed from their respective incubators and analyzed for aflatoxin production. Each tissue was thoroughly comminuted with an Oster blender. Thirty (30) ml of acetonitrile were added and samples reblended. The suspension, followed by a 10 ml acetonitrile wash of the Mason jar, was poured into a 250 ml fleaker and mechanically shaken for 30 minutes. Samples were filtered and evaporated to incipient dryness over a steam bath. Three replicates, each with 18 samples, were performed.

Sterile techniques were stressed throughout the experiment. A chamber for making transfers and inoculations was disinfected by: (1) wiping with ethanol, (2) wiping with a bleach and water solution [1 + 1], and (3) illuminating 30 minutes with an ultraviolet light. Gloves and arms were also washed with the bleach solution.

Experiment II

For the second experiment, a 750 ml batch of 20 percent sucrose supplemented yeast extract broth was utilized. Five (5) $\pm$ 0.2 g slices of fat or lean tissue were thoroughly comminuted with an Oster blender and incorporated into 75 ml portions of the broth. A second blending insured the mixture of maximum dispersion. Three 75 ml

control samples without incorporated tissue were employed for comparisons in each replicate. Each suspension or broth was transferred to a 500 ml Erlenmeyer flask. Samples were stoppered with cotton and sterilized for 15 minutes at 250 C and 15 psi in an autoclave.

The samples were cooled to ambient temperature and inoculated by passing the sterile 0.10 ml pipet containing the innoculant past the cotton to the broth. Samples were incubated 10 days at 32 C.

Extractions were made using 50 ml of the acetonitrile followed by 30 minutes of mechanical shaking. The extracts were filtered into fleakers, along with a 10 ml wash of acetronitrile. Examination of filtrates revealed two distinct layers -- water and acetonitrile. Thus, an additional separation with a 750 ml separatory funnel was employed. The water layer was discarded and the acetonitrile was evaporated to incipient dryness over a steam bath. The contents were redissolved in 10 ml chloroform and evaporated a second time.

II. EXTRACTION AND CLEANUP

The method for primary extraction of aflatoxin involved an equilibrium extraction rather than an exhaustive system. A common disadvantage of exhaustive systems noted by Pons and Goldblatt (1965) was the presence of large amounts of polar lipids, pigments and carbohydrates (in the primary extraction) which were not easily removed in subsequent extract purification steps. Lee (1965) noted a similar problem using chloroform in a 30 minute equilibrium shaker

extraction for peanut meal. Consequently, Eppley (1966) added an auxiliary extract purification system with chloroform and water for the equilibrium estimation of aflatoxin from peanut products.

Bullerman et al. (1969a) suggested an extraction technique employing chloroform and methanol. The system of extraction used in this study substituted acetonitrile for methanol and used chloroform after the primary extraction.

Acetonitrile and methanol are both highly polar solvents. However, the triple bonded cyanide (CN) group on the end of acetonitrile limits lipid solubilization by the solvent from the ham and thus subsequent carrying of the lipid with the aflatoxin extract. Hence, efficient defatting and aflatoxin extraction are conducted in a single extraction.

In each experimental system, the acetonitrile extract was decanted and filtered through five (5) grams sodium sulfate on Whatman No. 4 paper using a 100 cm diameter glass funnel. The high water content of the meats permitted direct extraction of aflatoxin without excess water as suggested by Lee (1965) for peanuts.

Each sample was evaporated to incipient dryness and concentrated over a steam bath. The residue was dissolved in 10 ml chloroform, transferred to a 50 ml beaker along with a 5 ml chloroform wash, and again evaporated to dryness over a steam bath.

Reagents

Acetonitrile - Analytical reagent grade from Fisher Scientific Company.

Acetone - Redistilled, analytical reagent grade from Fisher Scientific Company.

Chloroform - Analytical reagent grade from Fisher Scientific Company.

Sodium Sulfate - Analytical reagent grade, anhydrous, granular, from Fisher Scientific Company.

