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THE EFFECTS OF WITHDRAWAL AND PROCESSING UPON THE LEVELS OF AFLATOXINS IN THE TISSUES OF PIGS FED A CONTAMINATED RATION

By

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ABSTRACT

THE EFFECTS OF WITHDRAWAL AND PROCESSING UPON THE LEVELS OF AFLATOXINS IN THE TISSUES OF PIGS FED A CONTAMINATED RATION

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Two trials were conducted to determine the amount of time necessary for tissue clearance from pigs fed an aflatoxin contaminated diet. There were 20 pigs in each trial, with 4 being fed the control diet and 16 being used to determine the time necessary for tissue clearance after removal from the contaminated diet. The spiked diets of trials 1 and 2 contained 551 and 355 ppb of aflatoxins B_1 and B_2, respectively. The feed of the control pigs in trial 1 was naturally contaminated with 20 and 31 ppb of aflatoxins B_1 and B_2, respectively, while in trial 2 the control feed was free of aflatoxins. The initial phase of each trial, in which the pigs were fed the control and spiked diets lasted for 42 days.

In trial 1 there was no significant difference between weight gains and organ weights of the control pigs and those fed the aflatoxin spiked diet. The control pigs on trial 1 gained 40 percent less with a 24 percent reduction in feed intake as compared to the controls in trial 2. Even the low levels of aflatoxins occurring naturally in the control diet resulted in small amounts of aflatoxins in the liver and kidneys of the control pigs in trial 1.

In trial 2, the basal diet was uncontaminated with aflatoxins, so the pigs fed the spiked diet had 30 percent heavier livers, gained 45 percent less weight and had a 32 percent reduction in feed intake. Aflatoxins were found widely distributed in all tissues of the pigs fed the spiked diet. The blood showed the lowest level of residual aflatoxins, followed by the spleen, muscle and heart in that order. The highest concentration of aflatoxins was present in the liver and kidneys. Except for these organs, the levels of the M_1 and M_2 metabolites were much lower than the parent aflatoxins. The mean percentage retention of aflatoxins was calculated to be 0.03 and 0.04 percent for B_1 and B_2, respectively.

The withdrawal trial showed that one day after placing the pigs on an aflatoxin-free diet there was a significant decrease in the aflatoxin levels in all organs and tissues. Two days following withdrawal from the contaminated feed, tissues of only one pig contained trace amounts of aflatoxins. Four days after placing the pigs on an aflatoxin-free diet, there were no detectable levels of aflatoxins in any of the tissues.

Processing and cooking resulted in some reduction of the aflatoxins in the meat. The differences on comparing raw and processed tissues, however, were not statistically significant. Although cooking the fresh tissues had the greatest effect in reducing aflatoxin levels, it was still not very effective, with a mean maximum reduction of only

26 percent. Thus, processing and cooking were not very effective in removing the residual aflatoxins.

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INTRODUCTION

The aflatoxins are a group of closely related metabolites produced by Aspergilli, principally A. flavus and A. parasiticus. These metabolites can occur as natural contaminants in animal feeds, as well as in a wide variety of food material used for human consumption.

Experimentally, aflatoxins have been shown to be potent hepatocarcinogens, and epidemiologically they are believed to be important human carcinogens. Aflatoxicosis, the disease caused in animals upon consumption of aflatoxin-contaminated feeds, has been described extensively in the literature in connection with presumptive poisoning of experimental animals, such as cattle, swine, turkeys, ducks and other animals.

Effects of aflatoxins in vivo vary with the dose, the duration of exposure, the species and the nutritional status of the animal affected. The aflatoxins may be acutely toxic when given in large doses, whereas, sub-lethal doses produce chronic toxicity and low levels of chronic exposure may result in carcinogenesis. The LD_{50} values for most species of animals varies from 0.5 to 10 mg/kg body weight.

The transmission of aflatoxins through farm animals to the human food chain is very important since studies have

shown that ingested aflatoxins may be deposited as the original aflatoxin, or as one of its metabolites. The levels of aflatoxins found in the tissues are far lower than the levels found in the contaminated feed per se. However, the risk from chronic response of humans from eating contaminated meat can not be under-estimated since prolonged exposure to low levels of aflatoxins in the diet can cause liver tumors in a number of species, including trout, ducklings and rats. Trout and some species of rats, which are very susceptible to the carcinogenic effect of aflatoxins, develop liver tumors upon exposure to only a few parts per billion.

Studies on metabolism and tissue deposition of aflatoxins in different animal species have shown that most ingested aflatoxins are excreted within 24 hours, either as the original aflatoxin or as one of its metabolites. According to these studies most aflatoxins are biotransformed by the cytoplasmic or microsomal enzymes in the liver to more polar derivatives, which can undergo further conjugation with endogenous compounds, such as the active forms of glucuronic acid, sulfate, glutathione and amino acids. The aflatoxin metabolites have increased water solubility and are more efficiently removed from the body than the original aflatoxin.

The present study was designed to investigate various means of decreasing the levels of aflatoxins in the tissues of pigs and in meat products prepared from contaminated tissues. The amounts and kinds of aflatoxins carried over

into the tissues of pigs fed on an aflatoxin-contaminated diet were determined by analyzing the tissues at zero days withdrawal. The remaining pigs were placed on the uncontaminated control diet and killed at different time intervals, to determine the length of time required for tissue clearance. Finally, contaminated tissues were used to determine the effects of curing, smoking and cooking upon the levels of aflatoxins in hams and bacons.

REVIEW OF LITERATURE

Occurrence of Aflatoxins

The term mycotoxin is derived from the Greek words mykes meaning fungus and toxicum meaning poison or toxin (Goldblatt, 1972). Thus, the term literally means toxins from fungi. All fungi, including Aspergilli produce a large number of metabolites, which according to Steyn (1977) can be divided roughly into two categories: (1) molecules of primary metabolic and structural importance to the organism, and (2) those performing secondary or no obvious functions in the cells producing them. Mycotoxins are included under the latter category. Unlike bacterial toxins, mold toxins are not proteins, and in comparison to botulinal and other bacterial toxins, their chemical structure is simpler (Hesseltine, 1979).

As shown by Bu'Lock (1975) and cited by Steyn (1977), the mycotoxins are genotypically specific and are produced by a consecutive series of enzyme-catalyzed reactions from a primary pool of small molecules, such as acetate, malonate, mevalonate, pyruvate and amino acids. According to Weinburg, (1971) the most probable role of the secondary metabolites is for insuring an orderly disposal of intermediates that accumulate when the cell stops dividing. The process

provides a safety valve, without which resting cultures of cells would die.

Many secondary metabolites are extremely toxic to other forms of life. For example the antibiotics fall under this category. Others, such as the mycotoxins, are mutagenic, carcinogenic or teratogenic (Hesseltine, 1979). There is some question involving the magnitude of the diseases caused by mycotoxins. Recently, Hesseltine (1979) reviewed the occurrence of diseases in man and animals since the beginning of recorded history, and concluded that many diseases were caused by the metabolites of fungi.

The aflatoxins are a group of closely related metabolites produced by Aspergilli, principally by A. flavus and A. parasiticus (Hesseltine, 1968). They are by far the most extensively studied and the most important of the mycotoxins. Experimentally, aflatoxins have been shown to be potent hepatocarcinogens (Lancaster, 1961; Wogan, 1973). Epidemiologically, they may represent important human carcinogens (Campbell and Stoloff, 1974). Aflatoxicosis , the disease caused in animals upon consumption of aflatoxin-contaminated feeds, has been described extensively in the literature in connection with presumptive poisoning of experimental animals, such as cattle, swine, turkeys, ducks and a host of other animals (Newberne and Butler, 1969).

The discovery of aflatoxins started in England in the early 1960s with a severe toxic outbreak of a condition, which became known as turkey X disease because of the loss

of at least 100,000 turkey poults (Blount, 1961). In addition, ducklings and other young farm animals were also affected (Asplin and Carnaghan, 1961). Generally, the affected birds suffered loss of appetite, showed lethargic signs and wing weaknesses before dying. In most cases, postmortem examination revealed hemorrhages or pale necrotic lesions in the livers, and frequently engorged kidneys. Brazilian groundnut (peanuts) meal in feed given to birds was suspected to be the poisoning agent (Blount, 1961).

A similar incident occurred with poultry feed containing Brazilian groundnut meal in East Anglia, where 14,000 ducklings died within 4-5 weeks. No fatal cases, however, were observed on a ration from which the groundnut meal was removed (Asplin and Carnaghan, 1961). Later an outbreak of a disease occurred in pigs, which was apparently caused by an unknown toxic factor (Loosmore and Harding, 1961). A similar disease in cattle, reported to be indistinguishable from ragwort poisoning, was described the same year by Loosmore and Markson (1961). The cause of disease in these animals was traced to the presence of Brazilian groundnut meal in their rations (Loosmore and Markson, 1961).

The possibility that groundnut toxicity was not restricted to Brazilian groundnut meal was confirmed by Sargeant et al. (1961b), who gathered samples from 13 other countries. They detected toxicity in the samples from India, Uganda, Tanganyika, French West Africa, Nigeria, Gambia and

Ghana. Toxicity was also observed with maize meal by Allcroft and Carnaghan (1963) and in cotton seed cake by Loosmore et al. (1964).

Lancaster et al. (1961) fed rats with 20 percent Brazilian groundnut meal in a purified diet for 6 months and obtained multiple liver tumors in 9 out of 11 animals. Two animals also developed lung metastasis. These tumors did not occur in association with cirrhosis, cell necrosis, or cellular infiltration, thus it was assumed that the toxic agent directly affected the liver cells.

A fatal disease (exudative hepatitis) was observed in guinea pigs by Paget (1954). Symptoms of the disease were manifested by the appearance of small lesions on the liver, pancreas and lymphoid tissues, and production of ascites. However, the cause of the disease was attributed to a nutritional deficiency (Paget, 1954). The same disease was reported by Stalker and McLean (1957), and they suggested that it might be due to some contaminant in the diet. These reports were later shown by Patterson et al. (1962) to be the earliest cases of aflatoxin poisoning.

A. flavus had been implicated earlier as a producer of mycotoxins by Kulik (1957), who reported that extracts from peas contaminated with A. flavus were toxic to cats and rabbits. A. flavus was then implicated in the poultry mycotoxicosis when Forgacs et al. (1958) isolated a toxin-producing strain from grain, which produced the "hemorrhagic syndrome" in poultry.

A disease in dogs, referred to as hepatitis X (Seibold, 1953) was investigated by Newberne et al. (1955). It was traced to a diet containing peanut meal. Although the disease was reproduced by feeding the toxic meal, the exact nature of the etiological agent was not elucidated. Later, a fatal disease in swine and cattle characterized by liver lesions was reported by Burnside et al. (1957), who associated the disease with the incidence of moldy corn in the feed. They were able to isolate pure cultures of toxic strains of A. flavus and also of Penicillium rubrum. They found that sterile corn spiked with these molds and fed to animal produced similar symptoms to those observed in the disease, but the emphasis at this time was placed only in the P. rubrum culture.

Bailey and Groth (1959) later showed that the moldy corn poisoning of swine was the same as hepatitis X disease found in dogs. Wilson et al. (1967) described a similar toxicosis in swine produced by the administration of crystaline aflatoxins.

Isolation of Aflatoxins

Sargeant et al. (1961a) extracted the toxic principle in samples of Brazilian ground meal, and concentrated it 250 times. They used exhaustive soxhlet extraction of the sample with methanol, followed by further extraction of the methanol extract with chloroform and then defatted the final extract with petroleum ether. The final extract was fed by

intubation to turkey poults and ducklings and produced histological liver lesions identical to those seen in field outbreaks of turkey X disease.

The identity of the toxic substance in groundnut meal was unknown at this time, but Sargeant et al. (1961a) strongly suggested that it was of fungal origin. Furthermore, they ruled out the possibility that the toxic substance was a pyrrolizidine alkaloid or its N-oxide. The toxic principle was further purified by the same group (Sargeant et al., 1961b) using alumina chromatography. This step yielded colorless crystals. Although the preparation was not yet pure, it fatally affected 1-day-old ducklings within 24 hours on administering a dosage of 20 μg. Considerably less produced the characteristic histological liver lesions. The crystaline product isolated by Sargeant et al. (1961b), when chromatographed on Whatman no. 1 paper with 5 percent acetic acid in n-butanol, gave a single spot with an Rf value of 0.7, and emitted a bright-blue fluorescence under UV light. Of great significance was the fact that the amount of material present estimated visually corresponded with the toxicity of the samples as determined biologically (Sargeant et al., 1961a). Thus, a chemical assay for aflatoxin was developed.

Sargeant et al. (1961b) finally confirmed that the toxin was of fungal origin. First, they produced pure cultures of certain of the fungal species present in highly contaminated samples of peanuts. When an extract of

A. flavus was chromatographed on paper, a fluorescent spot with an Rf value of 0.7 was obtained. The material from this spot was toxic to ducklings and produced symptoms associated with turkey X disease. Therefore, Sargeant et al. (1961b) used paper chromatography to isolate a fluorescent material with an Rf value of 0.7 from the toxic peanut meal. It was then found to be identical to the material present in extracts from pure cultures of A. flavus. The toxic material was named aflatoxin in view of its origin.

It was soon found that the toxin obtained after paper chromatography was still a complex mixture. Nesbitt et al. (1962) using alumina chromatoplates with chloroform-methanol (98.5:1.5) were able to resolve the material from paper chromatography into two fluorescent spots under UV light. One had an Rf value of approximately 0.6 and exhibited a violet-blue fluorescence, while the other migrated slightly more slowly and exhibited a green fluorescence. For convenience, these authors referred to them as aflatoxin B and G, respectively. Nesbitt et al. (1962) also reported the melting points, chemical formulas and molecular weights, along with other physical and chemical characteristics of the aflatoxins B and G by using ultraviolet and mass spectrometry.

De Iongh et al. (1962) used silica gel column chromatography to purify a crude extract of groundnut meal isolated by the procedure of Sargeant et al. (1961a,b). The crude extract was sequentially eluted with chloroform and

methanol. Aflatoxins were only detected in the chloroform fraction, which was dried and transferred to Kieselgel TLC plates and developed with chloroform: methanol (98:2). This resulted in several spots, which exhibited different fluorescent colors under UV light. They named the different spots FB_1, FB_2, FB_3 etc. When the extracts of the spots from several plates were administered to ducklings alone or in combination together, some differences in the degree of toxicity were found.

Smith and McKernan (1962) also used silica gel Kieselgel G for the chromatographic separation of aflatoxins from extracts of highly toxic strains of A. flavus. They used chloroform-methanol-formic acid (95:5:1) as the solvent and separated at least 12 clearly defined spots, which fluoresced under UV light. Five of the spots caused the characteristic liver lesions in ducklings. These authors also introduced a confirmatory spray test for aflatoxins based on the change in fluorescence of the aflatoxins under UV light. The color changed from a blue or green color to a bright, yellow color when the chromatograms were sprayed with 50 percent sulfuric acid.

Hartley et al. (1963) were the first to report the isolation and characterization of the four main aflatoxins, which they named aflatoxin B_1, B_2, G_1, and G_2. A crude mixture from sterilized groundnut meal, which had been previously inoculated with a toxin producing strain of A. flavus, was resolved into several fluorescent spots. They used

silica gel G and chloroform-methanol (98:2) as the solvent. The four aflatoxins were identified on the chromatoplates, and further isolated and purified using silica gel G column chromatography. They concluded that the materials previously described as aflatoxin G (Nesbitt et al., 1962) and aflatoxin FB_1 (De Iongh et al., 1962) were identical to aflatoxin G_1 and B_1, respectively. Hartley et al. (1963) also demonstrated that the material originally called aflatoxin B by Nesbitt et al. (1962) was a mixture of aflatoxin B_1 contaminated with aflatoxin B_2.

Hartley et al. (1963) reported the isolation of the four main aflatoxins, Van der Merwe et al. (1963) demonstrated that aflatoxin B_2 is the dihydro-derivative of aflatoxin B_1. They synthesized aflatoxin B_2 by catalytic hydrogenation of aflatoxin B_1 with the uptake of one molar equivalent of hydrogen. Vander Merwe et al. (1963) also showed that aflatoxin G_2 is the dihydro-derivative of aflatoxin G_1 and can be produced in the same manner. They then put forward tentative structures for aflatoxins B_1 and G_1. The true structures of the aflatoxins, which differed slightly from those put forward by Van der Merwe et al. (1963), were finally elucidated and confirmed later by further studies (Asao et al., 1963, 1965). The structures of aflatoxins B_1, B_2, G_1 and G_2 are shown in Fig. 1.

The first indication of occurrence of aflatoxins other than B_1, B_2, G_1 and G_2 was reported by Allcroft and Carnaghan (1963). They found that extracts of milk from cows fed

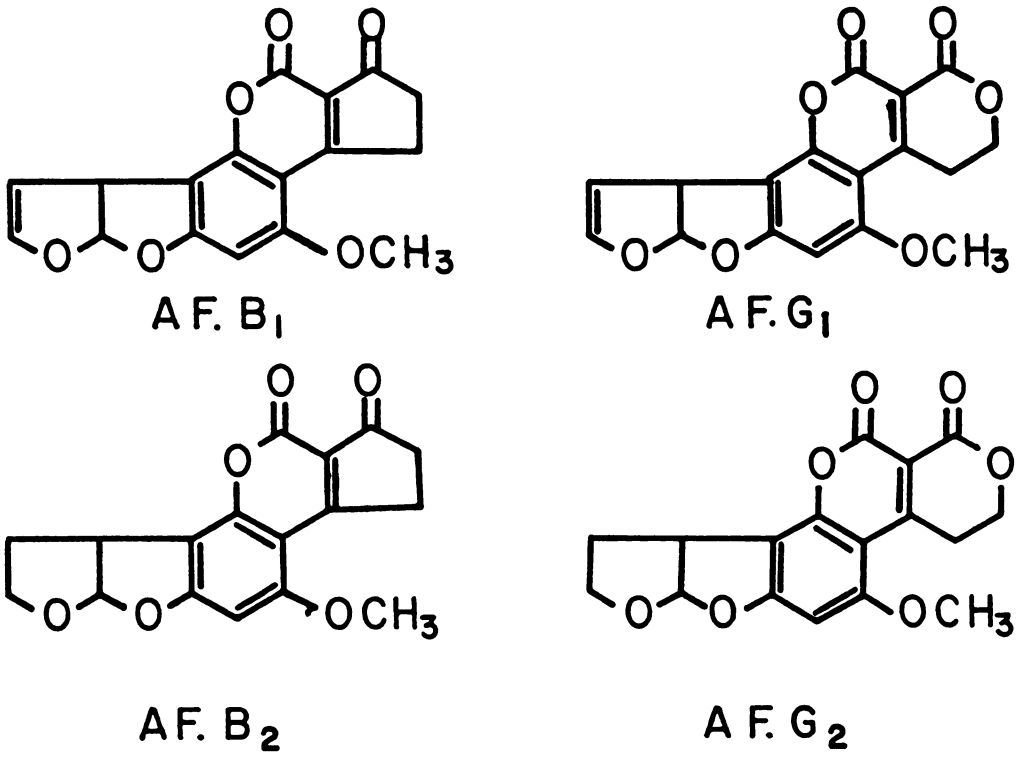

Figure 1 - Structures of aflatoxins B_1, B_2, G_1 and G_2.

aflatoxin containing toxic groundnut meal induced liver lesions identical to those caused by aflatoxin B_1. TLC examination showed that there was no aflatoxin B_1 present. The milk toxin, as it was named, was shown to be identical to a blue-violet fluorescent component also present in toxic groundnut meal (De Iongh et al., 1964). Allcroft and Carnaghan (1963) concluded that the toxic factor in milk resulted from metabolism of aflatoxin B_1 by the animal rather than from direct ingestion, since rats fed pure aflatoxin B_1 excreted the same metabolite found in milk. Allcroft et al. (1966), also isolated the milk toxin from the urine of sheep fed aflatoxin B_1 and confirmed its chromatographic equivalence to the milk toxin. They further proposed the generic name aflatoxin M for the toxin found in the milk.

Holzapfel et al. (1966), repeated the experiments of Allcroft et al. (1966), isolated aflatoxin M from the urine of aflatoxin-dosed sheep and separated it into two components using paper chromatography. The blue-violet component was named aflatoxin M_1 and the violet spot aflatoxin M_2. On the basis of ultraviolet, infrared, nuclear resonance and mass spectral data, these authors identified the structures of aflatoxins M_1 and M_2. Masri et al. (1967) later confirmed the structure of M_1. It was postulated that aflatoxin M_1 was the hydroxylated derivative of aflatoxin B_1, with the hydroxyl group in the C-4 position of the terminal furan ring, and that aflatoxin M_2 was the dihydro-derivative of aflatoxin M_1 resulting from the hydrogenation of aflatoxin

M_1. The structures of aflatoxins M_1 and M_2 are shown in Fig. 2.

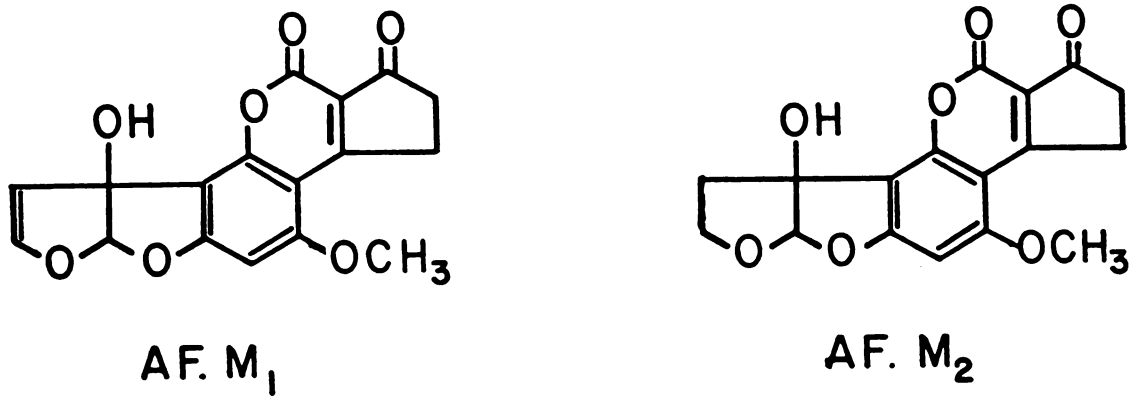

Figure 2 - Structures of aflatoxins M_1 and M_2.