Apparatus

Beakers - 100 ml

Chromatographic Tank - covered with aluminum foil.

Filter Flasks - 100 ml

Filter Paper - Whatman No. 4

Fleakers - 250 ml

Glass Funnels

Graduated Cylinders - 100 ml and 25 ml

Hamilton Syringe - 25 ml

Mason Jars - pint size with Oster Blending Heads.

Osterizer Blender

Oven

Pancake Gridle

Pipets - 10 ml, 1 ml and 0.1 ml

Separatory Funnels - 500 ml

Thin Layer Plates - Precoated Silica Gel G HR. Heat activate prior to use as directed by manufacturer.

III. THIN LAYER CHROMATOGRAPHIC ANALYSIS OF SAMPLES

Five (5) ml of chloroform were added to each sample at incipient dryness. A 10 µl aliquot was spotted on a silica gel G HR thin layer chromatographic plate with a Hamilton 25 µl syringe. Spotting employed taking up a small amount (4 µl) of chloroform, allowing an air gap and then taking up 10 µl of the sample solution. Standard aflatoxin solutions of 2 and 4 µl were spotted on the same thin layer plate containing the samples. Each plate was air dried 10 minutes.

Plates were placed in a dark, unsaturated chromatographic tank containing 300 ml of chloroform - acetone solution (9 + 1) as suggested by Eppley (1966) and Pons (1968). The solvent front traveled a distance of 75 cm. The plates were removed from the tank, air dried and held in a dark desicator until quantitated.

IV. QUANTITATION

The visual method of quantitation used by most experimentors in estimating the amount of aflatoxin present on plates was used to verify the presence of the toxin on the plate. Actual quantitation was attained with the use of a Schoeffel spectrodensitometer (SD 3000) with Schoeffel density computer (SDC 300). The spectrodensitometer was operated on natural fluorescens with the use of UV cutoff filter and SDA 334 wedge. The following settings were used to obtain maximum response: (A) Excitation wavelength, 365 nm; (B) Emission

wavelength, 430 nm; (C) Slit width, 1.5 mm; (D) Beam width, 0.5 cm; (E) Beam length, 1.0 cm; (F) O. D. units range, 0.4 - 10; (G) Density computer, positive - ratio - numerator.

A regression equation obtained from plotting standard aflatoxin of varying concentrations on each plate was used to calculate the quantity of aflatoxin in samples on the plates.

V. VALIDITY OF ANALYTICAL METHOD

Pons (1969), and Ayres and Sinnhuber (1966) reported acceptable aflatoxin recoveries in concentrations as low as 0.003 µg with the method employing methanol and chloroform as extractors.

In previous studies, quantitation by visual means had shown average recovery rates of 85 percent. Using the equilibrium method described and quantitation on the spectrodensitometer, recoveries in this study ranged from averages of 34.05 percent at 0.08 µg/50 g country ham to 70.48 percent at 0.4 µg/50 g country ham. Because of the low recovery rates at low concentration, concentrations greater than 0.04 µg/50 g country ham were necessary. In the present study, the accuracy, precision and sensitivity of the method were defined.

VI. METHOD EVALUATION

Standard Curve. Silica gel plates were spotted with standard purified aflatoxin solutions at concentrations ranging from 0.04 to 0.4 µg. The mean peak areas of two replicates were used to obtain a

standard curve as shown in Figure 2. A regression analysis of the standard curve was calculated to define the regression equation and has been presented in Table 5.

All concentrations produced spots which could be scanned by the spectrodensitometer. A correlation coefficient of 0.98 (significant at $p<0.05$) was obtained along with the regression equation of:

$$y = 0.0008\ (x) + (-0.02)$$

where x was area under the curve and y was µg of aflatoxin. Peak areas were sufficiently high so that background effects were negligible at high concentrations. However, at concentrations less than or equal to 0.04 µg/50 g country ham the background responses was equal to or exceeded the sample.

VII. DETECTION LIMIT

The detection limit was defined as twice the standard deviation of the thin layer plate background count. Five background areas were randomly selected from three replicate plates to obtain the values listed in Table 6 which establish the detection limit of the spectrodensitometer. The peak areas were substituted into the regression equation to yield the lowest limit of aflatoxin concentration detection.

The mean detection limit $\pm$ standard error of the mean for the three replicates was 0.04 $\pm$ .02 µg. Therefore, at three standard deviations, or a 99 percent confidence level, detection of 0.04 or less of aflatoxin was not possible.

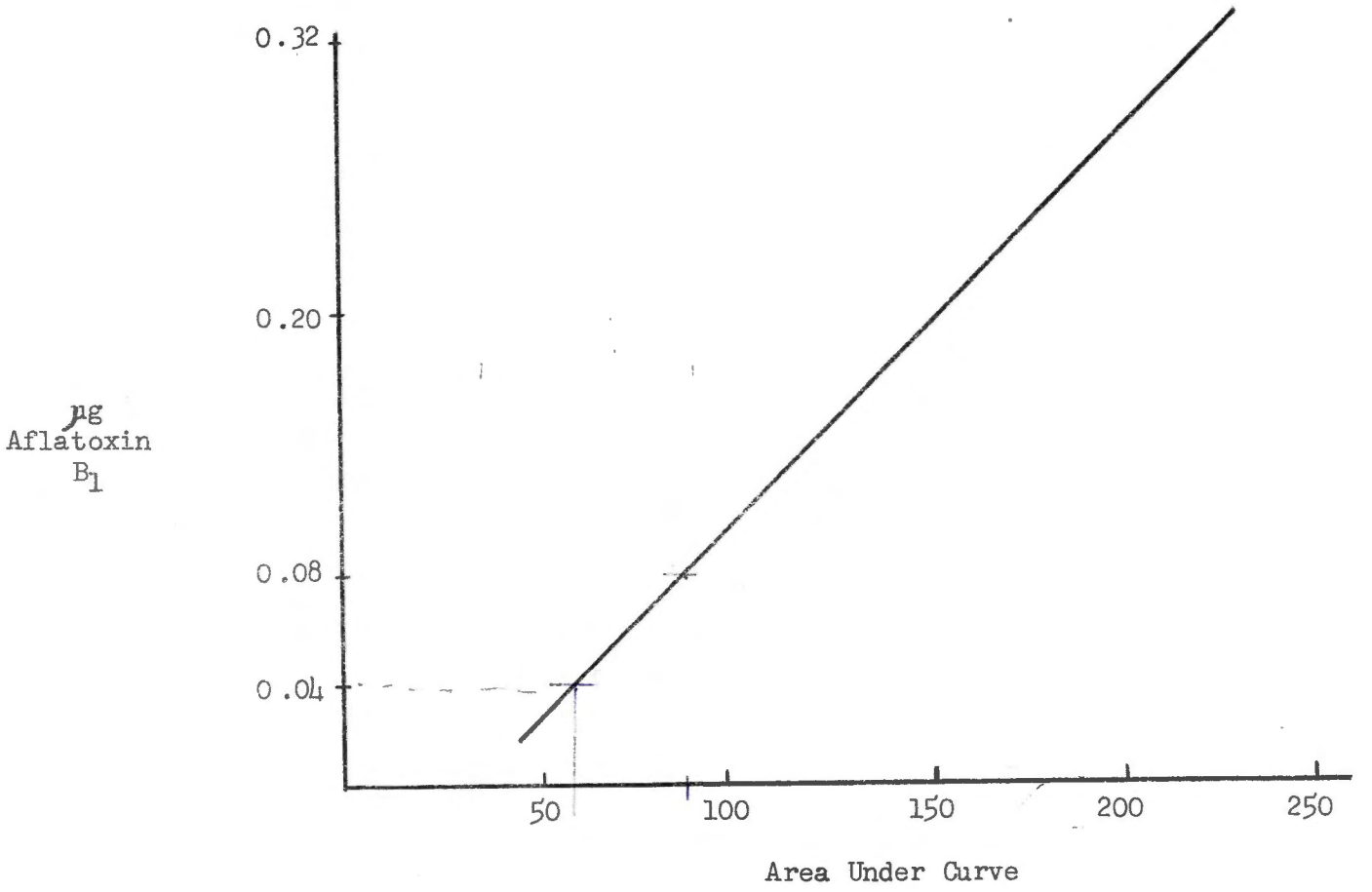

Figure 2. Standard curve from the mean peak areas of two replicate plates upon which were spotted and developed .04, .08, .20 and .32 µg of Aflatoxin.

TABLE 5

REGRESSION ANALYSIS OF STANDARD CURVE OBTAINED FROM THE MEAN PEAK AREAS OF TWO REPLICATE PLATES SPOTTED WITH AFLATOXIN STANDARDS

Concentration µg	Peak Area Plate #1	Peak Area Plate #2	Mean Peak Area
0.04	57.03	67.03	62.03
0.08	85.40	93.43	89.83
0.20	162.88	171.475	167.175
0.32	224.80	237.361	231.08

$r = 0.98$

$m = 0.0008$

$b = -0.02$

TABLE 6

DETERMINATION OF DETECTION LIMIT[a] FOR MEASURING AFLATOXIN AS A FUNCTION OF THIN LAYER PLATE BACKGROUND

Plate #1		Plate #2		Plate #3	
Peak Area	µg	Peak Area	µg	Peak Area	µg
37	.007	78	.04	32	.002
66	.03	31	.002	25	-.002
-40	-.05	29	.005	34	.004
-30	-.04	18	-.008	27	.001
73	.03	40	.009	42	.01
Detection limit (µg)		Detection limit (µg)		Detection limit (µg)	
0.06		0.02		0.008	

Mean detection limit = 0.04 ± 0.02

[a]The detection limit is defined as twice the standard deviation of the thin layer chromatographic plate background.