Two additional forms of aflatoxin were isolated by Dutton and Heathcote (1966) from cultures of A. flavus. They concluded that the two new aflatoxins were hydroxy derivatives of aflatoxins B_2 and G_2, and were thus, named aflatoxin B_{2a} and G_{2a}, respectively. Later they elucidated the structures and biochemical properties. They found that aflatoxins B_{2a} and G_{2a} were much less toxic to ducklings than the other aflatoxins (Dutton et al., 1968).

Subsequently, improvement of the analytical techniques for extraction of aflatoxins from animal tissue and development of TLC procedures with suitable solvent systems and the use of HPLC and mass spectrometry have enabled researchers to identify many other forms of aflatoxin metabolites: (1) Aflatoxin P_1 - a demethylation product of aflatoxin B_1 identified as a major metabolite of aflatoxin B_1 in rhesus monkeys

(Dalezios et al., 1971); (2) Aflatoxin Q_1 - isolated from post-mitochondrial liver preparations from rat, monkey and humans as well as from monkey urine (Masri et al., 1974; Adenkule, 1977; Buchi et al., 1974); (3) Aflatoxicol-isolated from post-mitochondrial liver preparation of humans, rabbits and several avian species, in addition to being the major metabolite in the plasma of rats dosed with aflatoxin B_1 (Patterson, 1973; Patterson and Roberts 1971, 1972a, 1972b; Salhab and Edwards, 1977; Wong and Hsieh, 1978); and (4) Aflatoxin B_1-epoxide-formed through the metabolic epoxidation of the 2,3 vinyl ether double bond of aflatoxin B_1. This metabolite has not yet been isolated, probably because of its instability and great reactivity, but studies have strongly supported its formation in vivo and in vitro by incubation of aflatoxin B_1 with post-mitochondrial liver fractions (Garner et al. 1971, 1972; Swenson et al., 1973, 1975, 1977; Roebuck et al., 1978).

Metabolism of Aflatoxins

Drugs and other foreign compounds enter the body mostly by absorption from the gastrointestinal tract, from which they are taken via the portal vein to the liver, where they may be chemically modified through different types of reactions, such as oxidation, reduction, hydrolysis and conjugation (Kappas and Alvares, 1975). Their essential effect is to convert lipophilic or fat-soluble compounds into hydrophilic or water soluble substances (Kappas and Alvares, 1975).

The modified products may either flow into the bile to be excreted in the feces or to the systemic circulation before being taken to the kidneys and voided in the urine. Other sites of drug metabolism are located in the lungs, kidneys, the skin and the gastrointestinal tract itself (Blumberg, 1978).

The microsomal mixed function oxygenase, an enzyme complex located in the endoplasmic reticulum of the cell, catalyzes the metabolism of various drugs, carcinogens, steroids, insecticides, and other compounds (Conney, 1967), including the aflatoxins (Garner et al., 1971, 1972). More than 200 compounds can influence the activity of this complex and these have been divided in two general classes (Conney, 1967): (1) the phenobarbital type, which enhances the formation of cytochrome P-450 and the activity of mixed function oxygenase toward several substrates; and (2) the polycyclic aromatic hydrocarbon type, exemplified by 3-methylcholanthrene and benzo(a)pyrene, which enhance the activity toward a limited number of substrates and stimulate the formation of different types cytochromes, cytochrome P_1-450 (Sladek and Mannering, 1966), and P-448 (Alvares et al., 1967). Evidence has accumulated to suggest that liver microsomal cytochrome P-450 is a mixture of several forms that can be identified on the basis of their electrophoretic patterns (Welton and Aust, 1974; Haugen et al., 1976), by their specificity for being induced by various chemicals (Conney et al, 1973) and by their catalytical and physical

properties as partially purified fractions (Ryan et al., 1975; Haugen et al., 1975). Aflatoxin B_1 is metabolized by the hepatic microsomal mixed function oxygenase system to a group of derivatives, such as aflatoxins M_1, Q_1, P_1, B_{2a} and aflatoxin B_1-epoxide (Campbell and Hayes, 1976). Aflatoxin B_1 can also be reduced by a cytoplasmic reductase to aflatoxicol (Wong and Hsieh, 1978).

Aflatoxin M_1 results from the ring hydroxylation of aflatoxin B_1 at the C-4 position (Fig. 3-pathway 1). It was one of the first aflatoxin metabolites to be discovered. It was first identified in milk from cows fed aflatoxin contaminated rations, thus aflatoxin M_1 was originally called "milk aflatoxin" (Allcroft and Carnaghan, 1963). Soon after Allcroft and Carnaghan (1963) discovered aflatoxin M_1 in milk, its presence was confirmed by De Iongh et al. (1964). It was later also shown to be present in the urine of animals (Holzapfel et al., 1966) and humans (Campbell et al., 1970), and in tissues of animals (Allcroft et al., 1966) ingesting rations containing aflatoxin B_1.

Aflatoxin P_1 is the phenolic metabolite resulting from the O - demethylation of aflatoxin B_1 (Fig. 3-pathway 2). Wogan et al, (1967) were the first to suspect that O - demethylation probably occurred during aflatoxin B_1 metabolism, in that approximately 25 percent of the radioactivity administered as ^{14}C-methoxy-labeled aflatoxin B_1 to rats appeared in the respired CO_2 within 24 hr. Shank and Wogan (1965) however, using ^{14}C-labeled ring carbons failed to demonstrate

its presence in the expired CO_2, which means that the ring cleavage either does not take place or that the products formed by the cleavage are not fully oxidized. The presence of aflatoxin P_1 was later confirmed by Dalezios et al. (1971) who identified it as the major metabolite in rhesus monkeys given a single intraperitoneal injection of ring labeled aflatoxin B_1.

Aflatoxin Q_1 is another hydroxylated derivative of aflatoxin B_1 and an isomer of aflatoxin M_1, with the hydroxyl on the beta carbon atom of the carbonyl of the cyclopentanone ring (Fig. 3-pathway 3). Aflatoxin Q_1 has been recently discovered in monkey (Masri et al., 1974) and rat (Adekunle et al., 1977) by liver incubations of aflatoxin B_1, and represents the major in vitro conversion of aflatoxin B_1 by human liver microsomes (Buchi et al., 1974).

Aflatoxin B_{2a} or aflatoxin B_1 hemiacetal results from the hydration of the 2,3 vinyl ether double bond in the aflatoxin B_1 molecule (Fig. 3-pathway 4). This transformation is accomplished readily by liver microsomes of a variety of domestic and laboratory animals (Patterson, 1973). Acid catalyzed addition of water to the vinyl double bond of aflatoxin B_1 also leads to the formation of aflatoxin B_{2a} (Pohland et al., 1968; Pons et al., 1972).

Aflatoxicol or aflatoxin R_o results from reduction of carbonyl group in the cyclopentanone ring of aflatoxin B_1 (Fig. 3-pathway 5). This pathway is especially prominent in the livers of rabbits and several avian species, however, unlike the previous metabolites, the reduction is not

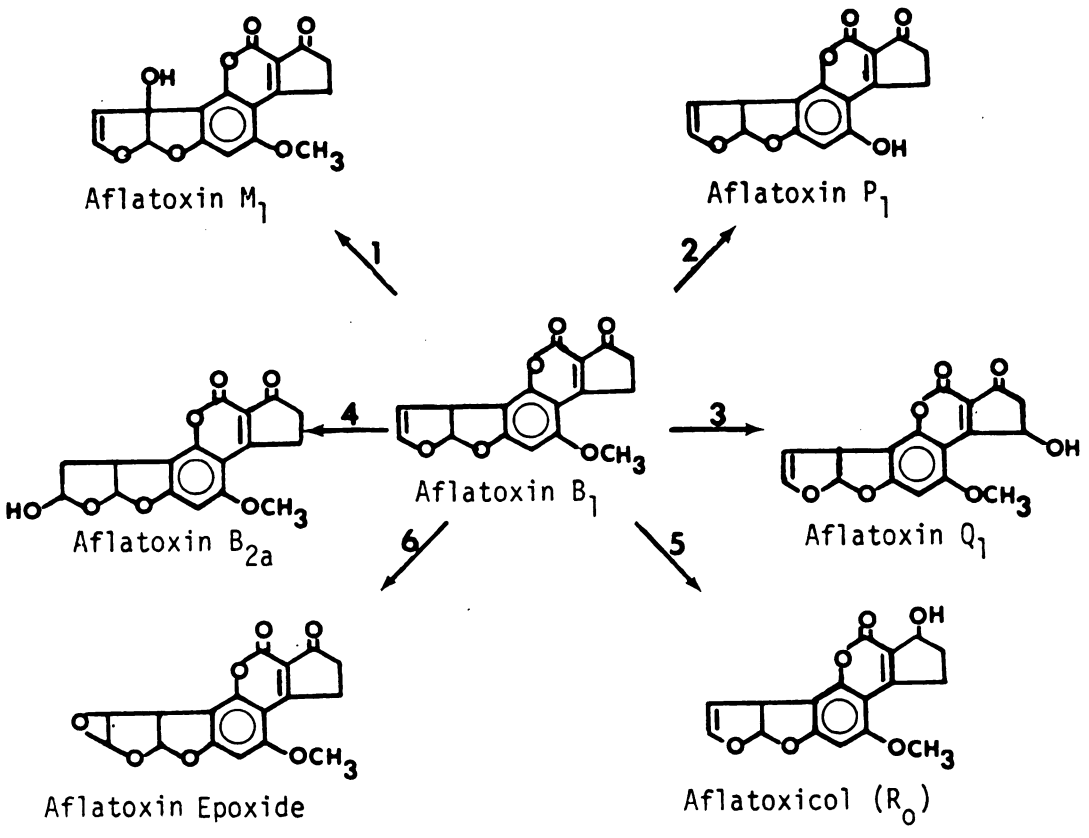

Figure 3.--Structural formulas for aflatoxin B_1 metabolites. Numbers indicate the pathways referred to in the thesis.

catalyzed by the microsomal mixed function oxidases, but by a NADP-linked dehydrogenase of the cytosol, which also has 17-ketosteroid dehydrogenase activity (Patterson, 1973; Patterson and Roberts 1971; 1972a; 1972b). Evidence for this pathway in post-mitochondrial liver preparations from humans has been recently presented by Salhab and Edwards (1977). Aflatoxicol has also been shown to be the major aflatoxin metabolite in the plasma of rats that are dosed orally or intravenously with ^{14}C-aflatoxin B_1 (Wong and Hsieh, 1978).

Aflatoxin B_1-2,3-oxide results from the metabolic epoxidation of the 2,3 vinyl ether double bond of aflatoxin B_1 (Fig. 3-pathway 6). Schoental (1970) was the first to suggest the possibility of formation of aflatoxin B_1-2,3 epoxide by hepatic microsomes. They developed this concept by analogy with the metabolic activation of polycyclic aromatic hydrocarbons. Although this labile molecular species has not been isolated, much indirect evidence has accumulated to substantiate its transient existence (Swenson et al., 1973; Croy et al., 1978).

Toxicity of Aflatoxins

A central issue in the toxicology of aflatoxins is the question of whether their biological activities are due to the effects of the toxins themselves, or indirect, and a consequence of structural alterations of the toxins per se during metabolism. According to Patterson (1973), experimental animals vary considerable in their abilities to

metabolize aflatoxins; the diversity of response suggesting that variation in metabolism may be important in determining the toxic action of aflatoxin B_1 in different species of animals.

Wong and Hsieh (1976) have studied the relative mutagenic potency of aflatoxins using the Ames test (Ames et al., 1975). They found that aflatoxin M_1 has only about 3 percent of the mutagenic potency of aflatoxin B_1. Except for aflatoxicol, which had 23 percent of the potency of aflatoxin B_1, all other metabolites were weaker mutagens than aflatoxin M_1. In the same study, Wong and Hsieh (1976) also reported that the activity of the aflatoxin metabolites, as found by the in vitro mutagenicity assay, correlated with their in vivo carcinogenic activity.

Recent studies on the metabolism and mode of action have provided evidence that aflatoxins require metabolic activation to elicit their carcinogenic effects. Wong and Hsieh (1976), using a Salmonella typhimurium mutant assay (Ames et al., 1975), have demonstrated that neither aflatoxicol nor aflatoxins M_1, Q_1, or B_{2a} possess activity in the absence of the activation factor from rat liver preparations. This indicated that none of these metabolites are the ultimate mutagenic and/or carcinogenic compounds. Magee (1974) reviewed the mechanism of activation of the carcinogenic compounds. He reported that many of these compounds require a metabolic activator to transform an inactive form into its ultimate mutagenic or carcinogenic species.

Pathway 6 in Fig. 3 (also shown in Fig. 4) seems particularly important in elucidating the mechanism of metabolic activation of aflatoxin B_1. Schoental (1970) first postulated that the formation of an epoxide intermediate of aflatoxin B_1 at its 2,3 double bond might account for its toxicity. Lijinsky et al. (1970) presented data showing the formation of nucleic acid and protein bound radioactive components after the administration of ^3H-aflatoxin B_1 to rats. They suggested that the aflatoxin B_1 or its metabolites might be covalently bound to these macromolecules.

Garner et al. (1971; 1972) demonstrated that incubation of rat hepatic microsomes with aflatoxin and a NADPH - generating system resulted in the formation of a reactive metabolite that was toxic to certain bacteria. The metabolite was not isolated but the inclusion of nucleophiles in the incubation medium, such as DNA and RNA, increased the survival of bacteria. Inhibition of the toxicity of the aflatoxin B_1 metabolite by the nucleic acids in the liver microsome mediated system suggested that RNA and DNA might covalently bind to the metabolite, since the complex did not dissociate when isolated and passed through a Sephadex column that would free any adsorbed aflatoxin.

Garner and Wright (1973) observed an increase in the amount of the lethal aflatoxin B_1 metabolite formed by phenobarbitone induced microsomes over those induced by 3-methyl cholanthrene or benzo(a)pyrene using the bacterial survival assay (Garner et al., 1971, 1972). Gurtoo and

Bejba (1974), using a different approach, reported that the epoxidation of aflatoxin B_1, measured as a DNA-alkylating metabolite, is enhanced by pretreatment of rats with phenobarbital, but not by 3-methyl cholanthrene. They also found that the addition of cyclohexene oxide, an epoxide hydrase inhibitor, did not increase binding of aflatoxin B_1 to form DNA adducts, but the possibility of an epoxide intermediate was not ruled out. Similarly, Garner and Wright (1973) did not detect any alteration in bacterial inhibition when cyclohexene oxide was added to the assay.

More evidence for the formation of the reactive aflatoxin B_1 derivative was reported by Swenson et al. (1973). They showed that on incubating aflatoxin B_1 in vitro with rat and hamster liver microsomes and RNA, a nucleic acid adduct was formed. Mild acid hydrolysis of the nucleic acid adduct liberated derivatives that were indistinguishable from 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 (Fig. 4). These observations strongly support the concept that aflatoxin B_1 is metabolically converted to an aflatoxin B_1-2,3-oxide, which binds covalently through its high electrophylic C-2 to nucleophilic sites on nucleic acids, probably at the N-7 or O position on the nucleotide residue. This hypothesis has been recently confirmed by Croy et al. (1978), who isolated the 2,3-dihydro-2-(^{7}N-guanyl)-3-hydroxy-aflatoxin B_1 as the principal product after hydrolysis of liver DNA of rats dosed with aflatoxin B_1.

These findings are in perfect agreement with Miller

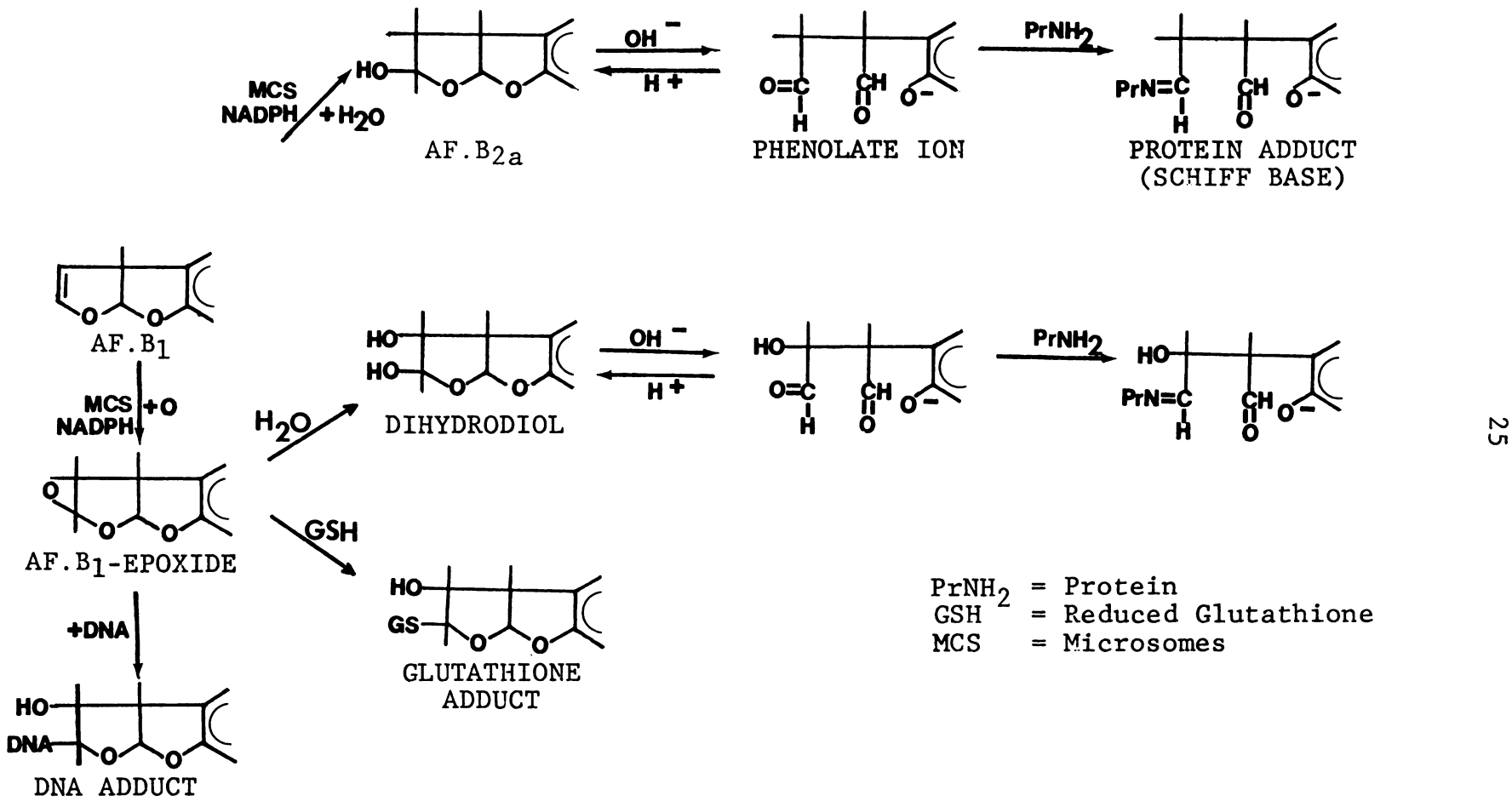

Figure 4 - Partial structures for aflatoxin B_1 metabolism showing two routes for metabolic activation to the hemiacetal (B_{2a}) and the 2,3 oxide (epoxide). Interactions of these metabolites with protein and DNA and inactivation of the epoxide by enzymatic reaction with reduced glutathione are also indicated (Patterson, 1977).

(1970), who analyzing the known or postulated forms of a variety of chemical carcinogens, made an assumption that most, and perhaps all, chemical carcinogens are either strong electrophilic reactants per se or are converted in vivo into potent electrophilic reactants. The electrophilic reactants then initiate the carcinogenic process through certain of their reactions with nucleophiles in crucial tissue components, such as the nucleic acids and proteins. Although the aflatoxin B_1-2,3-oxide has not yet been isolated or chemically synthesized, a more stable model compound, aflatoxin B_1-2,3-dichloride has been synthesized by Swenson et al. (1975). These authors have shown that the electrophilic analogue, aflatoxin B_1-2,3-dichloride, can mimic the biological effects of aflatoxin B_1-2,3-oxide in several biological and chemical systems.

Comparison of the carcinogenic activities of aflatoxins and the presence of the 2,3-vinyl ether double bond in the terminal furan ring seems to support the involvement of this functional group in toxicologic action. Roebuck et al. (1978) and Swenson et al. (1977) showed that aflatoxin B_2 is hepatocarcinogenic via dehydrogenation of the 2,3-carbon position in the terminal furan ring, thus generating aflatoxin B_1. Roebuck et al. (1978) also showed that the ratio of nucleic acid adduct formed from the two compounds was very similar to the ratio of their carcinogenic potencies, approximately 1:100 (aflatoxin B_2: aflatoxin B_1).

A recent study by Dagen and Neumann (1978) have

provided evidence for the existence of aflatoxin B_1-2,3-epoxide and also suggested an important role for glutathione (GSH) in the detoxification of the 2,3-epoxide (Fig. 4). These authors found that a glutathione conjugate, identified as 2,3-dihydro-2-(S-glutathionyl)-3-hydroxy aflatoxin B_1, was the major component in the bile from rats dosed with ^{14}C-aflatoxin B_1. They reported that the same conjugate was formed when a rat liver postmitochondrial supernatant was incubated with aflatoxin B_1 and ^3H-GSH.

The results of Dagen and Neumann (1978) were supported by Mgbodile et al. (1975), who showed that the depletion of the GSH levels in the livers of rats make these animals more susceptible to the toxic effects of aflatoxin B_1. Similarly, Allen-Hoffman and Campbell (1977) demonstrated that binding of aflatoxin B_1 metabolites to DNA is inversely related to the hepatic GSH levels.

Pathway 4 in Fig. 3 is also believed to be a mode for metabolic activation that depends upon the existence of the 2,3-vinyl ether double bond in the aflatoxin molecule. Patterson (1973) found that rabbit, duckling, guinea pig, mouse and chick liver microsomes all convert aflatoxin B_1 to B_{2a} at a rapid rate. However, rat liver microsomes were much less efficient. At physiological pH, aflatoxin B_{2a} rearranges itself to form a dialdehydic phenolate resonance bybrid (Fig. 4) which binds to protein by forming Schiff bases with free amino groups (Patterson and Roberts, 1970; 1972; Gurtoo and Campbell, 1974; Ashoor and Chu, 1975).