VIII. PERCENT RECOVERY

To determine if aflatoxin could be extracted and establish percent recovery rates from hams by acetonitrile - chloroform extraction, two levels (10 µl and 50 µl) of aflatoxin were added to 50 gram ham samples. Bullerman et al (1969a) noted no significant difference in recovery rates of aflatoxin treated samples held for 1 hour or 24 hours. Therefore, the samples were held one hour to permit distribution of aflatoxin through the product. The aflatoxin was extracted with acetonitrile and chloroform then spotted on TLC plates as previously described.

The percent recoveries obtained are listed in Table 7. There was significant improvement in percent recovery by increasing concentration from 0.08 µg/50 g country ham to 0.40 µg/50 g country ham. The mean percent recovery ± standard error of the mean for concentration of 0.4 µg/50 g country ham was 70.927 ± 2.20/as shown in Table 8.

IX. PRECISION

Precision was obtained by determining the standard error of the mean of measurements of 6 replicate spots containing a mean of 0.2845 µg aflatoxin. At no measurement was the deviation greater than 4.39 percent of the mean. The mean standard error of the mean as a percent of the mean was 2.45 ± 1.57 percent. At the 99 percent confidence level, greater than 92.84 percent precision can be expected with this method. The measurements are shown in Table 9.

TABLE 7

RECOVERY OF AFLATOXIN FROM HAM TISSUE USING ACETONITRILE-CHLOROFORM EXTRACTION

Actual Concentration µg/50g Ham	Calculated Amount µg/50g Ham	Percent Recovery
0.08	0.0256	32.00
0.08	0.0296	37.11
0.08	0.0264	33.04
0.40	0.2845	71.13
0.40	0.2892	72.30
0.40	0.2720	68.00

Mean percent recovery ± standard error of the mean = 52.323 ± 20.07 (for concentrations of both 0.08 and 0.4 µg).

Mean percent recovery ± standard error of the mean = 70.47 ± 2.22.

TABLE 8

PERCENT RECOVERY OF AFLATOXIN FROM HAM TISSUE USING CONCENTRATIONS OF 0.4 µg FOR SPIKING

Actual Concentration µg/50g Ham	Calculated Amount µg/50g Ham	Percent Recovery
0.4	0.2845	71.13
0.4	0.2892	72.30
0.4	0.2720	68.00
0.4	0.2906	72.66
0.4	0.2737	68.42
0.4	0.2922	73.05

Mean percent recovery $\pm$ standard error of the mean = 70.927 $\pm$ 2.20.

TABLE 9

PRECISION OF MEASUREMENT OF 0.2845 µg OF AFLATOXIN B_1

Calculated Amount of Aflatoxin (µg)	Deviation from 0.2845	Error of Mean as a Percent of the Mean
0.2845	0.0000	0.00
0.2892	+0.0047	1.65
0.2720	-0.0125	4.39
0.2906	+0.0061	2.14
0.2737	-0.0108	3.80
0.2922	+0.0077	2.71

Mean Standard Error of the Mean as a percent of the Mean = 2.45 ± 1.57

CHAPTER III

RESULTS

In the experimental system utilizing suspended ham portions, there was no growth of Aspergillus parasiticus noted on any of the samples. Subsequent extraction and cleanup procedures failed to reveal aflatoxin presence on TLC plates spotted with extracts from the ham tissues. Likewise, the spectrodensitometer could not detect aflatoxin presence on spotted areas of the plates other than standards.

In the second experiment, lean tissue increased aflatoxin production in sucrose supplemented yeast extract broth by 27.3 percent while the presence of fat supressed aflatoxin formation by 34.5 percent (calculated from Tables 10-13). The fungal mat of A. parasiticus was most evident in the lean tissue supplemented broth, and showed least growth with comminuted fat tissue. Visual estimation of TLC plates under ultraviolet light showed most brilliance in spots of standards followed by lean extract spots, control extract spots and fat extract spots in decreasing order of intensity. Spectrodensitometric evaluations gave similar results. The aflatoxin production in terms of µg/g substrate and µg/spore inoculate were calculated using:

$$\frac{\text{Area under curve}}{\text{Area under standard}} \times \frac{\mu\text{l sample}}{\mu\text{l spotted}} = \text{total } \mu\text{g aflatoxin present}$$

For µg/g substrate: divide by weight of sample (i.e.: 75 or 80).

TABLE 10

PRODUCTION OF AFLATOXIN IN SUCROSE SUPPLEMENTED YEAST EXTRACT BROTH WITH NO TISSUE. (μg AFLATOXIN/g MEDIA AND μg AFLATOXIN/SPORE INOCULATED)

Sample Number	I		II		III	
	μg/g	μg/spore	μg/g	μg/spore	μg/g	μg/spore
1	4.690	1.4×10^{-5}	6.647	1.99×10^{-5}	6.361	1.9×10^{-5}
2	5.445	1.63×10^{-5}	4.196	1.25×10^{-5}	11.633	3.48×10^{-5}
3	3.469	1.04×10^{-5}	9.075	2.72×10^{-5}	4.074	1.22×10^{-5}
Mean	4.535	1.36×10^{-5}	6.639	1.99×10^{-5}	7.347	2.2×10^{-5}

TABLE 11

PRODUCTION OF AFLATOXIN IN SUCROSE SUPPLEMENTED YEAST EXTRACT BROTH WITH FAT TISSUE. (µg AFLATOXIN/g MEDIA AND µg AFLATOXIN/SPORE INOCULATED)