According to Patterson (1973), the metabolic conversion of aflatoxin B_1 to its hemiacetal, B_{2a}, is characteristic in the liver of animal species susceptible to acute aflatoxin poisoning. It is this form that interacts with vital functions of liver cells leading to hepatocellular necrosis. The lack of oral toxicity of aflatoxin hemiacetal *in vivo* (Pohland *et al.*, 1968) may be explained by its high protein binding capacity, and thus, its sequestration and further elimination along with the desquamated epithelial cells before it can be absorbed (Patterson, 1973). Thus, according to Patterson (1977), the activation of aflatoxin B_1 by metabolic conversion to hemiacetal or aflatoxin B_{2a}, which binds to various key enzymes and metabolically important liver cell structures, is responsible for the acute toxicity of the aflatoxin B_1.

Recently, Wong and Hsieh (1978) have suggested that both *in vitro* and *in vivo* formation of aflatoxicol (Fig. 3-pathway 5) may be an indicator of species sensitivity to aflatoxin induced carcinogenesis, and may be useful in the prediction of human susceptibility. They found that aflatoxicol was the major metabolite in the plasma of Sprague-Dawley rats, that were dosed orally or intravenously with ^{14}C-aflatoxin B_1. Aflatoxicol however, was not detected in the plasma of similarly dosed mice and monkeys, which are both resistent to aflatoxin B_1 - induced carcinogenesis.

Theoretically, aflatoxicol could be toxic since its intact vinyl ether double bond can undergo direct

bioactivation by forming a reactive 2,3-epoxide. In addition, aflatoxicol is the most potent mutagen among the known aflatoxin B_1 metabolites (Wong and Hsieh, 1976). Furthermore, the transformation of aflatoxin B_1 to aflatoxicol has been shown to be reversible (Patterson and Roberts, 1972b; Salhab and Edwards, 1977). The reversibility has been suggested to function as a reservoir for aflatoxin B_1 and its metabolites, which prolongs the cellular exposure to the carcinogens, and hence enhances their carcinogenic effects (Patterson, 1973).

According to Wong and Hsieh (1976) the 2,3-vinyl ether double bond does not seem to be sole molecular site on the aflatoxin B_1 molecule that determines mutagenic activity. They found that alterations occurring elsewhere in the molecule invariably resulted in reduction of toxicity. Thus, alteration of the cyclopentanone ring, such as substitution by a terminal lactone ring as in aflatoxin G_1, 7-hydroxylation, as in aflatoxin Q_1, or reduction of the keto-group as in aflatoxicol, results in significant lowering of the mutagenic potential, despite the presence of an intact 2,3-double bond.

The experimental evidence accumulated thus far indicates that of all of the isolated aflatoxins and their animal biotransformations products, aflatoxin B_1 has the optimal molecular structure for acute toxicity, mutagenicity and carcinogenicity. Any changes in the bis-furan ring, the cyclopentanone ring, and the methoxy structure of aflatoxin B_1 would result in marked reduction in biological activity.

These changes in activity as a result of such structural alterations may be interpreted not only in terms of susceptibility to epoxidation, but also intracellular transport, conjugation, excretion, and affinity for active or target sites (Wong and Hsieh, 1976).

Excretion and Tissue Distribution of Ingested Aflatoxins

Patterns of tissue distribution and excretion of aflatoxin B_1 and its metabolites following oral or parenteral dosing have been studied in several species. As discussed elsewhere herein, most ingested aflatoxins are biotransformed by the cytoplasmic and microsomal enzymes in the liver cells to form oxidation or reduction products (Stoloff, 1980). All of these alterations result in the addition of one or more hydroxyl groups to the molecule. These various initial reactions have been called Phase I reactions (Campbell, 1977), because many of the products undergo further conjugation with endogenous compounds, such as the active forms of glucuronic acid, sulfate, glutathione, amino acids, and proteins, (Stoloff, 1980). Conjugates of this type have enhanced water solubility and reduced the solubility in other solvents, such as chloroform.

The distribution of ring and methoxy-labeled ^{14}C-aflatoxin B_1 in the rat after a single intravenous injection was studied by Shank and Wogan (1965) and Wogan *et al.* (1967). They found that 70 to 80 percent of the injected ^{14}C was

excreted in the urine, feces and exhaled CO_2 during the first 24 hours following dosing. In addition, the amount of ^{14}C excreted in urine was similar for both compounds. Approximately 20 percent of each dose was excreted by this route, and 50-60 percent of this amount appeared within the first hour after administration. Shank and Wogan (1965) and Wogan et al. (1967) also reported that the amount of ^{14}C excreted in the feces and respiratory CO_2 was significantly different for the two compounds. Nearly 60 percent of the ring labeled activity was excreted through the bile into the feces, but only 22 percent of the activity of the methoxy labeled compound was excreted by this route. The ^{14}C content of the exhaled CO_2 from animals dosed with the methoxy labeled compound amounted to approximately 27 percent of the dose, while only about 0.5 percent of the ring labeled ^{14}C appeared in the CO_2 fraction. The low levels of ^{14}C found in the CO_2 from the metabolism of the ring compound was indicative that ring cleavage either does not occur or that the products formed by cleavage are not fully oxidized. The high levels of ^{14}C in the exhaled CO_2 from the animal dosed with the ^{14}C-methoxy labeled aflatoxin B_1 indicated that O-demethylation represents an important pathway in the metabolism of aflatoxin B_1 for rats.

Wogan et al. (1967) also reported that the retained radioactivity was present mainly in liver and kidneys. Similar results have been reported by Arora et al. (1978), who monitored the tissue distribution of ^{14}C-aflatoxin B_1 in

mice using whole body autoradiography.

Bassir and Osiyemi (1967) also reported that bile is a major route of excretion of ring labeled ^{14}C-aflatoxin B_1 in rats. They showed that there was a latent period of 60 minutes between the time of injection and the first appearance of activity in the bile. The activity reached a maximum at 75 minutes after administration of the dose and then decreased rapidly. In the same study Bassir and Osiyemi (1967) isolated aflatoxins B_1 and M_1 from the urine and bile of the dosed rats. Aflatoxin M_1 was present in the urine and bile, mainly as glucuronide and taurocholate conjugates, respectively. Allcroft et al. (1966) in experiments with rats dosed with pure aflatoxin B_1 showed that the metabolism of aflatoxin B_1 to form milk toxin (aflatoxin M_1) occurs in the liver. Furthermore, they showed that the appearance of milk toxin in liver and systemic blood coincided within hours with the disappearance of aflatoxin B_1, after which the milk toxin also disappeared within a period of days.

Mabee and Chipley (1973) found approximately 8 percent of the administered radioactivity in the tissues of chickens dosed with ^{14}C-aflatoxin B_1. An important finding of these investigators was that the majority of radioactivity observed in the tissues was confined to the aqueous buffer extract, and only 9 percent of the total radioactivity was found to be extractable with chloroform. They reported that aflatoxin M_1 glucuronide was the major metabolite present. They also concluded that other metabolites of aflatoxin B_1 were

present, possibly as sulfate conjugates. In a similar experiment Chipley et al. (1974) found that protease treatment of the aqueous phase of tissue extracts from chickens fed ^{14}C-aflatoxin B_1 resulted in the release of 50 percent of the bound aflatoxin in the form of aflatoxin B_{2a}.

Sawhney et al. (1973) also studied the distribution of radioactive material in the tissues of laying hens at 1, 4 and 7 days following administration of ^{14}C-ring labeled aflatoxins. They found that 71 percent of the ^{14}C dose was eliminated in the excreta within 7 days following administration. The remaining ^{14}C activity was detected in the tissues and eggs at the various intervals following treatment. They also found that bile is a major route of excretion for the ingested aflatoxins. In the same study Sawhney et al. (1973) reported that the liver, crop, gizzard and fecal material were toxic when fed to ducklings. They also reported a half life of 66.8 hours for the ^{14}C activity retained in the body of laying hens.

Dalezios et al. (1971) identified aflatoxin P_1 as the principal urinary metabolite of aflatoxin B_1 in rhesus monkeys. They found that aflatoxin P_1 comprises approximately 60 percent of the urinary aflatoxin derivatives, with 50 percent being present as glucuronide, 10 percent as sulfate, and 3 percent as the unconjugated phenol. Together, these metabolites accounted for over 20 percent of injected dose of aflatoxin B_1.

Keyl and Booth (1971) reported that dairy cows fed a ration containing a daily dosage between 67 and 350 mg of aflatoxin B_1 secreted 70 to 154 ppb of aflatoxin M_1 in the lyophilized milk. They also observed that the aflatoxin disappeared rapidly from the milk after withdrawal from the ration.

Working with lactating cows fed a ration containing graded levels of aflatoxin B_1, Polan et al. (1974) found a minimum dosage of 46 ppb of aflatoxin B_1 was necessary in the ration before aflatoxin M_1 could be detected in the milk. They also reported that aflatoxin M_1 was not detected in the milk after 2 days following withdrawal of aflatoxin B_1 from the ration.

Stoloff (1980) has reviewed the occurrence of aflatoxin M_1 in milk of cows from several controlled feeding studies. He concluded that from 1 to 3 percent of the ingested aflatoxin B_1 is excreted as aflatoxin M_1 in milk. He also showed that aflatoxin M_1 begins to appear in milk about 12 hours after the cow consumes the toxin, reaches the maximum in 3 to 4 days and is present in milk for 4 to 5 days after the withdrawal of the contaminated feed. Stoloff (1980) also reported that the time required for disappearance of aflatoxin M_1 from the milk bore no relation to the level of aflatoxin in the feed or the level in the milk before withdrawal of the contaminated feed.

Occurrence of Aflatoxins in Foods of Animal Origin

According to Jarvis (1975) mycotoxins can enter the food chain in several ways, with the routes of contamination being either direct or indirect. Direct contamination occurs as the result of mold growth on the food material *per se*. Almost all foods are susceptible to mold growth during some stage of production, processing, storage or transport. Indirect contamination of foods by mycotoxin occurs as the result of contamination, such as occurs in the fermentation products of fungal origin or from animal products contaminated from eating a moldy feed containing aflatoxins.

Armbrecht (1971) and Rodricks and Stoloff (1977) have reviewed the occurrence of aflatoxins or their metabolites in the tissues and by-products from animals ingesting aflatoxin-contaminated feeds. According to these authors, the levels of aflatoxins found in the tissues of animals fed aflatoxin-contaminated feeds is far lower than the levels found in the contaminated feed *per se*. This is due to the efficient metabolism and excretion of absorbed aflatoxins by most animals. In addition, studies with ring labeled aflatoxins have shown that most of administered dose ends up in the tissues and excreta of the dosed animals, appearing mainly as water-soluble conjugated aflatoxin metabolites (Mabee and Chipley, 1973; Chipley *et al.*, 1974; Hayes *et al.*, 1977). The toxicological potential of these compounds has not been established experimentally. Thus, the hazard from ingesting these types of

animal products which contain aflatoxins is much lower than that from ingesting foods directly contaminated (Hayes et al., 1977).

A summary of the data from various studies is shown in Table 1 and presents the ratios for the level of aflatoxin B_1 in feeds in relationship to the level likely to be encountered in selected animal tissues (Rodricks and Stoloff, 1977).

Table 1 - Ratios of Aflatoxin B_1 Levels in the Feed in Relation to Aflatoxin B_1 or M_1 Levels in Edible Tissues (Rodricks and Stoloff, 1977)

ANIMAL	TISSUE	AFLATOXIN IN TISSUE	FEED TO TISSUES RATIO
Beef Cattle	Liver	B_1	14,000
Dairy Cattle	Milk	M_1	300
Swine	Liver	B_1	800
Layers	Eggs	B_1	2,200
Broiler	Liver	M_1	1,200

Most of the egg and milk data shown in Table 1 are based on a continuous feeding regimen. The liver data are based on slaughter 18 to 24 hours after the last exposure to aflatoxins. As shown in Table 1, milk is the animal product most vulnerable to aflatoxin contamination, particularly since feed consumption and lactation are concurrent events (Stoloff, 1979).

Allcroft and Carnaghan (1962, 1963) were the first to investigate the transmission of aflatoxins to the tissues of

animals fed a contaminated ration. Allcroft and Carnaghan (1962) showed that extracts of milk from cows fed aflatoxin-contaminated rations induced lesions identical to those produced by administration of aflatoxin B_1 directly to ducklings. The milk toxin, which was later named aflatoxin M_1, was finally isolated by Allcroft and Carnaghan (1963) and subsequently by De Iongh et al. (1964) from the milk of cows fed aflatoxin B_1.

Allcroft and Carnaghan (1963) using one-day-old ducklings for assay (Asplin and Carnaghan, 1961) failed to demonstrate toxicity from the livers and eggs of hens fed a ration containing 15 percent of toxic peanut meal, or from the clotted blood serum and livers from cows fed a concentrate ration containing 20 percent of toxic peanut meal, or from a pig liver taken from an animal with fatal aflatoxicosis. The sensitivity of the duckling assay was later established by Wogan (1964) as 2 μg during five days of treatment. Allcroft and Raymond (1966) using a chemical assay later found that the toxic peanut meal was contaminated with 10 and 0.2 ppm of aflatoxin B_1 and B_2, respectively.

Platonow (1965) was unable to demonstrate aflatoxins or their metabolites in extracts of liver or from the skeletal muscle of chickens fed a toxic peanut meal ration (3.1 ppm aflatoxin) during periods for as long as six weeks. He used ferrets for the biological assay. Samples of meat and livers of the chickens fed the toxic rations were also

extracted and analyzed according to the method of Heusinkveld et al. (1965). This method was originally proposed for analysis of aflatoxins in peanuts and peanut meal.

A systematic study was made by Kratzer et al. (1969) on the effect of graded levels of dietary aflatoxins on the performance of broilers under simulated practical conditions. No adverse effects were detected when a ration containing 400 ppb of aflatoxins was fed to chickens from one day to eight weeks of age. At higher levels (800 and 1600 ppb) adverse biochemical effects were detected in the liver. Using the method of Wiley (1966), Kratzer et al. (1969) found no evidence of aflatoxins in the meat, liver or blood of broilers fed 1600 ppb of aflatoxins for 60 days prior to slaughter. Similarly, they observed no aflatoxins in the eggs, meat, liver, or blood of hens fed a ration containing 2700 ppb of aflatoxins for a period of 48 days. The method of Wiley (1966) used for the chemical assay was a modification of the procedure of Pons and Goldblatt (1965) and was originally proposed for analysis of aflatoxins in cottonseed, peanuts, and a variety of other commodities. According to Kratzer et al. (1969) as little as 3-5 ppb of aflatoxin B_1 could be detected by this method.

Keyl and Booth (1971) also conducted a feeding trial with swine, beef cattle, dairy cattle and poultry to determine the the adverse effects of graded levels of aflatoxins in the ration. Samples of meat, eggs and milk from these animals were analyzed chemically by the method of Wiley

(1966) to determine if aflatoxins were transmited into these products. In growing-fattening swine, no evidence of toxic effects was observed at aflatoxin levels of 230 ppb or less. In a swine reproduction experiment, no adverse effects were detected in the pigs produced from sows fed 450 ppb of aflatoxins. No toxic effects were observed in beef steers fed aflatoxins at levels of 300 ppb or lower for 4.5 months. No adverse effects were discernible in broilers fed a ration containing 400 ppb of aflatoxins from one day to eight weeks of age. No aflatoxins were detected in the meat from swine and cattle fed rations containing 800 and 1000 ppb of aflatoxins, respectively. Lyophilized meat from broilers fed 1600 ppb of aflatoxins for eight weeks likewise contained no detectable aflatoxins. All the animals fed a high aflatoxin dosage exhibited signs of aflatoxicosis, including lowered feed conversion, organ enlargement, proliferation of fibrous tissue in the liver, reduced appetite, abnormal serum chemistry, histopathological deviations and high mortality.

Allcroft et al. (1966) were able to isolate aflatoxins M_1, B_1 and G_1 from liver, kidneys and urine of sheeps two hours after ingestion of a dose of 1 mg of mixed aflatoxins/kg of body weight in a ratio of 36:52:3:2 of B_1, B_2, G_1, G_2, respectively. They assayed the tissues according the procedure of De Iongh et al. (1964), which is a modification of the method developed by Broadbent et al. (1963).

Van Zytveld et al. (1970) extracted aflatoxins or aflatoxin metabolites from the livers and skeletal muscle

of chickens, which had ingested a daily dose varying between 0.09 and 0.61 mg of aflatoxin over a six weeks period. Aflatoxins or their metabolites were only detected in tissues from birds which were severely affected as a result of ingestion. The tissues were assayed according the method of Eppley (1966), which was originally used for analysis of aflatoxins in peanuts.

Mabee and Chipley (1973) administered ring labeled aflatoxin B_1 (0.1 mg/kg/day) to broiler chickens by crop intubation. The radioactivity in the liver, heart, gizzard, breast and leg accounted for 7.8 percent of the total ^{14}C administered. These authors prepared a pooled sample of lyophilized radioactive excreta, blood, organs and tissues and extracted them with sodium acetate buffer. According to analysis, 81,2 percent of the radioactivity observed in the combined sample was confined to the sodium acetate buffer extract. Testing of the sodium acetate buffer for the presence of conjugated aflatoxins, followed by treatment with β- glucuronidase and subsequent chloroform extraction revealed that 31.5 percent of the total radioactivity originally present in the buffer extract was transferred to the chloroform extract. The presence of aflatoxin M_1 was further confirmed by TLC of the chloroform extract. In the same study, Mabee and Chipley (1973) proposed that laying hens can metabolize the majority of aflatoxin B_1, if it is administered at relatively low levels. Aflatoxin conjugates were the predominating form of the metabolite. They also reported

that aflatoxin M_1 glucuronides constituted 38.9 percent of the total conjugates extracted by the sodium acetate buffer. They concluded that other forms of metabolites of aflatoxin B_1 were present, possibly as sulfate conjugates.

Allcroft and Roberts (1968) measured the amount of aflatoxin M_1 in milk from cows given diets containing various levels of aflatoxins. The daily intake of aflatoxin B_1 ranged from 0.875 to 24.5 mg, with excretion of aflatoxin M_1 being proportional to intake. Keyl et al. (1968) also reported a linear relationship between aflatoxin intake and the concentration of aflatoxin M_1 in the milk. McKinney et al. (1973) fed cows an aflatoxin B_1 contaminated ration which resulted in the consumption of 148.5 mg of aflatoxin per cow over a 14-day period. They reported aflatoxin B_1 levels of 0.1 ppb or less in the liver, a trace amount in the kidney and none in muscle or heart. For aflatoxin M_1, they found levels of 0.1 ppb in the liver, 0.05 to 0.30 ppb in the kidney, less than 0.05 ppb in heart and none in muscle.

Hayes et al. (1977) administered aflatoxin B_1 twice daily for 14 days to four lactating cows fed a concentrate ration containing either 10, 50, 250 or 1250 ppb of aflatoxin B_1. Twenty-four hour prior to slaughter, ^3H-labeled aflatoxin B_1 was included with the final feeding. They reported that only the animal fed 1250 ppb of aflatoxin B_1 showed detectable chloroform-soluble metabolites in the tissues. However, significant radioactivity was detected in the tissues of the cow fed 250 ppb of aflatoxin B_1. Based on

the recoverable radioactivity, the skeletal muscle and liver of the cow fed 1250 ppb of aflatoxin B_1 contained 1.7 and 0.3 ppb of the unidentified chloroform-soluble metabolite. The highest concentration of chloroform soluble metabolites was found in the kidneys, at a level below 1 ppb.

In the same study, Hayes et al. (1977) incubated ^{14}C-ring labeled aflatoxin B_1 with bovine liver preparations. They observed that about 15-22 percent of aflatoxin B_1 was metabolized to aflatoxins M_1, Q_1 and two unidentified metabolites. Some 61-64 percent of the original aflatoxin B_1 was found in the aqueous fraction, no aflatoxin B_{2a}, P_1 or aflatoxicol were found. The isolation of aflatoxin Q_1 in vitro, but not in vivo, led these authors to suggest that aflatoxin Q_1 might be conjugated in vivo, and thus, would be confined to the water-soluble fraction of the tissues.

Krogh et al. (1973) fed diets containing 300 and 500 ppb of aflatoxins B_1 plus B_2 to pigs for 120 to 230 days. During the growth period from 20 to 90 kg, the pigs on the aflatoxin-contaminated diets suffered impaired weight gains and lowered feed conversions. The majority of the animals exhibited typical signs of aflatoxicosis. Some of the animals died during the trial, showing severe liver degeneration. Aflatoxins B_1, B_2 and M_1 were found in the livers and kidneys of some pigs on the aflatoxin diet, mainly in those fed levels of 500 ppb. Heart, muscle and adipose tissue from some of the pigs also contained aflatoxins B_1, B_2 and M_1, but at very low levels. Aflatoxins were extracted from the

tissues according to the procedure of Pons and Goldblatt (1965), using a modified clean up step on a silica gel column. In the same work, Krogh et al. (1973) reported that aflatoxin B_1 added to homogenized liver at a level of 1 ppb showed a recovery of 100 percent. However, at 0.5 ppb the recovery was incomplete.

Murthy et al. (1975b) reported that the response of swine to aflatoxins depended on whether the aflatoxin-contaminated protein was fed separately or was incorporated into the total ration. They reported that pigs developed toxic symptoms when fed the aflatoxin source separately, and that aflatoxins B_1, B_2 and M_1 were found in the tissues. The pigs on the mixed diet did not develop toxic symptoms and no aflatoxin residues were found in the tissues of the only pig examined. Aflatoxin analysis of the tissues consisted of extraction of the toxins by methanol, a solvent partition of a methanol-water-chloroform solution, which was followed by silica gel chromatography as described by Brown et al. (1973). The extracts were further purified by liquid-liquid defatting with hexane, transferring the aflatoxins into chloroform followed by column chromatography on acidic alumina and anhydrous sodium sulfate.

Jemmali and Murthy (1976) proposed a modified method for the determination of aflatoxin residues in animal tissues, which was basically the same procedure used by Murthy et al. (1975a,b). The method consisted of extraction of aflatoxins from the sample with methanol and treatment of the

residue with a mixture of dimethoxymethane: methanol (4:1) to precipitate the proteins. Evaporation of the dimethoxymethane was followed by liquid-liquid defatting with hexane, and heating of the aqueous extract before transferring the aflatoxins into chloroform. The chloroform extracts were further purified by silica gel-acid alumina-anhydrous sodium sulfate column chromatography. The final dried extract was dissolved in chloroform, spotted and developed on TLC plates as described by Murthy et al. (1975a). Jemmali and Murthy (1976) analyzed the tissues of two adult pigs after they had been fed for 33 days on aflatoxin-contaminated peanut meal incorporated into a mixed ration. They divided each sample in two lots, one was assayed by the method of Brown et al. (1973) and the other by their own method. The values obtained by their procedure were higher than those obtained by the method of Brown et al. (1973). In addition, the modified method detected aflatoxin B_1, B_2 and M_1 in tissues, which were negative according to the procedure of Brown et al. (1973).

Monegue et al. (1977) worked with forty pigs to determine the minimum toxic level of aflatoxins and to monitor the aflatoxin residues in the tissues. Aflatoxin B_1 equivalent was added to the feed at 100, 200 and 300 ppb. They found that growth rate, feed consumption, feed efficiency and prothrombin time were not influenced at these levels. Similarly, the enzyme profiles indicated little liver damage. The liver weights were slightly elevated for the pigs

consuming aflatoxins, but kidney weights were not changed. In the same study, Monegue et al. (1977) did not find any residue of aflatoxins in the tissues of pigs fed an aflatoxin-contaminated ration. They concluded that 300 ppb of aflatoxins (B_1 equivalent) was below the minimum toxic dose under these experimental conditions. The aflatoxin analysis was carried out according the procedure of Brown et al. (1973).

Jacobson et al. (1978) found an appreciable amount of aflatoxin B_1 in the tissues from pigs fed 100, 200 and 400 ppb of aflatoxin B_1. Similarly, all the samples except two from muscle, contained a measurable amount of aflatoxin M_1. In the same study, Jacobson et al. (1978) established a linear relationship between the logarithmic plot of aflatoxin B_1 intake and the amount of residue in the tissues. They also suggested that liver is the best tissue to use for monitoring and demonstrating the transmission of aflatoxins into tissues. They assayed the tissued for aflatoxins according the procedure of Jacobson et al. (1971) with a modified clean up step as described by Wiseman et al. (1967).

Recently, Furtado et al. (1979) fed pigs a diet spiked with 662, 273, 300 and 285 ppb of aflatoxins B_1, B_2, G_1 and G_2, respectively. They reported that the pigs fed aflatoxins for 21 days had 36 percent heavier livers, gained 25 percent less weight, and ate 18 percent less feed than controls, but did not differ in efficiency of feed utilization. Inspection of the tissues of the pigs on the aflatoxin spiked

diet showed no observable gross of pathological lesions. Furtado et al. (1977) also reported that assay of liver, heart, kidney, spleen and muscle showed that there was some carry-over of aflatoxins B_1 and B_2 to all tissues, but G_1 and G_2 were not present. In addition, residues of the B_1 metabolites, aflatoxins M_1 and B_{2a}, were also found in all tissues of the pigs fed aflatoxins. They assayed the tissues for aflatoxins using the method of Trucksess and Stoloff (1979).

Shreeve et al. (1979) fed 4 cows a ration that contained either 385 or 1925 ppb of zearalenone and 20 ppb of aflatoxin B_1 or else 317 or 1125 ppb of ochratoxin A and 20 ppb of aflatoxin B_1. Although the concentration of aflatoxin B_1 was within the limits of the maximum allowance for aflatoxins in feeds (20 ppb), they found measurable amounts of aflatoxins B_1 and M_1 in milk, kidney and urine of cows fed the contaminated ration. For aflatoxin M_1, they found traces to 0.06 ppb in milk, traces to 0.2 ppb in kidney and 0.09 to 0.22 ppb in urine. Shreeve et al. (1979) suggested that interactions between the ingested mycotoxins might have contributed to the accumulation of aflatoxin M_1 in the tissues. For example, the mean level of aflatoxin M_1 detected in the kidneys of the cows fed aflatoxin B_1 plus ochratoxin A was at least twice as high as the levels found in the animals fed aflatoxin B_1 plus zearalenone.

Stoloff and Trucksess (1979) found detectable levels of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 in the livers of 4

cows which were receiving 0.29 mg of aflatoxins/kg body weight on 2 consecutive days. The cows were slaughtered 24 hours after receiving the second dose. Levels varied from 2.8 to 5.6 ppb and from 1.8 to 4.6 ppb for aflatoxins B_1 and M_1, respectively. In the same study, Stoloff and Trucksess (1979) assayed the livers of 4 pigs that had died after 35 to 42 days on a ration containing 1.5 ppm of aflatoxin B_1. They reported that aflatoxin B_1 was found at a level of 0.08 ppb in the liver of only one pig and no aflatoxin M_1 was detected.

The severe drought occurring in the southeastern U.S. in 1977 resulted in a decreased corn yield and also stressed the crop so it was more vulnerable to attack by insects, resulting in a heavy infestation of A. flavus before harvesting. A 1977 FDA survey in southeastern U.S. showed that only 40 percent of the corn crop met the FDA guideline of 20 ppb for total aflatoxins, in comparison to an average of 70 percent during the 1969-1976 period (Stoloff, 1979). Wilson et al. (1979) surveyed corn samples for aflatoxins from 31 counties in Georgia in the summer of 1977. They reported that before harvest 78 percent of the corn samples contained over 100 ppb of aflatoxins. Ten percent of the crop contained 0-22 ppb, 12 percent contained 21-100 ppb, 42 percent contained 101-400 ppb, 20 percent contained 410-1000 ppb, while 16 percent contained over 1000 ppb.

The unusually heavy contamination of the corn crop in southeastern U.S. provided evidence that cow's milk may

contain measurable levels of aflatoxin M_1. A 1977 survey showed that aflatoxin M_1 was found in milk at detectable levels (>0.05 ppb) in 63 percent of all southeastern U.S. samples, in 80 percent of the samples from the state of Georgia, and in one or more samples from 84 percent of all bottling plants in the sample area (Stoloff, 1980).

In Arizona in 1978, milk from cows fed cottonseed meal containing aflatoxins at the ppm level was found to have apreciable amounts of aflatoxin M_1 (Stoloff, 1980). Similar incidents were observed in 6 adjacents states due to the use of contaminated cottonseed meal before State and Federal regulatory authorities could control the situation (Stoloff, 1980). This culminated in the FDA establishing a maximum allowance of 0.5 ppb for aflatoxin M_1 in fluid milk (FDA 1977).

Stability of Aflatoxins in Foods

Although aflatoxins are fairly stable molecules, heat processing and storage may reduce the initial levels found in foods. Coomes et al. (1966) reported that autoclaving moist peanut meal for 4 hours at 120° C reduced the amount of aflatoxin from 7,000 to 350 ppb. Biological assay of the autoclaved meal showed a corresponding decrease in toxicity. In the same study, Coomes et al. (1966) autoclaved pure aflatoxin B_1 for 4 hours at 120° C. They also refluxed pure aflatoxin B_1 with water for 10 hours. They then compared the isolated products from the two model systems with the material formed after roasting. Based on the UV spectra

of the isolated compounds, they conclude that heating aflatoxin B_1 in presence of moisture causes hydrolytic opening of the lactone ring on the aflatoxin B_1 molecule, which can further be decarboxylated, leading to non-toxic breakdown products.

Mann et al. (1967) made a more detailed study of the effect of heat and moisture on aflatoxins in oil seed meals. They found that temperatures of 60 to 80° C have little effect on aflatoxins in oil seed meals, while substantial amounts of aflatoxin are degraded at 100° C. They reported that increasing the moisture content and/or the heating time caused a proportional decrease of aflatoxins in the meal. For example, heating meal containing 20 percent moisture for 2 hours at 100° C resulted in degradation of approximately 80 percent of the aflatoxin. Reducing the time to 1 hour at 100° C resulted in doubling of the residual aflatoxin level. Reducing the temperature to 80° C and heating for 1 hour resulted in a further doubling of the residual aflatoxin level. Peers and Linsel (1975), however, observed that aflatoxin B_1 was extremely stable in peanut and corn oil. They found that aflatoxin B_1 was not degraded in these oils until the temperature approached 250° C, which is close to the melting point of aflatoxin B_1.

Lee et al. (1969) studied the stability of aflatoxins under conditions simulating commercial dry and oil roasting of peanuts artificially contaminated with 130 to 6300 ppb of total aflatoxins. They found an average reduction of aflatoxin concentration ranging from 45 to 83 percent, depending on roasting conditions and initial aflatoxin levels. Oil

roasting times varied from 3 to 7 minutes with the temperatures varying from 325 to 345° F. Dry roasting times varied from 5 to 30 minutes, with the temperatures varying from 250 to 400° F. Similar results were obtained by Waltking (1971) upon roasting commercially rejected peanuts on a pilot plant scale under conditions simulating those used for making peanut butter. The aflatoxin levels for raw peanuts varied from 500 to 627 ppb of total aflatoxins. They reported an average loss of 40 to 50 percent and 20 to 40 percent for aflatoxins B_1 and G_1, and B_2 and G_2, respectively.

Conway et al. (1978) also obtained similar results roasting aflatoxin-contaminated corn. They showed a 40 to 80 percent reduction in aflatoxins could be obtained by a single passage of corn through a continuous roaster (temperatures ranging from 145 to 165° C). In a second experiment they combined the effects of the heat and ammonia treatments. They reported a reduction of 57 percent in the aflatoxins from corn tempered to 20 percent moisture with an aqua ammonia concentration of 0.5 percent NH_3 on a dry weight basis when passed through the corn roaster. When the corn was retempered as described above and again passed through the roaster, a further reduction in the aflatoxin concentration resulted. Reductions of over 90 percent were achieved by this process.

Ammoniation has been sucessfully used in the decontamination of several agricultural products contaminated with aflatoxins. A full account of the use of ammonia and other

methods of detoxification of aflatoxin-contaminated products are given by Marth and Doyle (1979).

Allcroft and Carnaghan (1963) reported that toxic milk from cows fed rations containing 20 percent toxic peanut meal did not show a reduction in toxicity after treatment by pasteurization or by roller-drying. They used the one-day-old duckling assay test (Asplin and Carnaghan, 1961) to measure the toxicity of the milk before and after processing. However, the duckling assay test for aflatoxin M_1 is not a particularly sensitive test (Wogan, 1964). Similar results were obtained by Stoloff et al. (1975) and Van Egmond et al. (1977), who observed no loss of aflatoxin M_1 from milk after different forms of heat treatments. In contrast to these reports, Purchase et al. (1972) showed that processing of milk reduces its aflatoxin M1 content, and that the higher the temperatures used the lower the amount of aflatoxins. Using chemical analyses, they reported a reduction of aflatoxin M_1 in milk of 33 percent by pasteurizing at 62° C for 30 minutes, and 80 percent upon sterilization at 80° C for 45 seconds. Similarly, they reported a reduction in the levels of aflatoxin M_1 from freeze-dried and spray-dried milk, as indicated by both chemical and the ducklings assay tests.

McKinney et al. (1973) reported that aflatoxin M_1 in liquid raw milk disappears very rapidily during storage. They found that in samples with low aflatoxin M_1 levels, approximately 40 percent of the initial aflatoxin M_1 was not

detectable after a period of 4 days, and approximately 80 percent disappeared during 6 days storage at 0° C. However, Stoloff et al. (1975) did not observe any decrease of aflatoxin M_1 levels in raw milk stored over a period of 17 days at 4° C. On the other hand, they observed a 45 percent reduction of aflatoxin M_1 in milk after a period of 120 days frozen storage (-18° C), with detectable changes starting at 68 days. McKinney et al. (1973) found an 87 percent reduction of aflatoxin M_1 in milk after 120 days storage under similar conditions, with detectable changes starting at 30 days.

Strzelecki (1973) reported that recovery of aflatoxin from raw ham, cured ham and salami decreased with storage time. Using different time periods and storage conditions, they found recoveries of 19 percent from salami, 16 percent in raw ham, and 7 percent for cured ham with the amount retained being related to the aflatoxin level added initially to the products. Murthy et al. (1975) also reported that there was a decrease in recovery of aflatoxin B_1 injected into beef with increased holding periods during storage. The total recovery of injected aflatoxin B_1 dropped from 98 percent after 20 days of storage to 79 percent after 183 days. They suggested that incomplete extraction due to interactions of aflatoxins with the meat constituents during storage probably accounted for the losses.

EXPERIMENTAL

Feeding Trial

Preparation of the Diet

In order to prepare the spiked ration, the standard aflatoxins were first extracted with chloroform and were then diluted to 2 liters in a volumetric flask. The chloroform solution was then divided into two equal fractions and each of them was slurried with 1 kg of finely ground feed (screened through a 20 mesh screen and dried over night in an oven at 100°C). The chloroform was allowed to evaporate in the dark over night with forced air under a hood.

Each sample of aflatoxin spiked feed was then homogenously mixed with additional feed in a 4-speed Reynolds Mixer (Reynolds Electric Co.) to give a final weight of about 7 kg of feed. Then each of the aflatoxin premixed feed samples was separately transferred to a stainless steel Wenger horizontal mixer (Wenger Mixer Mnfg. Co.), and homogenously mixed with additional feed to give a final weight of about 49 kg. The 49 kg lots were then transferred to a horizontal mixer (Bryant Poff. Inc.) and mixed with additional feed plus the vitamin and mineral supplements to give a final weight of 1400 kg. The two lots of aflatoxin-spiked feed totaled 2800 kg. The feed was packed in 23 kg bags and stored at

approximately 0° C until fed. The basal ration used for the control group was handled the same, except that it was not spiked with aflatoxins.

Experimental Animals

Forty crossbred pigs were used in two trials to determine the amount of time necessary for tissue clearance after feeding an aflatoxin contaminated diet. The pigs were housed in pens with aluminum slotted floors in an atmosphere controlled building. Each pen was equipped with a self-feeder and nipple-type waterer. Feed and water were offered ad libitum. The pigs were weighed at 7 day intervals except for the last weigh period, which was 6 days in length. Feed consumption was also recorded weekly. The basal diet consisted of corn and soybean meal and was fortified with vitamins and minerals. The composition of the diet is given in Table 2.

There were 20 pigs in each trial, with 4 being fed the control diet and 16 being used to determine the time necessary to obtain tissue clearance after removal from the contaminated diet. The spiked diets contained 551 and 355 μg of aflatoxin B_1 and B_2 per kg of feed, respectively. Since we had not previously found aflatoxins on analysis of the feed, the basal ration was assumed not to contain any contamination. However, analysis revealed that the basal diet on trial 1 contained 20 and 31 μg of aflatoxin B_1 and B_2 per kg of feed, respectively. In trial 2, the basal diet was uncontaminated with aflatoxins. The analysis of feed for

aflatoxins was carried out using a modification of the A.O.-A.C. method (1975).

In trial 1, preliminary data on the length of time required to obtain tissue clearance after removal of the aflatoxin contaminated diet was determined. The pigs averaged 9.6 kg initial weight and they were randomly assigned in three groups based on litter, weight and sex. There were 4 pigs in the control group and 8 pigs in each of the two experimental groups. At the end of the experimental feeding period four experimental pigs and four controls were slaughtered. The remaining twelve pigs were placed on the uncontaminated control diet and held for 1,2,4,8,16 and 32 days. At the end of each period two pigs were killed and the tissues were examined for aflatoxins.

Once the length of time for tissue clearance was established a second trial was carried out using larger members of animals to confirm the previous results and to give more detailed information.

The pigs on trial 2 averaged 9.6 kg initially and they were assigned into four groups according to litter, weight and sex. There were 4 pigs on the control group with 5,5 and 6 pigs in the experimental groups. As in trial 1, four controls and four experimental animals from trial 2 were killed after 42 days of feeding an aflatoxin-contaminated diet. The remaining 12 pigs were fed an aflatoxin-free diet for 1,2 and 4 days. After each period, 4 pigs were killed and the tissues were examined for aflatoxins. Analysis of the tissues

for aflatoxins were carried out using a modification of the method of Trucksess and Stoloff (1979).

Table 2 - Composition of the Ration[a,b]

Ingredients	Percentage
CORN-GROUND	75.35
SOYBEAN MEAL	21.85
MINERAL MIXTURE[c]	2.30
VITAMIN PREMIX[d]	0.50

[a]Feed analysis: protein, 16.5%; lysine, 0.80%; methionine + cystine, 0.55%; tryptophan, 0.19%; calcium, 0.67%, and phosphorus, 0.505%. [b]Digestible energy, 3436 kcal/kg. [c]Composition of mineral mixture as percentage of diet: Sodium chloride, 0.50; limestone, 1.00; dicalcium phosphate, 1.00; and the following in ppm: Se, 0.1; Zn, 74.8; Mn, 37.4; I, 2.7; Cu, 9.9; and Fe, 59.4. [d]The vitamin premix supplied the following per kilogram of ration: vitamin A, 3300 IU; vitamin D, 660 IU; vitamin E, 5.5 IU; vitamin K compound, 2.2 mg; riboflavin, 3.3 mg; niacin, 17.6 mg; D-pantothenic acid, 13.2 mg; choline, 110.0 mg; and vitamin B_{12}, 19.8 μg.

Slaughtering and Collection of Samples

The pigs were taken to the MSU Meat Laboratory at approximately 5 p.m. on the day preceeding slaughter. They were held off feed until approximately 6:30 a.m. the following day, when they were slaughtered. After slaughtering, the tissues were examined for possible gross lesions by a Michigan State Department of Agriculture Meat Inspector. Samples of blood, heart, kidneys, liver, muscle and spleen were collected from the pigs. The samples were weighed,

frozen and stored at -20°C for later analysis.

Processing of Meat Tissues

Preparation for Processing

Tissue samples were also taken to study the influence of different forms of cooking upon the stability of aflatoxins and to determine the effects of curing, smoking and cooking upon the levels of aflatoxins in hams and bacon. Hams, loins and bellies from both sides of the carcass from the 4 experimental animals in trial 1 (slaughtered at zero day withdrawal period) were collected and processed as follows: One of the cuts was divided in two halves, one half was used as the control and the other half was cooked. The other cut was cured, smoked and then divided in two portions, one of which was analyzed before cooking and the other after cooking. Using this procedure the effects of cooking and processing on the level of aflatoxins in the samples were determined by analyzing the meat, both before and after processing.

Curing Procedure

Ham Curing

The fresh hams were stitch pumped to 10 percent by weight. The pickling brine was prepared using the following: 6 lbs salt, 3 lbs sugar, 28 g sodium nitrite and 33 lbs of cold water. The solution was thoroughly mixed before pumping into the meat. After pumping, the hams were submerged

and held in an identical brine solution for seven days in a cold room at a temperature of approximately 6°C.

Bacon Curing

The fresh bellies were rubbed with a dry curing mixture and placed in plastic boxes inside a cooler at a temperature of approximately 6°C. The dry curing mixture was prepared using the following ingredients: 6 lbs. salt, 2.3 lbs. sugar and 7 g of sodium nitrite, per 100 lbs. of meat. The curing ingredients were thoroughly mixed by hand before rubbing the meat. The bellies were held in the curing room for a period of 7 days to allow for good distribution of the cure throughout the tissues.

Smoking-Cooking Schedule

After the seven days cure, both the hams and bellies were transferred to a Elek-Trol laboratory smokehouse (Drying Systems Inc.). They were then smoked-cooked to an internal temperature of 66°C using the schedule shown in Table 3.

Table 3 - Smoking-Cooking Schedule for Bacon and Ham

Time (min.)	Temperature (°C) Dry Bulb	Wet Bulb	Relative Humidity (%)
135	49	32	30
75	54	38	35
60	60	44	46
165	71	66	76

Smoke was applied throughout cooking using a midget size Mepaco smoke generator (Meat Packers Equip. Co.) utilizing mixed hard wood sawdust.

Cooking of Raw Hams

In preparing samples for cooking, the raw frozen hams were cut into slices about 2.5 cm thick using a hand meat saw. The frozen slices were thawed and then cooked in stainless steel pans at an oven temperature of 176°C. The final internal temperature was 76°C. The temperature was monitored by placing a thermometer in the meat tissues. To assure uniform cooking, the position of the meat pieces inside the oven was switched at regular intervals. The cooked tissues were then ground and stored as described earlier herein. The drip from each sample was collected and stored for subsequent analysis.

Cooking of Cured Ham Samples

Slices of cured ham were prepared similar to those described above. The ham slices were cooked in an electric oven at about 163°C, until an internal temperature of 71°C was reached. The cooked tissues were ground and stored as described earlier. The drip from the various samples was pooled and stored as outlined above.

Frying Belly and Bacon Samples

The belly and bacon samples were sliced into thin strips about 2.5 mm thick before frying. To assure good slicing, the samples were semi-frozen and then immediately sliced using an electric bacon slicing machine (Hobart Mnfg. Co.). The meat strips were then cooked for 3 minutes on each side using an electric fry pan (Sunbeam Appliance Co.) with the temperature set at 171°C. After cooking, the samples were ground and stored as described earlier. The drip was collected and the frying pan was washed thoroughly after cooking each sample in order to prevent any cross contamination.

Broiling of Loin Samples

The loin samples were placed in stainless steel pans and cooked with the oven set for broiling. They were then broiled until the top of each chop was lightly brown, at which time they were turned over until the other side was browned to the same extent. The internal temperature of the meat was monitored with a thermometer and the samples were removed from the oven at an internal temperature of 76°C. The cooked samples were ground and then stored as described earlier.

General Methods of Analysis for Aflatoxins

Sample Preparation for Extraction

The tissue samples were deboned and the collagenous tissues and excess fat were removed from the lean portion. All tissues, except for the internal organs weighing less than 100 g, were passed twice through a meat grinder (The Hobart Mnfg. Co.), using a plate having 3/16 inches diameter holes. The ground tissues were then thoroughly mixed before removing samples for analysis. The tissues were stored at -20 °C until analyzed. The internal organs (kidneys, hearts and spleens) were cut into small pieces without thawing and placed directly in the blender.