Sample Number	I		II		III	
	µg/g	µg/spore	µg/g	µg/spore	µg/g	µg/spore
1	4.660	1.49×10^{-5}	4.356	1.39×10^{-5}	4.537	1.45×10^{-5}
2	2.268	7.2×10^{-6}	6.610	2.11×10^{-5}	3.283	1.05×10^{-5}
3	2.268	7.2×10^{-6}	6.537	2.09×10^{-5}	1.846	5.9×10^{-6}
Mean	3.065	9.7×10^{-6}	5.834	1.86×10^{-5}	3.234	1.03×10^{-5}

TABLE 12

PRODUCTION OF AFLATOXIN IN SUCROSE SUPPLEMENTED YEAST EXTRACT BROTH WITH LEAN TISSUE (µg AFLATOXIN/g MEDIA AND µg AFLATOXIN/SPORE INOCULATED)

Sample Number	I		II		III	
	µg/g	µg/spore	µg/g	µg/spore	µg/g	µg/spore
1	3.902	1.24×10^{-5}	12.917	4.12×10^{-5}	8.943	2.85×10^{-5}
2	2.000	6.36×10^{-6}	11.722	3.74×10^{-5}	8.300	2.65×10^{-5}
3	5.800	1.84×10^{-5}	10.006	3.2×10^{-5}	7.166	2.29×10^{-5}
Mean	3.901	1.24×10^{-5}	11.548	3.39×10^{-5}	8.136	2.6×10^{-5}

TABLE 13

PRODUCTION OF AFLATOXIN IN SUCROSE SUPPLEMENTED YEAST EXTRACT BROTH WITH AND WITHOUT TISSUE (MEAN VALUES)

Replication Number	Tissue Type: Lean µg/g	Lean µg/spore	Fat µg/g	Fat µg/spore	None µg/g	None µg/spore
I	3.901	1.24×10^{-5}	3.065	9.7×10^{-5}	4.535	1.36×10^{-5}
II	11.584	3.69×10^{-5}	5.834	1.86×10^{-5}	6.639	1.99×10^{-5}
III	8.136	2.6×10^{-5}	3.234	1.03×10^{-5}	7.347	2.2×10^{-5}
Mean of the Means	7.136	2.51×10^{-5}	4.044	1.28×10^{-5}	6.174	1.85×10^{-5}

For µg/spore inoculate: divide by number of spores in innoculate (i.e.: 2.5×10^6). The results of these data are presented in Tables 10, 11 and 12. A summary of means is presented in Table 13. In all replicates, the mean toxin production was highest in lean supplemented broth, intermediate in broth with no tissue present, and lowest in broth supplemented with fat tissue.

The analysis of variance shown in Table 14 indicated that there was a highly significant difference among treatments ($p<0.01$). In addition, however, the F-value for replication was significant ($p<0.01$).

TABLE 14

SUMMARY OF ANALYSIS OF VARIANCE FOR TREATMENT[a] VS. REPLICATION[b] FOR AFLATOXIN PRODUCTION IN HAM SUPPLEMENTED YEAST EXTRACT BROTH

Parameter	Value
SSA	66.009263710
k-1	2.000000000
SSB	79.003083230
n-1	2.000000000
SSAB	36.492339210
(n-1)(k-1)	4.000000000
SSE	67.788764700
kn(m-1)	18.000000000
A	33.004631855
B	39.501541615
AB	9.123084802
C	3.766042483
FA	8.763743903
FB	10.488873078
FAB	2.422459343
SST	249.293450850

[a]Denoted as A.

[b]Denoted as B.

CHAPTER IV

DISCUSSION

The results of the first experiment indicated that there was no mold growth or toxin production on fat or lean ham tissues inoculated with A. parasiticus spores and incubated at 10, 21 and 32 C. Wilson and Jay (1975) found that A. parasiticus and A. flavus had the capacity to produce aflatoxin under certain conditions. Bullerman and Ayres (1968) demonstrated that molds on country cured hams included species of the A. flavus group which had toxin forming abilities. He therefore postulated that the presence of these molds on meats could constitute potential health hazards to human health.