Extraction of Aflatoxins from Tissues

Extraction and analysis of aflatoxins from raw tissues and the processed samples were carried out according to a modification of the procedure of Trucksess and Stoloff (1979). About 100 g of raw tissue or 60 g of cooked tissue were blended for 2 minutes in a Waring blender at moderate speed with 42 ml of NaCl - citric acid solution (35 g NaCl + 4.8 g citric acid/100 ml H_2O). Then 300 ml of acetone was added to the homogenate while washing the sides of the blender jar. The tissue was blended for an additional 3.0 minutes at moderate speed followed by 2 minutes at high speed. The material was then filtered through fast filtering prefolded filter paper (Whatman 114 v), and the filtrate was collected

in a 250 ml graduate cylinder. After filtration was completed, the meat residue was discarded.

A total of 235 ml of the filtrate was transferred to a 500 ml Erlenmeyer flask. Then 20 ml of $Pb(OAc)_2$ solution (200 g $Pb(OAc)_2.3H_2O$ in 500 ml H_2O containing 3 ml of acetic acid and diluted to 1 liter with H_2O) and 150 ml of H_2O were added. The solution was stirred for 0.5 minutes using a magnetic stirring device, and then 10 g of $(NH_4)_2SO_4$ were added. The stirring was continued for an additional minute while 10 g of diatomaceous earth was added to the solution. The solution was allowed to stand for about 5 minutes before filtering through fast filtering folded filter paper into a 500 ml graduate cylinder. The residue on the filter paper was discarded.

Purification of the Aflatoxin Extract

Liquid-Liquid Partition

Exactly 325 ml of the filtrate were transferred to a 500 ml separatory funnel. Then 100 ml of petroleum ether (30-60°C b.p.) were added. The separatory funnel was shaken vigorously for about 1 minute. The layers were allowed to separate, and the lower aqueous-acetone layer was drained into a second 500 ml separatory funnel. The petroleum ether layer was then discarded. Then 50 ml of chloroform were added to the aqueous-acetone solution and the separatory funnel was shaken as before. After the layers separated, the

lower chloroform acetone layer was collected in a 500 ml flask. Aflatoxin extraction from the aqueous-acetone layer was repeated one more time using 50 ml of chloroform-acetone (1:1). The aqueous layer remaining after the chloroform extraction was discarded. In some of the samples, especially in cooked tissues, a small amount of water was trapped within the chloroform-acetone extract. The water was removed by passing the chloroform-acetone extract through a column packed with 10 g of anhydrous sodium sulfate. After the second chloroform-acetone extract was passed through the column as before, it was washed with an additional 50 ml of chloroform. The eluates were collected in a 500 ml flask. The chloroform-acetone extract was then evaporated to dryness in a rotary evaporator (Buchi, Switzerland) using a water bath setting of 40°C.

Silica Gel Column Chromatography

Chloroform was added to a 22x300 mm chromatographic column until the tube was 2/3 full. Then a ball of glass wool was placed in the bottom of the tube and approximately 5 g of anhydrous sodium sulfate were added to give a base for the silica gel column. Then 10 g of silica gel 60 (70-230 mesh ASTM-EM Laboratories Inc.), which had been previously slurried in 50 ml of chloroform, were added to the column. The silica gel was allowed to settle and then the chloroform was drained to about 2 cm above the top of the silica gel. About 2 cm of anhydrous sodium sulfate was layered

slowly on top of the silica gel. The excess of chloroform was then drained from the column until it reached the level of the upper layer of sodium sulfate.

The aflatoxin extract was dissolved in approximately 5 ml of chloroform-hexane (1:1) and then transferred to the column with a disposable glass pipet. The sides of the flask were washed three more times with 5 ml of chloroform-acetone (1:1) and the washings were added to the column. After each addition of chloroform-hexane extract, the column was drained to the top of the packing and the eluate was discarded. Any interferring substances were eluted from the column in 100 ml of the ether-hexane (3:1). The eluate was then discarded.

The aflatoxins were eluted from the silica gel column with 160 ml of chloroform-methanol (97:3). The eluate was collected in a 250 ml flask and evaporated to near dryness in a rotary evaporator as described earlier herein. The sample extract was dissolved in dichloromethane-acetone (1:1) and quantitatively transferred to 2 dram vials using a disposable glass pipet. The dichloromethane-acetone solution was evaporated to dryness on a N-Evap evaporator (Organomation Assoc.) under a gentle stream of nitrogen using a water bath setting at 50°C. Special care was taken to avoid overheating of the dry extract. The aflatoxin extract was then dissolved in a 100 μl of benzene-acetonitrile (9:1) and the vial was sealed with a teflon lined screw cap. For samples extracts with high aflatoxin concentrations, the volume of the benzene-acetonitrile (9:1) was adjusted to give the

proper concentration of aflatoxins for densitometric analysis. The vial containing the aflatoxins was shaken vigorously for about 1 minute on a vortex shaker before removing the samples for analysis.

Thin Layer Chromatography

As shown in Figure 5,6 and 7 three different sizes of TLC plates were used to carry out the aflatoxin analysis: (1) 10x10 cm, (2) 10x20 cm, and (3) 20x20 cm.

The 10x10 cm TLC plates (Figure 5) were made by cutting a precoated 20x20 cm silica gel plate (Sil-G-HR-25, Brinkman Instruments Inc.) into four equal parts. The TLC plates were cut using a glass cutter (Sargent-Welch, #S-39885). The 10x10 cm TLC plates were used for qualitative analysis of aflatoxins extracted from the raw tissues.

The plates were scored and spotted as shown in Figure 5. A 20 μl sample of the aflatoxin extract was applied to the sample spot with a 25 μl Syringe (Hamilton Co). Standards of 2.51, 0.88, 2.0 and 2.0 ng of aflatoxin B_1, B_2, M_1 and M_2, respectively, were spotted on the plates as standards in both directions of development.

The plates were developed in the first direction with chloroform-acetone (3:2) in an unlined and unequilibrated 1.5 liter beaker, which was tightly covered with aluminum foil. After the development in the first dimension was completed, the plates were removed from the beaker and dried under a hood for about 2 minutes. The plates were then

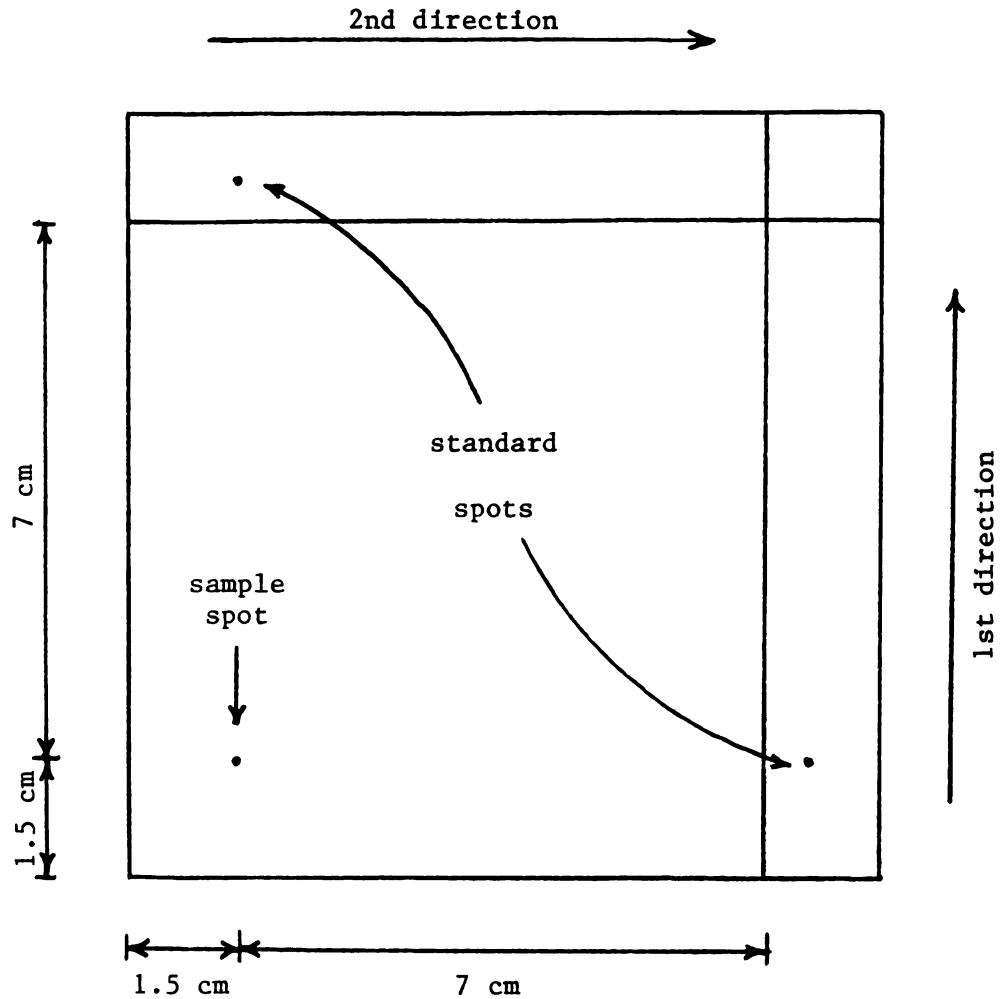

Figure 5 - Spotting and Scoring Pattern for Two-Dimension 10x10 cm TLC Plates.

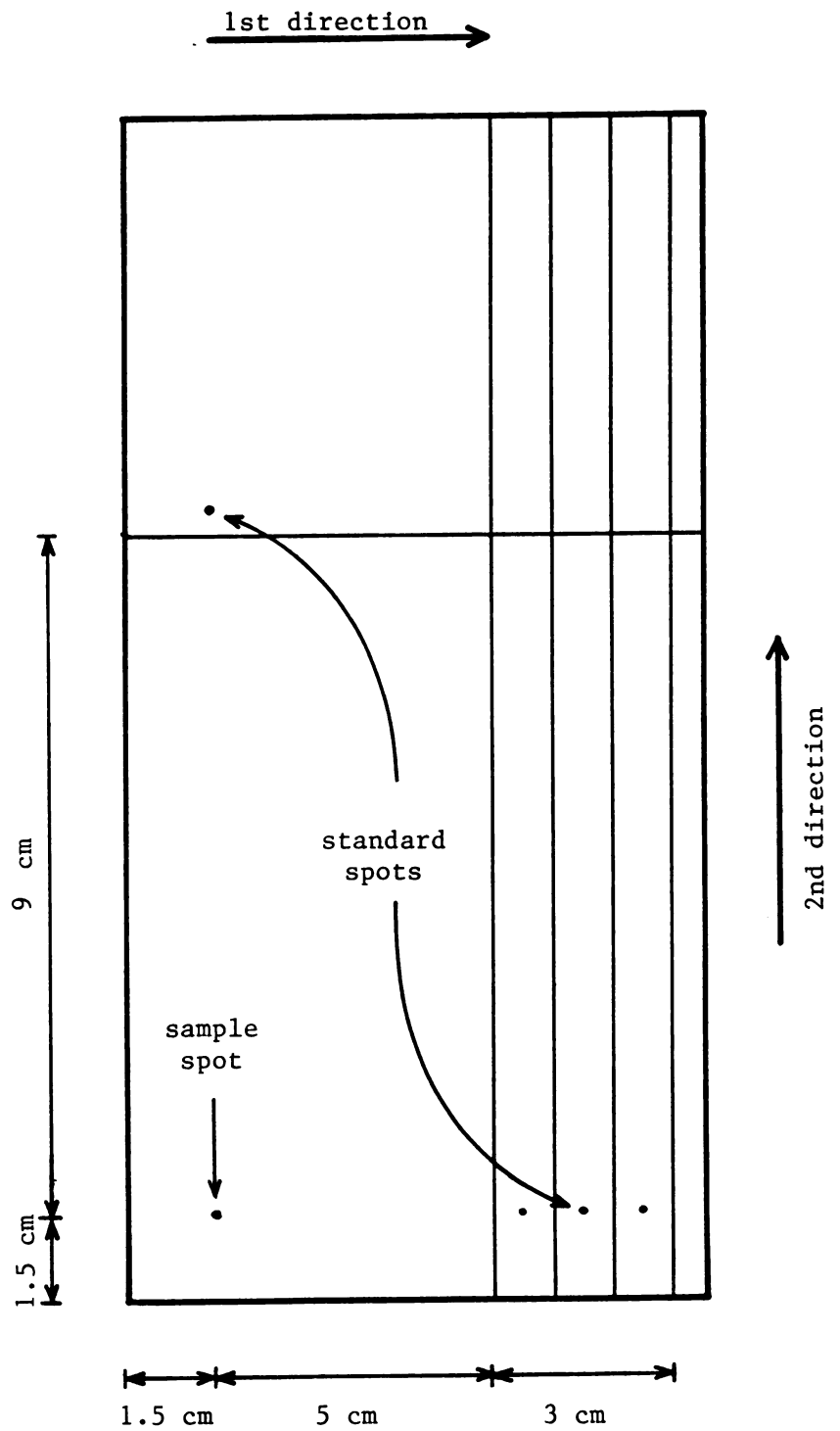

Figure 6 - Spotting and Scoring Pattern for Two-Dimension 10x20 cm TLC Plates.

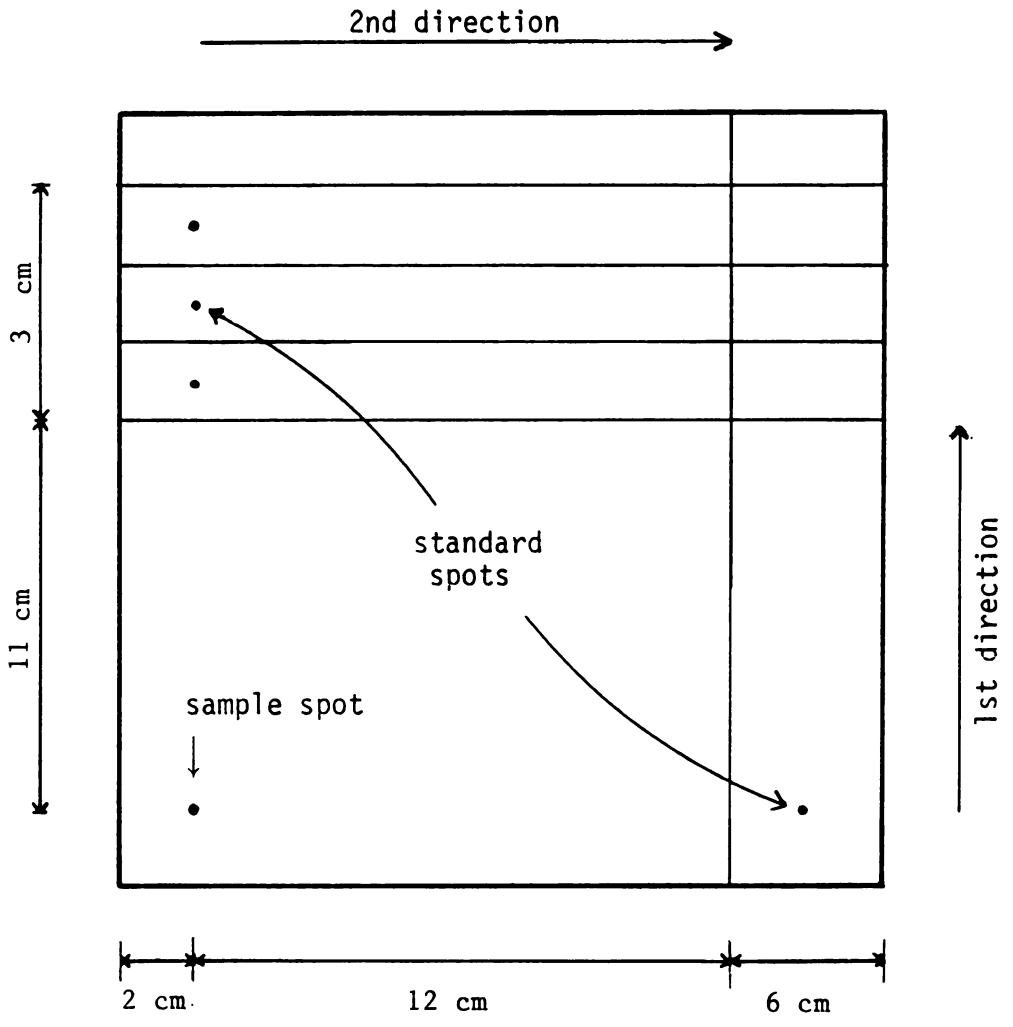

Figure 7 - Spotting and Scoring Pattern for Two-Dimensional 20x20 TLC Plates.

transferred to an oven with the temperature set at 50° C and dried for an additional 1 minute under a stream of nitrogen. After the plates were removed and allowed to cool for approximately 1 minute, they were developed in the second direction with anhydrous diethylether-methanol-water (90:8:2) as described earlier. After development was complete, the plates were removed, dried under a hood and then examined in a UV cabinet (Ultra-Violet Products, Inc.). The sample spots were compared with the reference standard spots in order to determine their chromatographic equivalence.

The 10x20 cm TLC plates were obtained by cutting a 20x20 cm precoated TLC plate in two halves as shown in Figure 6. These plates were used for quantitative analysis of aflatoxins extracted from raw tissues. The plates were scored and spotted as indicated in Figure 6 and as described earlier herein.

The plates were developed in the first direction with chloroform-acetone-isopropanol (85:10:5) in a sealed and unequilibrated tank. After the development in the first direction was completed, the plates were removed from the tank and dried as before. After evaporation of the solvent, the plates were developed in the second direction with anhydrous diethylether-methanol-water (95:4:1). After development in the second direction, the plates were dried 1 minute under a hood and prepared for densitometric analysis.

The 20x20 cm precoated silica gel plates were scored and spotted as shown in Figure 7. These plates were used

for quantitative analysis of aflatoxins extracted from the raw and cooked bellies, bacon and other cured and cooked tissues. The plates were developed in the first dimension with chloroform-acetone-isopropanol (87:10:3). After the development, the plates were dried as described earlier. The plates were then developed in the second dimension with anhydrous diethylether-methanol-water (95:4:1). They were then dried and prepared for densitometric analysis.

Densitometric Analysis of Aflatoxins

A double beam spectrodensitometer SD 3000-4 (Schoeffel Instruments) equipped with a 3380-A integrator (Hewlett-Packard) was used for quantifying the aflatoxin spots on the TLC plates. The plates were scored prior to spotting as shown in Figure 6 and 7 (Schoeffel Scoring Device - SDA 303), providing 10 mm strips. The average of three readings of the aflatoxin reference standards (spotted within the three strips parallel to the second direction of development) was used for densitometric comparison in calculating the concentrations of the sample spots. For the analysis of the standards spots, the scanning beam was focused on each of the three stips and scanning was carried out. In scanning the sample spots, the plates were viewed under UV light, and each spot was localized within two pencil marks made on the silica gel layer. The marks were about 1 cm apart and located approximately 3 mm ahead of the sample spot along the second dimension of development. The plates were then

placed on the plate carrier and with the beam of light set up at 540 nm (green light) so that the position of each spot on the plate was recorded by focusing the light beam within the two marks. Then each spot was scanned by driving parallel to the second dimension with the scanning beam. The spectrodensitometer was operated in the reflectance mode. For excitation, the monochromator was set to 365 nm and a secondary filter (430 nm band) was used to collect the emitted fluorescence around 425 nm. The secondary filter only permits the emitted or visible fluorescent light to pass into the phototube so that all ultraviolet light from the lamp is screened out.

Aflatoxins B_1, B_2, M_1 and M_2 concentrations were calculated according to the following formula:

$$\mu g/kg = (BxYxSxV)/(ZxXxW)$$

were:

B = Area of the aflatoxin peak in the sample spot,

Y = Concentration of aflatoxin standard in μg/ml,

S = μl of the aflatoxin standard,

V = Dilution of sample extract in μl,

Z = Area of aflatoxin standard peak (average of three replications),

X = μl of sample extract spotted on the plate,

and W = Grams of sample in the final extract.

The weight of tissue represented in the final aflatoxin extract (the value W in the formula above) was calculated based on the amount of water in the sample tissue, the

volume of extracting solution added to the tissue homogenate and the volume of filtrate collected in the two filtration steps. For bacon and belly samples, the volume of fat in the tissue was considered since fat also is soluble in the solvents. For lean tissues, the fat content was not accounted for in the calculation. The moisture and fat content of the tissues were determined by the A.O.A.C methods (1965).

The sample weight in grams (W) represented in the final aflatoxin extract, was calculated according to the following formula:

$$W = \frac{235 \text{ ml}}{(300\text{ml}+42\text{ml}+\text{ml of water in sample})} \times \frac{325 \text{ ml}}{(20\text{ml}+150\text{ml}+235\text{ml})} \times \text{initial weight of sample (g)}$$

Using the formula above, values of 235 and 325 ml are the volumes of the first and second filtrate, respectively. The other values refer to the volumes of extracting solutions added to the tissue homogenate.

Analysis of Aflatoxins in the Feed

Analysis of the feed was carried out according to a modification of the A.O.A.C. method (1965). A 50 g sample of feed was weighed into a 500 ml Erlenmeyer flask. Then, 25 ml water, 25 g of diatomaceous earth and 250 ml of chloroform were added. The stopper was secured with masking tape and the flask was shaken for 30 minutes on a wrist action shaker (Burrel Corp.). The material was then filtered through prefolded filter paper (Whatman 2 V) and the first

50 ml of filtrate were collected, dried and transferred to a 10 g silica gel column as described earlier herein. The aflatoxins were eluted from the column, spotted in 20x20 cm TLC plates and quantified as described before for analysis of aflatoxins in tissue samples.

Analysis of Aflatoxins from Drip of Cooked Meat

The analysis of drip from the cooked tissues was carried out using a modification of the method of Trucksess and Stoloff (1979). The drip was weighed and transferred to a 500 ml Erlenmeyer flask. Then, 200 ml acetone plus 40 ml NaCl-citric acid solution was added. The flask stopper was secured with masking tape, and the flask was shaken for 30 minutes using a wrist action shaker. Then 150 ml of water, 20 ml of lead acetate solution, 10 g of ammonium sulfate and 10 g of diatomaceous earth were added to the flask. The solution was stirred for about 1 minute using a magnetic stirring device and then filtered through a fast filtering filter paper. A total of 325 ml of the filtrate was transferred to a 500 ml separatory funnel and the solution was defated as before. The chloroform extracts were purified by silica gel column chromatography as described earlier herein. The final extract was spotted, developed and quantified using 20x20 cm TLC plates and the same solvent system that was used for analysis of cooked tissues.

Confirmatory Tests for Aflatoxins

Aflatoxin B_1

Twenty μl of sample extract were applied about 4 cm from both edges in the left corner of a precoated TLC plate (20x20 cm Sil-G-HR-25, Brinkman Instruments, Inc.). Approximately 2.5, 0.5, 2.0 and 2.0 ng of aflatoxins B_1, B_2 and M_1 and M_2 standards, respectively, were spotted in the same location on right corner of the plates. Then the plates were developed in a closed, unlined and unequilibrated tank using anhydrous diethylether-methanol-water (96:3:1). After development, the plates were air dried under a hood for about 2 minutes and then dried in a 50 °C oven under a stream of nitrogen for about a minute.

The spot corresponding to aflatoxin B_1 was identified by comparison with the aflatoxin B_1 standard. Then it was marked in the silica gel on the left with a pencil along the direction of the development. Another pencil mark was made about 1 cm apart to the left of the first. The second mark was used as a guide to apply about 2.5 ng of aflatoxin B_1 standard close to the aflatoxin B_1 sample spot. Then 2 μl of trifluoroacetic acid (TFA): chloroform (1:1) were applied to both of the aflatoxin B_1 spots. The plate was allowed to stand in the dark for about 5 minutes at room temperature. Then the plate was dried in oven for 10 minutes at 45°C. After cooling the plate in the dark at room temperature, another 2.5 ng of aflatoxin B_1 standard was applied about

1 cm to the left of the second pencil mark. The plate was developed in the second direction with chloroform-acetone-isopropanol (87:10:3) the same way as described before. After the plate was developed and dried, the chromatogram was examined for the formation of aflatoxin B_{2a}, which has a lower R_f than the unreacted aflatoxin B_1 standard. The chromatographic equivalence of the sample and the aflatoxin B_1 standard spot after treatment with TFA was used as a confirmatory test for identifying aflatoxin B_1.

Aflatoxin M_1

The TLC plate was spotted, developed in the first direction and dried as described earlier herein in order to carry out the aflatoxin B_1 confirmatory test. Then the aflatoxin M_1 spot in the sample was marked in the silica gel on the left with a pencil along the direction of the development. Another pencil mark was made about 3 cm apart to the right of the first and close to the aflatoxin M_2 spot. The second mark was used as a guide for applying about 2.0 ng of aflatoxin M_1 standard. Two μl of TFA: chloroform (1:1) were then applied to both the sample spot and the aflatoxin M_1 standard. The plate was allowed to stand in the dark for about 5 minutes at room temperature. Then the plate was dried in a chromatographic oven for 5 minutes at 75°C. The plate was then cooled in the dark at room temperature and another 2.0 ng of aflatoxin M_1 standard was spotted about 1 cm to the right of the second pencil mark. Then the plate

was developed perpendicular to the first direction using chloroform: acetone: isopropanol (85:10:5) for development. After development, the plate was dried and examined under UV light for the formation of the aflatoxin M_1 derivative in order to ascertain if the R_f was lower than that of the unreacted aflatoxin M_1 standard. The chromatographic equivalence of the sample and the aflatoxin M_1 standard spot after treatment with TFA was used as a confirmatory test for the identity of aflatoxin M_1.

General Confirmatory Test for Aflatoxins

This technique offers additional confirmation for the presence of aflatoxins at low levels (Przybylski, 1975). The technique is as follows: after development of the TLC plate and identification of the spots under UV light, the plate was sprayed with 25% sulfuric acid (v/v). Then the plate was dried in a chromatographic oven at 45°C under a stream of nitrogen. Changes in the characteristic fluorescence of aflatoxins B_1, B_2, M_1 and M_2 from blue to yellow after the H_2SO_4 treatment was used as an additional confirmatory test for the presence of aflatoxins.

Three-Dimensional Chromatography

This test is based on comparison of the chromatographic mobilities of aflatoxins from the sample extract with reference to authentic standards after three-dimensional chromatography using three different solvent systems. The

aflatoxin spot in the sample was eluted from the plate and spotted along with the aflatoxin standard in the same place in a 10x10 cm TLC plate. The presence of only one spot after three-dimensional chromatography was used as an additional confirmatory test for the identity of the aflatoxins in the sample extract.

The aflatoxin spot in the sample was eluted from the plate using the following procedure: A ball of glass wool was applied into a 5 3/4 inches long glass disposable pipet (Scientific Products). Then a layer of about 0.5 cm of anhydrous sodium sulfate was added. The tip of the glass pipet was then attached to rubber vacuum tubing connected to a water aspirator. The spot on the TLC plate was visualized with a UV cabinet and the aflatoxin spot to be extracted was marked on the silica gel layer using four pencil marks to define its boundary. Then about 2 μl of water were applied to the aflatoxin spot to displace the aflatoxins from the silica gel adsorbent. The silica gel layer containing the aflatoxin was then removed by scraping it from the plate with the edge of the prepared glass pipet. The vacuum generated inside the pipet was adequate to aspirate and collect the flaked silica gel spot. Once the spot area was removed from the silica gel plate, the glass pipet was disconnected from the aspirator and clamped to a metal support. The aflatoxin was then eluted from the silica gel using 3 ml of acetone, and the eluate was collected in a 1 gram vial. The acetone was then evaporated under a gentle stream of

nitrogen as described earlier hearin. The aflatoxin extract was dissolved in 40 μl of chloroform. The entire chloroform extract was then spotted at a point located about 1.5 cm from both edges in the left corner of a 10x10 cm silica gel plate. About 2 ng of the aflatoxin standard was then superimposed over the sample spot. The plates were then developed in 1.5 liter beaker tightly covered with aluminum foil. After development in one direction, the plate was dried and then developed perpendicular to the first direction as explained earlier herein. The following solvent systems were sequentially used for three dimensional development: (1) chloroform-acetone (85:15); (2) anhydrous diethylether-methanol-water (90:8:2); and (3) chloroform-acetone-isopropanol (85:10:5).

This technique was specially developed for confirmatory identification of aflatoxin B_2 and M_2, which can not be identified using chemical reagents to form derivatives. It can also be used as a confirmatory test for aflatoxin B_1 and M_1, using the TFA procedure if the background in the sample extract is too intense to permit good visualization of the spots. In this case after quantitation of the sample spots, the B_1 or M_1 spot was removed from the plate and respotted on a 20x20 cm TLC plate as described before. Then the corresponding aflatoxin standard spot was applied close to the sample spot and followed by the TFA treatment, drying and development of the TLC plates as described herein in the confirmatory test for aflatoxins B_1 and M_1.

Preparation of Aflatoxin Reference Standards

Aflatoxins B_1, B_2, M_1, M_2 and B_{2a} used as reference standards were obtained from Applied Science Division. The aflatoxins B_1 and B_2 used in the preparation of the spiked diets were purchased from CAL Biochem.

Aflatoxin reference standards were prepared according to the AOAC method (1975) and contained 0.501, 0.175, 0.4 and 0.4 μg/ml of aflatoxins B_1, B_2, M_1 and M_2, respectively. A solvent mixture of benzene-acetonitrils (98:2) was used as the solvent for aflatoxins B_1 and B_2, while chloroform was utilized as the solvent for aflatoxins M_1 and M_2. The aflatoxin standard solutions were stored at - 20°C.

Fat and Moisture Analysis

Moisture Content

The A.O.A.C. (1965) procedure for determining moisture was used. Five grams of tissue were accurately weighed to four decimal places into a previously dried and tared aluminum dish (100°C for at least 1 hour). The sample plus the dish were then dried overnight for 18-24 hours in an air convection oven at 100°C. The dried sample was cooled in a desiccator and weighed to four decimal places. Loss in weight was reported as moisture for each hundred grams of meat. Three replicates were run for each sample.

Fat Content

The fat content was determined using the Goldfisch extraction method of the A.O.A.C. (1965). The same sample was used following moisture analysis. The aluminum dish containing the dried meat sample was carefully folded into a porous thimble and clipped into a Goldfisch apparatus. The fat was extracted with anhydrous diethylether for approximately 8 hours into a previously dried and tared beaker. The extract was then dried for 1 hour at 100°C in an air convection oven, cooled in a desiccator and weighed as before. The percent fat was calculated as grams of fat extracted from each one hundred grams of tissue. Three replicates were run per sample.

Safety Procedures

All glassware and vials in contact with aflatoxins were soaked either with 5-6% NaOCL (household bleach) or with sulfuric acid-dichromate solution (120 g $Na_2Cr_2O_7.2H_2O$ + 1600 ml conc. H_2SO_4 and diluted to a volume of 3 liters with water) to destroy any residual aflatoxins. Plastic disposable gloves were worn routinely during all work with aflatoxins. Respirator masks were worn when mixing and handling the spiked rations. Surface work areas were routinely scanned with a UV lamp and any contaminated areas were treated by washing thoroughly with 5.6% NaOCl solution. All TLC plates used in aflatoxin analysis were thoroughly soaked in

NaOCl solution before discarding. Filter papers and tissue residues resulting from aflatoxin analysis were thoroughly soaked with concentrated ammonium hydroxide solution overnight before discarding. These waste materials, after treatment with ammonia, were collected in plastic bags and placed inside tightly closed containers and labeled properly until removed by the MSU Animal Waste Disposal Unit. All work involving scraping of the plates was done under a hood. Similarly any work involving the use of toxic solvents, such as benzene, chloroform and acetonitrile, were also performed under the hood. This involved preparation of silica gel columns, spotting, development and drying of the TLC plates.

RESULTS AND DISCUSSION

Feeding Trial

The response of the animals to aflatoxins was determined by growth, feed consumption and organ weights. Aflatoxin intake by the pigs was expressed as the amount of the toxin ingested per unit of body weight per day, which is the daily dosage rate (DR). Armbrecht et al. (1971) concluded that DR gives the best measure of the response of the exposed animals to aflatoxins.

Except for the feed of the control pigs on trials 1 and 2, the experimental conditions for both trials 1 and 2 were identical. The feed of the control pigs on trial 1 was naturally contaminated with 20 and 31 ppb of aflatoxins B_1 and B_2, respectively, while in trial 2 the feed was free of aflatoxins.

In trial 1 the low level of contamination in the basal diet turned out to be a fortuitous circumstance. It was found that there were no significant differences between the weight gains and organ weights of the control pigs and those fed the aflatoxin spiked diet. This is in contrast to the comparison between the aflatoxin free controls and pigs

fed a spiked diet in trial 2. The control pigs in trial 1 exhibited depressed growth and on the average gained 40 percent less weight than the controls in trial 2 over the same feeding period (Table 4). Furthermore, the controls in trial 1 also showed an average of 24 percent reduction in feed intake as compared to the controls in trial 2 (Table 5). The data from Furtado et al. (1979) were also in contrast to those in trial 1 as they found significative differences in gains between the controls and pigs fed aflatoxins.

Results indicate that even at the low levels of aflatoxins found in the naturally contaminated diet, deposition of small amounts of aflatoxins occurred in the liver and kidneys of the control pigs (Table 8). Although no visual lesions were observed in the liver and kidneys of control pigs in trial 1, results suggest that exposure to levels as low as 20 to 31 ppb of aflatoxins B_1 and B_2, respectively, was adequate to cause adverse effects on the performance of the pigs.

In trial 2, the basal diet was uncontaminated with aflatoxins, so that there was a significant advantage in liver weights and live weight gains for the controls over the group fed the spiked diet (Tables 4 and 6). The results showed that the pigs fed aflatoxins exhibited depressed growth, and on the average gained 45 percent less weight than the control animals over the 42 day trial (Table 4). Although feed consumption data could not be statistically analyzed, on the average the pigs fed aflatoxins showed a 32 percent reduction in

TABLE 4 - Summary of Feeding Trial for Pigs from Trails 1 and 2.[a]

		Starting Weight (kg)			Final Weight (kg) *			Weight Gain (kg) *		
		Group [c]			Group [c]			Group [c]		
		1	2	3	1	2	3	1	2	3
Experimental:	Mean	9.5	9.7	-	20.8	19.8	-	11.3	10.1	-
	S.D.[b]	1.16	1.11	-	4.59	3.49	-	3.67	2.73	-
Control:	Mean	9.5	-	-	24.7	-	-	15.2	-	-
	S.D.	1.11	-	-	6.24	-	-	6.15	-	-
Experimental:	Mean	9.6	9.8	9.4	25.3[d]	23.4[d]	20.6[d]	13.9[f]	16.3[f]	11.2[f]
	S.D.[b]	0.59	0.66	0.95	4.48	5.88	3.10	4.25	5.36	2.67
Control:	Mean	9.6	-	-	34,8[e]	-	-	25.2[g]	-	-
	S.D.[b]	0.39	-	-	1.22	-	-	1.11	-	-

* Mean values between experimental and control treatments followed by different superscripts were significant at $P < 0.01$

[a] Data are taken from Appendix Tables 15 and 17.

[b] S.D. = Standard deviation.

[c] Trial 1 - Control 1 group had 4 pigs; Experimental groups 1 and 2 were replicates with 8 pigs per group; Trial 2 - Control 1 contained 4 pigs; Experimental groups 1, 2 and 3 were replicates with 5, 5 and 6 pigs per group, respectively.

TABLE 5 - Average Feed Intake, Average Aflatoxin Consumption, Feed Efficiency and Daily Dosage Ratio for Pigs on Trials 1 and 2

Group No.	Avg. Feed Intake Per Pig (kg)	Avg. Aflatoxin Consumption Per Pig (mg)	Daily Dosage Rate (DR) [a]	Feed Efficiency (Feed/Gain)
		Trial 1		
Control #1	38.5	2.0	3	2.5
Exptl. #1	28.9	26.2	41	2.6
Exptl. #2	28.1	25.5	41	2.8
		Trial 2		
Control #1[b]	50.6	0	0	2.0
Exptl. #1	34.5	31.3	45	2.5
Exptl. #2	34.5	31.3	45	2.5
Exptl. #3	33.5	30.4	48	3.0

[a] Amount of aflatoxins calculated from formula DR = Wa/0.5(Ws + We)t (Armbrecht et al., 1971) Where, (Wa) μg of aflatoxin ingested during the interval of time (t) in days, starting weight (Ws) and ending weight (We) in kg.

[b] The feed of the control pigs on Trial 2 was free of aflatoxins.

TABLE 6 - Summary of Organ Weights Expressed as Percent of Live Body Weight for Pigs on Trials 1 and 2.

Organ	Control		Experimental	
	Mean[ab]	S.D.[c]	Mean[ab]	S.D.[c]
		Trial 1		
Heart	0.35[e]	0.09	0.43[e]	0.06
Kidneys	0.44[f]	0.10	0.52[f]	0.09
Liver	2.36[g]	0.6	2.92[g]	0.45
Spleen	0.18[h]	0.03	0.18[h]	0.01
		Trial 2		
Heart	0.38[i]	0.03	0.38[i]	0.04
Kidneys	0.49[j]	0.06	0.49[j]	0.07
Liver	2.15[k]	0.25	2.80[l]	0.3
Spleen	0.17[m]	0.02	0.18[m]	0.03

[a] Data are taken from values in Appendix Tables 16 and 18.

[b] Mean values on the same line having identical superscripts are not significantly different ($P < 0.05$).

[c] S.D. = Standard deviation.

feed intake as compared to the controls. Feeding the pigs aflatoxins also adversely effected efficiency of feed utilization (Table 5).

Results from trial 2 in the present study agree with those reported by Keyl and Booth (1971) and Krogh et al. (1973), in which they reported decreased growth and feed efficiency for pigs fed diets containing 810 and 500 ppb of aflatoxins, respectively. These results also agree with those reported earlier by Furtado et al. (1979), except for the fact that they found aflatoxins did not decrease feed efficiency over a 21 day feeding period. Long-term exposure and the use of young animals in the present experiment and in previous studies (Keyl and Booth, 1971; Krogh et al., 1973) may account for the differences found upon comparing these data with the earlier work (Furtado et al., 1979). Monegue et al. (1977) found that levels of 100, 200 or 300 ppb of aflatoxin B_1 did not significantly effect gains and feed consumption or efficiency of young pigs fed a contaminated diet up to market weight.

The pigs fed the aflatoxin-spiked rations in the present study had an average dosage rate (DR) of 44 μg/kg (Table 5). According to Armbrecht et al. (1971) this level of intake is near the transition point between clinical and subclinical aflatoxicosis for pigs over a 16-week feeding trial. In addition, they reported that pigs fed on a similar DR exhibited subnormal body weights and altered feed conversion over a 6-week treatment. These data provide additional

support for the results obtained in trial 2. The control group in trial 1, which was fed the naturally contaminated ration, had a DR value of only 3 μg/kg.

Results indicate that under the conditions of trial 1 levels of aflatoxins as high as 551 and 355 ppb of aflatoxins B_1 and B_2, respectively, did not significantly influence average weight gains over the control groups during a 42 day feeding period. The low levels of contamination of the control diet in trial 1 may explain why there was no significant difference between the controls and the pigs fed the spiked diets. On the other hand, in trial 2 the control diet was uncontaminated, so that there was a significant growth supression in the pigs receiving the aflatoxin contaminated feed. Overall results would suggest that even the low level of natural contamination was high enough to adversely effect growth, feed consumption and feed efficiency.

Gross Observations On Tissues

At slaughter, the pigs were inspected for gross pathological lesions by a Michigan State Department of Agriculture Meat Inspector. The weights of the internal organs (hearts, kidneys, livers and spleens) were recorded (Tables 16 and 18).

Tissues and organs from the control and experimental pigs in trial 1 and 2 were normal in appearance, except the livers of two pigs, one from trial 1 (slaughtered at zero day withdrawal) and the other from trial 2 (killed after withdrawal for two days). Livers from these two pigs

exhibited yellow discoloration, appeared quite fatty, and were condemned for human consumption by the meat inspector. All the other organs, even the kidneys, and all carcasses were free of other lesions, and were not rejected for human consumption by the meat inspector. The changes observed in the livers of the two pigs in this study agree with the report of Krogh et al. (1973), who observed similar liver lesions in some pigs fed 300 and 500 ppb of aflatoxins B_1+B_2 for 120 - 231 days.

There was no significant difference in organ weights expressed as percentage of body weight for the control and experimental pigs on trial 1 (Table 6). In trial 2, however, a significant difference was observed between liver weights of the control and experimental groups. The pigs fed aflatoxins exhibited, on the average, 30 percent heavier livers than the controls (Table 6). The other internal organs, however, were not affected by the treatment, with no significant differences between the controls and the pigs fed aflatoxins (Table 6). The natural contamination of the control diet with aflatoxins, even at low levels, may account for the differences observed between trials 1 and 2.

The results of trials 2 agree with those of Furtado et al. (1979), who observed liver enlargement but no other effects on the organs of pigs fed an aflatoxin-contaminated diet. Similarly, Keyl and Booth (1971) observed liver enlargement in pigs fed rations containing 450 ppb of aflatoxins or higher. Keyl and Booth (1971) and Armbrecht et al.

(1971) have also reported kidney enlargement in some of the animals fed aflatoxins although to a lesser extent than for the livers.

Localization of the ingested aflatoxins and their metabolites in the liver and kidney may explain the predominance of pathological lesions in these two organs. In contrast to other reports, however, no alteration was observed in the kidneys of the pigs fed aflatoxins in the present study. A possible explanation may be that the aflatoxin exposure was adequate to produce hepatic tissue enlargement, but was not high enough to produce abnormalities in the kidneys. This confirms the fact that the liver is more susceptible to aflatoxin damage and is the best internal organ to use in monitoring the pig's response to aflatoxin (Jacobson et al., 1978; Furtado et al., 1979).

Gross pathological lesions were observed in only two pigs. All other organs and carcasses seemed normal in appearance. In trial 2, however, feeding aflatoxins to the pigs resulted in a significant increase in liver weights over the controls. In contrast, on trial 1, pigs fed an identical aflatoxin-spiked diet had no significant increase in liver weights. Low levels of natural contamination of the control diet in trial 1 seemed to be adequate to cause liver enlargement in the controls and may account for the differences observed. This suggests that growing pigs are very susceptible to aflatoxin toxicity, even when exposed to low levels in the diet.

Aflatoxin Residues in the Tissues of Pigs Fed a Contaminated Diet

Results of aflatoxin analysis for the tissues of pigs slaughtered following removal from contaminated feed (no withdrawal period) from trials 1 and 2 are presented in Table 7. Measurable amounts of aflatoxins B_1 and B_2 were carried over to all tissues of the pigs fed the contaminated feed, with the highest levels being found in the livers and kidneys. The blood showed the lowest levels of aflatoxins followed by muscle, spleen and heart, in order of increasing levels. Aflatoxins M_1 and M_2, the metabolites of aflatoxins B_1 and B_2, respectively, were also found in most of the tissues of pigs fed the contaminated ration. Similar to B_1 and B_2, the livers and kidneys were the organs showing the highest residual levels of aflatoxins M_1 and M_2. The lowest levels were present in the blood and spleen.

The high capacity of the liver and kidneys to concentrate the aflatoxins may be related to the fact that these organs are very important in the metabolism and elimination of drugs. Although the precise mechanism by which these organs remove toxicants from the blood has not been established yet, active transport or binding to tissue components is believed to be involved (Klaassen, 1980).

Except for the liver and kidneys, the levels of aflatoxins M_1 and M_2 were much lower than the levels of B_1 and B_2. This is probably due to the higher polarity and increased water solubility of these hydroxylated metabolites,

TABLE 7 - Aflatoxin Residues (μg/kg) Detected in Selected Pig Tissues at Zero Time Interval Following Removal of Contaminated Diet.