In a study similar to the present experiment, Bullerman et al. (1969c) found that A. flavus and A. parasiticus could grow and produce aflatoxins on country cured hams during the aging process. Although mold growth and aflatoxin production was inconsistent, the researchers suggested ideal growth conditions. The most likely time for growth and toxin production was when the aging temperature increased to 28 to 30 C, but before the water activity of the hams became too low (below 0.65) to permit growth of the toxin molds. Conversely, low temperatures (10 C) and low humidities (65 to 70 percent) as well as high salt concentrations during aging of the hams would prevent mold growth and aflatoxin production.

In this experiment, the jars were sealed to create a micro-environment which would prevent gas exchange of any type. Atmospheric air contains 20.97 percent oxygen (Buckman, 1971) which is much higher than the 0.01 percent which has been reported to support growth of the mold, A. flavus (Follstad, 1966). Similarly, atmospheric air contains 0.03 percent CO_2 by volume (Buckman, 1971). Only at levels above 80 percent has CO_2 been shown to interfere markedly with mold growth and toxin production (Landers, et al. 1967). Therefore the environmental conditions of atmosphere in the individual jars should have been sufficient to support mold growth and toxin production and should not have inhibited either at the time the experiment was initiated. However, various oxidative reactions could have consumed the available oxygen and limited mold growth later in the experiment. The relative humidity requirement for mold growth and toxin production has been estimated at around 80 percent, but varies and must be established on different substrates (Diener and Davis, 1967). Bullerman et al. (1969c) used no humidity controls and reported growth and toxin production on country cured ham lean tissue under atmospheric conditions with undefined humidities. The A_w of tissues from hams used was initially 0.96 and 0.84 for lean and fat tissue respectively. As the time of incubation progressed, the tissues lost water and would have passed the optimum A_w of 0.64 established for toxin production.

Spores of A. parasiticus (2.5×10^6) were used in all inoculations to insure spore germination, 1.5×10^6 spores had been used in earlier experiments (Bullerman et al. 1969c).

The results of the second experiment demonstrated the possibility that ham tissue inhibits mold growth and aflatoxin production. Intermediates are known to build up during fat metabolism by fungi (Austwick and Ayerst, 1963). Competition between the fungi and these intermediates for the substrate can reduce or restrict the amount of aflatoxin formed in the system (Diener and Davis, 1969). This explanation could possibly account for the suppression of mold growth and toxin production noted in the broth incorporated with adipose tissue. However, the possibility that the fat tissue was merely an inert material and diluted the media cannot be ruled out as a possible explanation for decreased aflatoxin production in broth to which fat was added.

The analysis of variance demonstrated the difference in production of aflatoxin as influenced by the presence of different tissue types or the absence of tissue. However there was also a significant difference among replicates of various treatments. Therefore, although the experiment did demonstrate a significant difference in toxin production in broth containing various tissues, it is not possible from these data to clearly define the nature or extent of those differences.

CHAPTER IV

SUMMARY

This experimental study was undertaken to determine the effects of ham tissue type on growth of Aspergillus flavus and production of aflatoxin. Two experiments were involved, one utilized suspended ham portions, the other employed comminuted ham in control broth. The variables consisted of the different tissues, fat or lean, in each experiment. However, an additional variable, temperature, was also part of the first experiment.

The results of the first experiment suggested that ham tissue does not support growth of the mold or formation of the toxin. The second experiment demonstrated that tissue type does influence aflatoxin formation in a control media.

In view of these results, it has been suggested that the lack of aflatoxin production on fat tissue may be the result of inhibition of aflatoxin biosynthesis or lack of adequate nutrients. On the other hand, the lack of mold growth and toxin production on lean tissue is more likely the result of some other presently unknown factor. However, these data could also be artifactual and the result of low oxygen and water activity levels in the stored samples.

It would appear that further research is necessary before a proper assessment could be made concerning potential public health

hazards of molds present on the hams. Only then could the possible toxin producing molds be regarded harmless on hams for human consumption.

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