Pig No.[a]	Levels of Aflatoxins[b]							
	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Blood				Liver			
1	0.10	0.14	tr	0	0.31	0.89	0.27	0.28
2	0.17	0.13	0.06	0	0.25	0.41	0	0
3	0.21	0.17	0.08	0.09	1.52	1.02	0.53	1.08
4	0.12	0.05	0.10	0	0.36	0.33	0.42	0.57
5	0.10	0.16	tr	tr	0.86	0.73	0.26	0.67
6	0.26	0.24	tr	0	2.07	1.95	0.19	0.33
7	0.56	0.53	tr	tr	0.98	1.06	0.27	0.98
8	0.27	0.35	tr	tr	0.41	0.41	0.33	2.17
Mean	0.22	0.22	tr	tr	0.85	0.85	0.28	0.76
	Heart				Muscle			
1	0.13	0.15	0	0	0.20	0.24	0	0
2	0.24	0.44	0.09	0	0.34	0.50	0.12	0
3	0.25	0.33	0.14	0	0.45	0.38	0.17	0.14
4	0.09	0.14	0.08	0	0.18	0.14	0.10	tr
5	0.89	0.42	tr	0	0.28	0.15	0.07	0
6	0.20	0.14	tr	tr	0.06	0.05	0.05	0.14
7	1.32	1.05	0.09	tr	0.66	0.60	0.07	tr
8	0.62	0.60	tr	tr	0.46	0.35	tr	tr
Mean	0.47	0.41	0.05	tr	0.33	0.30	0.07	0.04
	Kidney				Spleen			
1	0.40	1.07	0.29	0.38	0.26	0.14	0	0
2	0.30	0.45	0.13	tr	0.32	0.64	0.15	0
3	0.59	0.73	0.39	0.62	0.32	0.27	tr	0
4	0.28	0.34	1.10	0.59	0.69	0.12	0	0
5	1.02	0.96	1.13	0.94	0.51	0.44	tr	0
6	0.18	0.21	0.53	1.79	tr	tr	tr	0
7	1.14	2.05	0.55	0.60	0.94	0.94	tr	tr
8	0.90	1.45	0.50	0.85	0.46	0.48	tr	tr
Mean	0.60	0.91	0.58	0.72	0.44	0.38	tr	tr

[a] Numbers 1 to 4 refer to pigs from trial 1 and numbers 5 to 8 to those from trial 2.

[b] tr = trace amounts, visible but too small of an amount to quantitate (< 0.05 μg/kg).

which are more easily removed from tissues either as the original compound or as aflatoxin conjugates after being bound to various endogenous compounds (Bassir and Oziyemi, 1967; Dalezios et al., 1971).

The data from this study agree with those reported by Jacobson et al. (1978) in that they found aflatoxins B_1 and M_1 in the livers, kidneys and muscles of pigs fed an aflatoxin B_1 contaminated diet. Similarly, they found that most of the residual aflatoxins deposited in the tissues were localized in the liver and kidneys. In previous work, Furtado et al. (1979) reported similar results after feeding pigs for 3 weeks on a diet which contained aflatoxins at approximately the same levels used in the present experiment. They found aflatoxin B_1, B_2 and M_1 in the tissues of the pigs, although the levels were lower than those in the current study. This may be explained by the fact that younger animals and longer exposure times were used in the present study. Young animals are more susceptible to aflatoxin toxicity than adults (Wogan, 1968). In addition, longer exposure to aflatoxins increases the risk of liver and kidney lesions, which then leads to impaired metabolism and excretion of the aflatoxins.

Furtado et al. (1979) tentatively identified aflatoxin B_{2a}, a metabolite of B_1 (Figure 3), in the tissues of pigs fed aflatoxins. However, the spot identified as aflatoxin B_{2a} was not positively identified due to the unavailability of B_{2a} and M_2 standards. Since these standards were

available in the current study, the sample of Furtado et al. (1979) was rechromatographed using three-dimensional chromatography with three different solvent systems and compared with authentic M_2 and B_{2a} standards. The spot previously identified as B_{2a} did not co-chromatograph with the authentic B_{2a} standard in the three-dimensional chromatography confirmatory test, but was positively identified as M_2.

Aflatoxins B_{2a} and M_2 are similar chemically and structurally (Figures 1 and 3), differing only by position of the hydroxy group in the molecule. Thus, they have similar chromatographic characteristics in the solvent systems utilized. Furthermore, both compounds exhibit blue fluorescence under UV radiation, which accounts for the incorrect identification earlier (Furtado et al. 1979).

The feed of the control pigs in trial 1 was found to be naturally contaminated with 20 and 31 ppb of aflatoxins B_1 and B_2, respectively. The contamination of the feed with aflatoxins produced some interesting results. First, it was shown that even the low levels in the diet resulted in small amounts of aflatoxins in the liver and kidneys (Table 8). This is in contrast to earlier studies where low levels (100-300 ppb) of aflatoxins did not result in detectable levels in the tissues of pigs (Booth, 1969; Monegue et al., 1977). Keyl and Booth (1971) did not detect aflatoxins in the meat and livers from swine and cattle fed rations containing aflatoxin levels as high as 800 and 1000 ppb, respectively. On the other hand, Shreeve et al. (1979) found

TABLE 8 - Aflatoxin Residues (μg/kg) Found in Selected Tissues of Control Pigs from Trial 1.[a]

Pig No.	Levels of Aflatoxins			
	B_1	B_2	M_1	M_2
		Blood		
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
		Heart		
1	0	0	0	0
2	0	0	0	0
3	tr	tr	0	0
4	0	0	0	0
		Kidney		
1	tr	tr	0.05	0.20
2	0	0	0.05	0.15
3	0	0	0	0
4	0	0	0.10	0.11
		Liver		
1	tr	tr	tr	0.09
2	tr	tr	tr	0.08
3	tr	tr	tr	tr
4	0	0	0	0
		Muscle		
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
		Spleen		
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0

[a] The control ration was found to be naturally contaminated with 20 and 31 ppb of aflatoxins B_1 and B_2, respectively. The control diet of trial 2 was free of aflatoxins.

measurable amounts of aflatoxins B_1 and M_1 in milk, kidneys and urine of cows fed only 20 ppb of aflatoxin B_1. These authors, however, suspected that interactions with other mycotoxins, which were present in the feed, may have contributed to their accumulation.

Another interesting observation found upon feeding the pigs low levels of aflatoxins B_1 and B_2 in the current study was that the aflatoxins were present mainly as M_1 and M_2. This suggests that the metabolism of aflatoxins by the pigs is more efficient when the aflatoxins are ingested at low levels.

Other controlled studies of aflatoxin transmission from feed to animal tissues have shown less conclusive and less consistent data as compared to those from the present experiment. Jemmali and Murthy (1976), using an improved method, found aflatoxins B_1, B_2 and M_1 in some gall bladder, kidney, liver, muscle and spleen samples from two pigs fed a diet containing high levels of aflatoxins. On repeating the analysis, using the methods of Brown et al. (1973), however, they obtained either negative results or lower values than were obtained by the improved method. Earlier investigations using a biological assay (Allcroft and Carnaghan, 1963; Platonow, 1965) or methods primarily designed for assaying contamination in plant materials (Keyl et al., 1968; Kratzer et al., 1969; Keyl and Booth, 1971) failed to detect aflatoxins, even in animals showing confirmed signs of aflatoxicosis.

The sensitivity of the analytical procedures may be implicated in the conflicting and negative results of the previous studies on transmission of aflatoxins to tissues of animals ingesting contaminated feeds. According to Rodricks and Stoloff (1977) a number of other factors may also be implicated, the most important of which are: (1) the species and breed of animals, (2) the level of exposure, (3) the diet and state of health of the animals, and (4) the time interval after cessation of aflatoxin exposure, slaughter and collection of samples for analysis.

As shown in Table 9, 648 was the ratio between the aflatoxin B_1 level in the feed and the B_1 level in the liver. This is below the value of 800 predicted by Rodricks and Stoloff (1977), which was calculated as an average for the data from several studies (Table 1). Thus, there was a 19 percent increase in the efficiency of aflatoxin B_1 deposition in the livers of the pigs from this experiment as compared to that estimated by Rodricks and Stoloff (1977). In addition Table 9 shows that the ratio between aflatoxin B_2 in the feed and B_2 or M_2 levels in the kidney and liver is much lower than the ratio between aflatoxin B_1 in the feed and the levels of B_1 and M_1 deposited in the same tissues. This indicates that metabolism and/or clearance of aflatoxins B_2 and M_2 in the livers and kidneys of the pigs is somewhat impaired in relation to that of aflatoxins B_1 and M_1.

TABLE 9 - Ratios of Aflatoxins B_1 or B_2 Levels in the Feed in Relation to Aflatoxins M_1 or M_2 Levels in Kidneys and Livers

Tissue	Aflatoxin in Tissue	Feed to a Tissue Ratio [a]
Kidney	B_2	390
	M_2	493
Liver	B_2	417
	M_2	467
Kidney	B_1	918
	M_1	605
Liver	B_1	648
	M_1	1967

[a]Higher values indicate more efficient tissue clearance.

Aflatoxins M_1 and M_2 are similar molecules resulting from the ring hydroxylation of aflatoxins B_1 and B_2, respectively, at the C-4 position (Figure 2). *In vivo* and *in vitro* studies have provided evidence that aflatoxin B_{2a} and aflatoxin B_1-2,3-epoxide are two major metabolites in several animal species. Gurtoo and Campbell (1974) reported that aflatoxin B_{2a} was the major metabolite identified upon incubating rat liver microsomes with aflatoxin B_1. They also reported that aflatoxin B_{2a} was found to be covalently bound to the microsomal proteins. Patterson and Roberts (1970a) also found that metabolism of aflatoxin B_1 to M_1 was a minor pathway when compared to metabolism of B_1 to B_{2a} by liver preparations from rabbits, ducklings, guinea pigs and rats. The formation of aflatoxin B_1-2,3-epoxide was shown to be a

significant pathway for metabolism of aflatoxin B_1 on using microsomal liver preparations from humans and rats (Swenson et al., 1974; Croy et al., 1978). If the pig behaves similarly, the metabolism of aflatoxin B_1 by the liver microsomal enzymes would lead to formation of both aflatoxin B_{2a} and the 2,3-epoxide, in addition to aflatoxin M_1. Therefore, partitioning of aflatoxin B_1 into these two metabolites (aflatoxin B_{2a} and 2,3-epoxide) may contribute to a decrease in the levels of aflatoxins B_1 and M_1. This may be a significant metabolic route, which is dependent on the presence of the 2,3 ether vinyl double bond. This route does not exist for aflatoxin B_2 and may account for the higher levels of B_2 and M_2 in the livers and kidneys of the pigs.

Aflatoxicol and aflatoxins Q_1 and P_1 are other metabolites of aflatoxin B_1. Although they have not yet been found in the tissues of food-producing animals, it it not unlikely that one or more of these compounds may be present (Rodricks and Stoloff, 1977). They have been found in the tissues and excreta of animals, mainly in the conjugated form. Such aflatoxin conjugates have enhanced water solubility and reduced solubility in other solvents, such as chloroform; thus they are not extracted by classical methods (Mabee and Chipley, 1973).

Aflatoxin P_1 represents a major urinary metabolite of aflatoxin B_1 in rhesus monkeys according to Dalezios et al. (1971) and accounts for about 20 percent of the administered dose. It is excreted predominantly in the form of its water-

soluble glucuronide and sulfate. Hayes et al. (1977) found that upon incubation of ^{14}C-aflatoxin B_1 with bovine liver preparations about 60 percent of the original activity was transformed into water-soluble material, and approximately 10 percent was metabolized to aflatoxins M_1, Q_1 and two unidentified metabolites. Aflatoxin Q_1, however, was not isolated from the tissues of cows fed aflatoxin B_1 contaminated feed, which led these authors to suggest that aflatoxin Q_1 might be conjugated in vivo, and thus, would be confined to the water-soluble fraction of the tissues. Mabee and Chipley (1973) found that aflatoxin M_1 glucuronide was the major metabolite isolated from the tissues of chickens dosed with ^{14}C-aflatoxin B_1.

The present study suggests that only a small fraction of the aflatoxins consumed is deposited in the tissues of the pig, either as the original aflatoxins or their metabolites. The mean percentage of retention of aflatoxin dosage expressed as B_1 and B_2 equivalents, was calculated to be 0.03 and 0.04 percent for B_1 and B_2, respectively (Table 10). The highest levels of residual aflatoxins were present in the livers and kidneys, with the value for the combined aflatoxins being below 3 ppb. The total aflatoxin levels found in the muscle samples were even lower than those found in the liver and kidneys and averaged less than 1 ppb.

Wogan (1968) reported that the LD_{50} of aflatoxin B_1 for most animal species varied from 0.5 - 10 mg/kg body weight. The 1-day-old duckling was shown to be the most

TABLE 10 - Average Calculated Value for Aflatoxins Found in Selected Pig Tissues[abcd]

Tissue	Average Weight of Fraction(g)	Total Amount of Aflatoxin (μg)	
		B_1	B_2
Blood	1,400	0.35	0.32
Heart	74	0.04	0.03
Kidneys	92	0.11	0.15
Liver	522	0.59	0.84
Muscle	8,800	3.52	2.99
Spleen	33	0.02	0.01

[a] Data calculated from values in Tables 4, 5, 7, 16 and 18.

[b] Aflatoxin B_1 is expressed as B_1 equivalents = $B_1 + M_1$, and aflatoxin B_2 is expressed as B_2 equivalents = $B_2 + M_2$.

[c] Blood volume is based on an average live weight of 22 kg per pig based upon an average of 6.5 percent blood on the live weight basis (Cornegay et al., 1964). Lean tissue values are based on an average of 22 kg live weight with lean comprising 40 percent of live weight (Guidelines for Uniform Swine Improvement Program - USDA, In Press.).

[d] The mean percentage retention of aflatoxin dosage was calculated to be 0.03 and 0.04 percent for B_1 and B_2, respectively.

susceptible species with an LD_{50} of 0.5 mg/kg body weight. On the basis of the LD_{50} estimates (Wogan, 1968), there would appear to be little risk of acute toxicity to humans from eating the tissue from the pigs in the present study, even at zero day withdrawal.

While the LD_{50} data are useful as an index of specie susceptibility, they do not give information on the effects of prolonged intake at low levels. For example, Wogan (1968) showed that feeding male rats a purified diet containing aflatoxin B_1 at a level of 1 ppm resulted in an incidence of 100 percent of the rats having tumors within 41 weeks. On the other hand, a period of 68 weeks was necessary to produce similar effects using 15 ppb of aflatoxin B_1.

Carnaghan (1965) fed a diet containing 30 ppb of aflatoxin B_1 to ducklings and found tumors in 8 out of 11 animals after 14 months. Ashley *et al.* (1965) using rainbow trout (a sensitive species) reported that levels as low as 0.5 to 2 ppb of aflatoxins caused a significant incidence of liver tumors on feeding for 10 months to 2 years.

Of the various aflatoxins and metabolites, all have been shown to be considerabily less toxic than aflatoxin B_1 (Wong and Hsieh, 1975). Purchase (1967) and Holzapfel *et al.* (1966) reported that the LD_{50} of aflatoxin M_1 and M_2 in ducklings was comparable to that of aflatoxin B_1 and B_2, respectively. Similar lesions were also produced by these compounds. Sinnhuber *et al.* (1970) found that M_1 was carcinogenic to rainbow trout, but was only about one-third as

potent as B_1 in inducing liver tumors. Recently, Wong and Hsieh (1975) using a Salmonella typhimurium mutant assay (Ames et al., 1975) reported that aflatoxin M_1 was only 3 percent as mutagenic as aflatoxin B_1.

Although no acute responses from residual aflatoxins would be expected in humans consuming meat from pigs exposed to the levels of aflatoxins used in this study, the chronic or long-term toxicity of such residues needs to be studied in more depth. In addition, the data did not account for the aflatoxin conjugates, which have been shown to comprise a major fraction of the total aflatoxins accumulated in animal tissues (Bassir and Osiyemi, 1967; Dalezios et al., 1971; Mabee and Chipley, 1973).

The toxicity of the aflatoxin conjugates is unknown. There is, however, evidence to suggest that they can be broken down in vivo, since aflatoxins M_1 and B_{2a} have been sucessfully liberated from conjugates by liver, stomach and pancreatic enzymes in vitro (Mabee and Chipley, 1973; Chipley et al., 1974). Thus, the release of aflatoxins from the bound form may occur in vivo, and suggests that the aflatoxin conjugates should be considered as an additional source of contamination. Absorption of the free aflatoxins along with those released from their conjugates may contribute to the risk of a chronic toxicity during long-term ingestion of contaminated tissues.

Withdrawal Trial

Results of the withdrawal trial showed that pigs can metabolize and efficiently remove aflatoxins from the tissues. One day after withdrawal from the contaminated feed, low levels of aflatoxins B_1 and B_2 were present in the blood, muscle and spleen from 2 out of 6 pigs (Table 11). Aflatoxins M_1 and M_2 were not detected in these tissues or in the hearts of any of the pigs. Although some of the pigs still showed appreciable amounts of aflatoxins B_1 and B_2 in the heart, liver and kidney one day after removal of aflatoxins from the diet, only trace amounts of aflatoxins M_1 and M_2 were detected in these tissues.

The heart of one pig had 4.76 ppb of aflatoxin B_1 one day after withdrawal of the aflatoxin-contaminated diet, an unusually high value not normally observed in the tissues of pigs slaughtered after feeding on the contaminated feed. Except for this, however, the aflatoxin levels found in the kidneys, hearts and livers of pigs after one day on an aflatoxin-free diet were far lower than the levels found in the tissues of pigs killed without any withdrawal period.

Thus, results show that aflatoxins M_1 and M_2, the hydroxylated metabolites of aflatoxins B_1 and B_2, respectively, are more efficiently removed from tissues than the parent compounds. This is probably due to their higher polarity and increased water solubility.

TABLE 11 - Aflatoxin Residues (μg/kg) Detected in Selected Pig Tissues at One Day Interval Following Removal from the Contaminated Diet.

	Levels of Aflatoxins[b]							
Pig No.[a]	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Blood				Liver			
1	0	0	0	0	tr	tr	tr	tr
2	0	0	0	0	0	tr	0	0
3	0	0	0	0	0.10	0.08	tr	tr
4	0	0	0	0	0	0	0	0
5	tr	tr	0	0	0.17	0.26	tr	tr
6	0.17	0.12	0	0	0.42	0.53	0.07	0.15
Mean	tr	tr	0	0	0.12	0.15	tr	tr
	Heart				Muscle			
1	4.76	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0.05	tr	0	0	0	tr	0	0
4	0	0	0	0	0	0	0	0
5	0.12	0.06	0	0	0.07	0.03	0	0
6	0.39	0.23	0	0	0.13	0.12	tr	0
Mean	0.89	0.05	0	0	tr	tr	0	0
	Kidney				Spleen			
1	tr	tr	tr	tr	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0.13	0.06	0.14	0	0	0	0	0
4	tr	tr	tr	tr	0	0	0	0
5	0.24	0.12	tr	0	tr	tr	0	0
6	0.38	0.41	0.26	0.23	0.16	0.23	0	0
Mean	0.13	0.10	0.07	tr	tr	tr	0	0

[a] Numbers 1 to 4 refer to pigs from trial 1 and numbers 5 to 8 to those from trial 2.

[b] tr = trace amounts, visible but too small of an amount to quantitate ($< 0.05\ \mu$g/kg).

The withdrawal trial showed that 5 out of 6 pigs were free of detectable aflatoxins in all tissues after 2 days on the aflatoxin-free diet (Table 12). The one pig, which still showed traces of aflatoxins after 2 days, had severe liver necrosis, which apparently decreased his ability to metabolize these toxic compounds. Similarly, the liver of one pig on the aflatoxin-containing diet slaughtered immediately after withdrawal (pig #2, Table 7) exhibited liver necrosis. The liver appeared quite fatty and uniformly pale. Analysis showed that only aflatoxins B_1 and B_2 were present. The absence of M_1 and M_2 in the necrotic liver of this pig (slaughtered at zero time) suggests that its ability to metabolize aflatoxins was decreased, since aflatoxins M_1 and M_2 were seen in the livers of all other pigs slaughtered at the same time period. Furthermore, the livers of the remaining pigs were normal in appearance. Similarly, Van Zytveld et al. (1970) only detected aflatoxins in the liver and skeletal muscle of chickens after they became severely affected as a result of feeding an aflatoxin-contaminated ration over a 6 week period.

Four days after putting the pigs on an aflatoxin - free diet, there were no detectable levels of aflatoxins in any of the tissues (Table 13). This suggests that placing pigs on an aflatoxin-free diet for 4 days prior to slaughter is adequate to remove detectable levels of free aflatoxins and their metabolites from the tissues of pigs that had previously been fed a highly contaminated diet. The data shown

TABLE 12 - Aflatoxin Residues (μg/kg) Detected in Selected Pig Tissues at Two Days Interval Following Removal From the Contaminated Diet.

Pig No.[a]	Levels of Aflatoxins[b]							
	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Blood				Liver			
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	tr	tr	0	0	0.05	tr	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
	Heart				Muscle			
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	tr	0	0	0	tr	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
	Kidney				Spleen			
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	tr	tr	0	0	0.37	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0

[a] Numbers 1 to 4 refer to pigs from trial 1 and numbers 5 to 8 to those from trial 2.

[b] tr = trace amounts, visible but too small of an amount to quantitate (< 0.05 μg/kg).

TABLE 13 - Aflatoxin Residues (μg/kg) Detected in Selected Pig Tissues at Four Days Interval Following Removal From the Contaminated Diet.

Pig No.[a]	Levels of Aflatoxins[b]							
	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Blood				Liver			
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
	Heart				Muscle			
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
	Kidney				Spleen			
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0

[a] Numbers 1 to 4 refer to pigs from trial 1 and numbers 5 to 8 to those from trial 2.

[b] tr = trace amounts, visible but too small of an amount to quantitate ($< 0.05\ \mu$g/kg).

for aflatoxin clearance from the tissues of pigs in the present work is similar to aflatoxin clearance studies with other animal species. Shank and Wogan (1965) and Wogan et al. (1967) found that rats dosed with ^{14}C labeled aflatoxin B_1 excreted approximately 80 percent of the administered dose within 24 hours following treatment. Similarly, they showed that most of the absorbed aflatoxins were found in the kidneys and livers. Polan et al. (1974) found no detectable level of aflatoxins in the milk of cows 2 days following withdrawal from the contaminated feed. In a similar study, McKinney et al. (1973) reported that the milk of lactating cows fed an aflatoxin-contaminated ration were essentially free of aflatoxins at 72 hours following withdrawal from the contaminated feed. On the other hand, Keyl and Booth (1971) still found aflatoxin M_1 residues in the milk of cows 7 days after replacement of the contaminated feed with an aflatoxin-free diet.

Other studies (Bassir and Osiyemi, 1967; Dalezios et al., 1971; Mabee and Chipley, 1973) using ring labeled aflatoxins have shown that most of the administered dose appears in the tissues and excreta of the dosed animals as water-soluble conjugated aflatoxin metabolites. Due to their increased molecular weight and the presence of two or more aromatic rings in the molecule, the aflatoxin conjugates are preferentially excreted through the bile similar to other exogenous and endogenous compounds (William et al., 1965; Milburn et al., 1967). The aflatoxins have been found in

the bile either as sulfate, glutathione, glucuronide or taurocholate conjugates (Bassir and Osiyemi, 1967; Dalezios et al., 1971; Mabee and Chipley, 1973). Due to their high polarity and enhanced water solubility, these compounds would not be extracted by the solvents used in this study. On the other hand, they should be more easily cleared from the body than the nonconjugated aflatoxin metabolites. Although the data shown in the clearance study refers solely to the free or nonconjugated aflatoxins, the possibility that aflatoxins conjugates may remain in the tissues of the pig following withdrawal from the contaminated feed is far more unlikely than is the case for the free aflatoxins.

The absorbed aflatoxins may also exist in the tissues of the dosed animals as covalent bound residues. These aflatoxin adducts bound to vital cell macromolecules constitute the critical initiating events of aflatoxin toxicity ultimately leading to altered cell function and death. The active species involved in the covalent binding to tissue macromolecules are either the electrophilic aflatoxin B_1-2, 3-epoxide, which binds to various nucleophiles (Garner et al. 1971, 1972; Swenson et al., 1973, 1975, 1977; Roebuck et al. 1978) or the aflatoxin B_1 hemiacetal, aflatoxin B_{2a}, which readily forms Schiff bases with free amino groups at physiological pH (Patterson and Roberts, 1970, 1972; Gurtoo and Campbell, 1974; Ashoor and Chu, 1975).

These aflatoxin adducts will probably remain in the tissues longer than the free or conjugated aflatoxins. They

are eventually removed from the cell either through the cellular repair mechanism or are eliminated along with the associated molecule by cellular catabolism. Although a short withdrawal period was used in this study, it was believed to be adequate to obtain total tissue clearance of free and conjugated aflatoxins. However, it is possible that the aflatoxin adducts may still remain in the tissues of the aflatoxin-dosed animals. For example, Sawhney et al. (1973) using ring labeled aflatoxins found that approximately 29 percent of the administered dose still remained in the tissues of chickens 7 days after treatment. Swenson et al. (1974) showed that 9 percent of an administered dose of ^{3}H-aflatoxin B_1 was covalently bound to the macromolecules in rat liver. They also reported that the specific activity of DNA and rRNA were 15 and 20 times higher, respectively, than those of total protein. Hayes et al. (1977) found that about 14 percent of the initial ^{14}C labeled aflatoxin B_1 incubated into bovine liver microsomes was bound to the microsomal protein. Furthermore, Gurtoo and Campbell (1974) reported that aflatoxin B_{2a} was the major metabolite formed upon incubation of aflatoxin B_1 with rat liver microsomes. Aflatoxin B_{2a} formed in this way was found to be covalently bound to the microsomal fraction.

Studies have shown that dissociation of the covalent ligand between aflatoxins and other cellular components can occur under appropriate conditions, and result in the release of bound aflatoxins. Swenson et al. (1973) showed

that mild acid hydrolysis of the RNA bound aflatoxin B_1 resulted in formation of the dihydrodiol, the same compound resulting from spontaneous hydration of the aflatoxin B_1-2, 3-epoxide (Figure 4). Aflatoxin B_{2a} has also been isolated after enzymatic digestion of tissue extracts from chickens dosed with aflatoxin B_1 (Chipley et al., 1974).

The release of aflatoxins B_{2a} and dihydrodiol from the covalently bound form may also occur if contaminated meat is ingested. This is believed to be true since the aflatoxin adducts may be hydrolyzed under acidic conditions or by the enzymes of the digestive tract (Chipley et al., 1974). If the release of aflatoxin B_{2a} and dihydrodiol occurs under these conditions, it may, however, represent no health hazard because aflatoxin B_{2a} has been shown to be essentially non-toxic when ingested orally (Pohland et al., 1968). This may be due to its high protein binding capacity, and thus, sequestration and elimination along with the desquamated epithelial cells (Patterson, 1977). The dihydrodiol should be equally non-toxic under the same conditions, since it is structurally and chemically similar to aflatoxin B_{2a}. Thus, the mechanism of toxicity should follow the pattern similar to that of aflatoxin B_{2a} as shown in Figure 4. Although the data accumulated on metabolism and tissue distribution of aflatoxins suggest that their conjugated and bound forms are more unlikely to be risk hazards, more data are needed to substantiate or disprove this theory. Thus, more information on the toxicity and metabolism of aflatoxin adducts is

needed to fully determine the importance of these residues in meat for both man and other animals.

Results indicate that the aflatoxins are efficiently removed from the tissues of the pig after putting the animals on an aflatoxin-free diet. Such post-exposure depletion data could be used as a guide in feeding experiments utilizing contaminated feed in situations where the capability for control exists. Thus, the clearance study suggests that placing the pigs on an aflatoxin-free diet for 4 days prior to slaughter is adequate to remove detectable levels of aflatoxins and their metabolites from the tissues of pigs (Table 13).

Effects of Processing and Cooking upon the Levels of Aflatoxins in Pig Tissue

Broiling of Fresh Tissue

The aflatoxins present in naturally contaminated pig tissues (zero days withdrawal) were fairly stable under the conditions used in this experiment, which simulated commercial processing of meat. For example, contaminated loin tissues broiled to an internal temperature of 76° C resulted in an average of 23 and 28 percent reduction in aflatoxin B_1 and B_2 levels, respectively (Table 14). The differences observed between the cooked and raw tissues, however, were not statistically significant due to the relatively small number (4) of samples. There was also considerable animal to animal variation in the aflatoxin levels.

Processing of Hams

Cooking raw hams at 176° C to an internal temperature of 71° C caused an average of 30 and 12 percent reduction in the levels of aflatoxins B_1 and B_2, respectively (Table 14). The differences, however, were not statistically significant due to the large amount of variability and the limited number of samples. Similarly, curing of hams followed by smoking-cooking to an internal temperature of 66° C reduced the levels of aflatoxins B_1 and B_2 by an average of 24 and 18 percent, respectively. Further cooking of the smoked-cured hams at 171° C to an internal temperature of 71° C, which would be representative of cooking hams in the home, resulted in an average of 17 and 9 percent reduction in the levels of aflatoxins B_1 and B_2, respectively, over the raw hams (Table 14). However, the differences observed were not statistically significant. Under these conditions, the aflatoxins were quite stable, thus, curing, smoking and cooking were not very effective in reducing aflatoxin levels.

Processing of Bellies

Frying the raw belly strips for 3 minutes on each side at a temperature of 171° C caused a slight increase in the average residual levels of aflatoxins over the raw samples (Table 14). The mean levels of aflatoxins B_1 and B_2 in the raw-fried bellies were 10 and 4 percent higher, respectively, than the uncooked samples. These differences were small and

TABLE 14 - Aflatoxin Levels (μg/kg) Expressed as Dry Weight Basis Found in Pig Tissues Processed in Different Ways

Sample[b]	Levels of Aflatoxins[a] B_1 Range	B_1 Mean±S.D.[c]	B_2 Range	B_2 Mean±S.D.[c]	% Losses[d]
Loin					
Raw	0.67-1.91	1.12±0.46	0.44-1.78	1.18±0.48	0
Cooked	0.54-1.41	0.86±0.33	0.50-1.25	0.85±0.28	-26
Hams					
Raw	0.66-2.05	1.15±0.52	0.66-1.41	0.95±0.33	0
Raw-Cooked	0.54-1.35	0.80±0.27	0.41-1.29	0.83±0.29	-22
Cured-Smoked	0.59-1.54	0.87±0.34	0.36-1.24	0.78±0.33	-21
Cured-Smoked-Cooked	0.44-1.70	0.95±0.45	0.43-1.51	0.86±0.42	-14
Bellies					
Raw	0.31-0.75	0.45±0.20	0.25-0.57	0.45±0.14	0
Raw-Cooked	0.33-0.77	0.50±0.19	0.26-0.63	0.47±0.15	+8
Bacon-Smoked	0.34-0.66	0.46±0.15	0.32-0.67	0.47±0.15	+3
Cooked-Smoked Bacon	0.27-0.53	0.35±0.12	0.20-0.68	0.37±0.22	-20

[a] Data are taken from Appendix Tables 19, 20 and 21.
[b] Ham and Loin values are averages of 8 replicates and Bellies are averages of 4 replicates.
[c] S.D. = Standard deviation.
[d] Refers to total aflatoxin losses or gains ($B_1 + B_2$).

were not statistically significant. Similarly, a slight increase in average aflatoxin levels was observed upon processing raw bellies into bacon followed by smoking-cooking to an internal temperature of 66° C. The differences in the aflatoxin levels as result of the processing were not statistically significant. In contrast, cooking smoked-cured bacon samples under the same conditions used for raw bellies resulted in an average of 22 and 18 percent reduction of aflatoxin B_1 and B_2 levels, respectively, over the raw bellies. The differences, however, were still not statistically significant due to the small number of samples and large amount of variability.

Overall results suggest that the internal cooking temperatures achieved and the short duration of cooking did not cause a major reduction in the levels of aflatoxins in the meat. Although curing, smoking-cooking, and cooking per se caused some degradation of the aflatoxins, these differences were not statistically significant on comparing processed and raw tissues.

Most studies on the degradation of aflatoxins using heat treatments have been concerned with feed stuffs. Only a few investigations have studied the effects of processing on the stability of aflatoxins in foods, and these have been with dairy products. Allcroft and Carnaghan (1963) reported that milk naturally contaminated with aflatoxins did not show a reduction in toxicity after treatment by pasteurization or by roller-drying. They used the one-day-old

duckling assay test (Asplin and Carnaghan, 1961) to measure the toxicity of the milk before and after processing. However, the duckling assay test for aflatoxin is not a particularly sensitive test (Wogan, 1964).

Using chemical analysis, Stoloff et al. (1975) and Van Egmond et al. (1977) reported similar results to those of Allcroft and Carnaghan (1963). They observed no loss of aflatoxin M_1 from milk after different types of heat treatments. In contrast to these reports, Purchase et al. (1972) claimed that processing of milk under conditions similar to those used by Stoloff et al. (1975) and Van Egmond et al. (1977) reduced its M_1 content. In the same study, Purchase et al. (1972) also reported that the higher the temperature used the lower the amount of residual aflatoxins. On using chemical analysis, they reported a reduction of aflatoxin M_1 in milk by 33 percent on pasteurization at 62° C for 30 minutes, with a 45 percent reduction upon sterilization at 72° C for 45 seconds and 64 percent reduction upon sterilization at 80° C for 45 seconds.

Under the conditions used for processing and cooking of the contaminated meat in the present study, the internal temperature of the cooked samples was always below 80° C, except in the case of the bacon and belly strips. The mild effects of the heat treatment on the aflatoxins in the meat agree with the results of Mann et al. (1967), who found that temperatures of 60 to 80° C have little effect on aflatoxins in oil seed meals. On the other hand, they reported a

substantial amount of aflatoxins are degraded at temperatures of 100° C or more.

In the same study, Mann et al. (1967) reported that increasing the moisture content and/or the heating time caused a proportional decrease of aflatoxins in oil seed meals. Thus, temperature, heating time and moisture may be important factors influencing the stability of aflatoxins during cooking. For example, Peers and Linsel (1975) found that aflatoxin B_1 was not degraded in peanut and corn oils until the temperature reached 250° C, which is close to the melting point of aflatoxin B_1. In contrast, Coomes et al. (1966) reported that autoclaving moist peanut meal for 4 hours at 120° C reduced the amount of aflatoxins from 7000 to 350 ppb. In the same study using model systems, Coomes et al. (1966) concluded that moisture and heat are necessary to cause the hydrolytic opening of the lactone ring on the aflatoxin B_1 molecule, which can then be further decarboxylated leading to formation of non-toxic breakdown products. Thus, the decreased moisture levels of smoked-cured products as compared to fresh tissues and the higher fat content of bacon and bellies may have a protective effect during cooking. Evidence for this is seen in that the influence of cooking was even lower in bellies, bacons and cured hams than in the fresh tissues (Table 14).

Results demonstrate that bacon had considerably lower aflatoxin levels than hams and loins, apparently because the aflatoxins are localized in the lean tissues. Analysis of

drippings collected upon cooking showed them to contain only trace amounts of aflatoxins, thus, further suggesting that the aflatoxins are not fat soluble.

The present study shows that curing and smoking-cooking did not greatly influence the levels of aflatoxins in bacons and hams. Cooking raw samples showed the maximum reduction of aflatoxins in meat, and broiling was even more effective than roasting. Although the meat samples were cooked to a same internal temperature, broiling was done at higher temperatures than roasting. Thus, results verify the greater effect of high temperatures upon destruction of aflatoxins. The smaller size of the loin roasts may have also resulted in greater exposure of the aflatoxins to heat. The results also indicate that the losses of aflatoxin B_1 during processing were higher than for B_2. This suggests that aflatoxin B_1 is less stable to the heat treatment than B_2. Processing had the least effect on aflatoxins present in the bellies and bacons, except for the fried-smoked bacon strips. Although the same frying schedule was used for the bacon and bellies, the fried-smoked bacon strips appeared to be overcooked in relation to the other samples. This may explain the greater destruction of aflatoxins in these samples.

Although processing had some effect in lowering the levels of aflatoxins in meat, the differences on comparing raw and processed tissues were not statistically significant. This was probably the result of the relatively small number of samples. There was also considerable variability in the

aflatoxin levels from sample to sample as each was taken from a different pig. Cooking fresh tissue, which had the greatest effect in reducing the aflatoxin levels, was still not very effective with a mean maximum reduction of 26 percent. Never-the-less, most of the aflatoxins originally present in the tissue were unaffected by cooking and/or curing. Thus, processing meat under the conditions used in this experiment was not effective in reducing aflatoxin levels of contaminated tissues to within safe limits.

SUMMARY AND CONCLUSIONS

Two feeding trials were conducted to determine the amount of time necessary for tissue clearance of pigs fed an aflatoxin contaminated diet. There were 20 pigs in each trial, with 4 being fed the control diet and 16 being used to determine the time necessary for tissue clearance after removal from the contaminated diet. The pigs on both trials 1 and 2 were fed the control diet spiked with aflatoxins to give a concentration of 551 and 355 μg of B_1 and B_2, respectively, per kg of feed. The feed for the control pigs on trial 1 was found to be naturally contaminated with 20 and 31 μg of aflatoxins B_1 and B_2, respectively, per kg of feed, while in trial 2 the control feed was free of aflatoxins. The initial phase of each trial in which the pigs were fed either the control or spiked diets lasted for 42 days.

Results demonstrated that even the low levels of aflatoxins found in the naturally contaminated diet of the control pigs in trial 1 were high enough to adversely affect the pig's performance and organ weights. In trial 1, there were no significant differences between the weight gains and organ weights for the control pigs in comparison to those fed the spiked diets. The control pigs on trial 1 exhibited depressed growth and on average gained 40 percent less weight than the controls in trial 2 over the same feeding

period. Furthermore, the controls in trial 1 had a 24 percent reduction in feed intake as compared to the controls in trial 2. Thus, overall results suggest that growing pigs are very susceptible to aflatoxin toxicity, even when exposed to low levels in the diet.

In trial 2, the basal diet was uncontaminated with aflatoxins, so that there was a significant difference in rate of gain and feed consumption between the controls and the group fed the aflatoxin spiked diet. The pigs fed aflatoxins exhibited depressed growth and on the average gained 45 percent less weight than the control animals over the 42 day feeding trial. On the average, the pigs fed aflatoxins showed a 32 percent reduction in feed intake as compared to the controls. Feeding the pigs aflatoxins also adversely effected the efficiency of feed utilization. There was also a 30 percent increase in the liver weights of the pigs fed aflatoxins in comparison to the control group.

Tissues and organs from the control and experimental pigs in trial 1 and 2 were normal in appearance, except the livers of two pigs, one from trial 1 (slaughtered at zero day withdrawal) and the other from trial 2 (killed after withdrawal for two days). Livers from these two pigs exhibited yellow discoloration, appeared quite fatty, and were condemned for human consumption. All other organs, including the kidneys, and all carcasses were free of other lesions, and were passed for human consumption.

Analysis of the tissues of pigs at zero day withdrawal showed that only a small fraction of the aflatoxins consumed was deposited in the tissues, either as the original compound or as their metabolites. The aflatoxins were found to be widely distributed in all tissues. The blood showed the lowest level of residual aflatoxins, followed by spleen, muscle and heart, in that order. The highest levels of aflatoxins were present in the liver and kidneys, with the mean value for the combined aflatoxins being less than 3 ppb. The total aflatoxin levels found in the muscle samples were even lower than those found in the liver and kidneys and averaged less than 1 ppb.

Except for the liver and kidneys, the levels of aflatoxins M_1 and M_2 were much lower than B_1 and B_2. The mean retention of aflatoxins B_1 and B_2 were calculated to be 0.03 and 0.04 percent, respectively. Even the low levels of aflatoxins occurring naturally in the control diet resulted in small amounts of aflatoxins in the liver and kidneys of the control pigs in trial 1.

Results from the withdrawal trial showed that the pigs metabolized and efficiently removed aflatoxins from the tissues. Four days after placing the pigs on an aflatoxin-free diet, there were no detectable levels of aflatoxins in any of the tissues.

Processing and cooking of the contaminated tissues (zero day withdrawal) did not cause a major reduction in the levels of aflatoxins in the meat. Broiling raw loins to an

internal temperature of 76° C reduced the mean aflatoxin levels by 23 and 28 percent for B_1 and B_2, respectively. Cooking raw hams to an internal temperature of 71° C resulted in an average of 30 and 12 percent reduction in the levels of aflatoxins B_1 and B_2, respectively. Similarly, curing of hams followed by smoking-cooking to an internal temperature of 66° C reduced the levels of B_1 and B_2 an average of 24 and 18 percent, respectively. Further cooking of the smoked-cured hams to an internal temperature of 71° C resulted in an average reduction of 17 and 9 percent in the levels of aflatoxins B_1 and B_2, respectively.

Frying the raw belly strips caused a slight but insignificant increase in the levels of aflatoxins B_1 and B_2, which was also true for the bacon smoked during processing. In contrast, cooking of the smoked-cured bacon following processing resulted in an average reduction of 22 and 18 percent for aflatoxin B_1 and B_2, respectively.

APPENDIX A

STATISTICAL ANALYSIS

Analyses of Variance

APPENDIX A

STATISTICAL ANALYSIS

Analyses of Variance

k = No. of Treatments n = No. of Observations

1. Mean of observation in the i^{th} sample (i = 1, 2, ... k)

$$\bar{X}_i = \frac{1}{n_1} \sum_{j=1}^{n_1} X_{ij}$$

2. Standard deviation of observation in the i^{th} sample

$$S_1 = [(\sum_{j=1}^{n_1} X_{ij}^2 - n_1\bar{X}_1^2)/n_1-1]^{1/2}$$

3. $$\text{Sum}_1 = \sum_{j=1}^{n_i} X_{ij}$$

4. Total sum of squares

$$TSS = \sum_{i=1}^{k} \sum_{j=1}^{n_i} X_{ij}^2 - \frac{(\sum_{i=1}^{k} \sum_{j=1}^{n_i} X_{ij})^2}{\sum_{j=1}^{k} n_1}$$

5. Treatment sum of squares

$$TrSS = \sum_{i=1}^{k} \frac{(\sum_{j=1}^{n_1} X_{ij})^2}{n_i} - \frac{(\sum_{i=1}^{k} \sum_{j=1}^{n_1} X_{ij})^2}{\sum_{i=1}^{k} n_i}$$

6. Error sum of squares

$$ESS = TSS - TrSS$$

7. Treatment degrees of freedom

$$df_1 = k - 1$$

8. Error degrees of freedom

$$df_2 = \sum_{i=1}^{k} n_i - k$$

9. Total degrees of freedom

$$df_3 = df_1 + df_2 = \sum_{i=1}^{k} n_i - 1$$

10. Treatment mean square

$$TrMS = \frac{TrSS}{df_1}$$

11. Error mean square

$$EMS = \frac{ESS}{df_2}$$

12. The F ratio

$$F = \frac{TrMS}{EMS} \quad \text{(with degrees of freedom } df_1, df_2)$$

APPENDIX B

SUPPLEMENTAL TABLES

APPENDIX B

SUPPLEMENTAL TABLES

TABLE 15 - Performance Data for Six Weeks Feeding Experiment of Pigs from Trial 1 Slaughtered at Zero Day Withdrawal

Pig No.	Starting Weight (kg)	Final Weight (kg)	Weight gain (kg)
	Control Group		
1	10.9	23.9	13.0
2	8.6	29.5	20.9
3	9.8	29.1	19.3
4	8.6	16.1	7.5
Mean	9.5	24.7	15.2
	Aflatoxin Fed Group 1		
5	8.2	14.8	6.6
6	8.9	16.1	7.2
7	10.5	25.5	15.0
8	9.8	23.4	13.6
9	10.0	18.4	8.4
10	8.6	21.1	12.5
11	8.6	19.1	10.5
12	11.6	28.0	16.4
Mean	9.5	20.8	11.3
	Aflatoxin Fed Group 2		
13	8.2	13.6	5.4
14	11.1	23.4	12.3
15	8.9	19.3	10.4
16	8.6	19.3	10.7
17	9.1	19.3	10.2
18	10.5	24.5	14.0
19	10.0	17.3	7.3
20	10.9	22.0	11.1
Mean	9.7	19.8	10.1

TABLE 16 - Internal Organ Weights in Grams and as Percentages of Body Weight (Values in Parenthesis) of Pigs from Trial 1 Slaughtered at Zero Day Withdrawal

Pig No.	Heart	Kidneys	Liver	Spleen
		Control Group		
1	101 (0.42)	123 (0.51)	672 (2.81)	43 (0.18)
2	111 (0.38)	141 (0.48)	780 (2.64)	44 (0.15)
3	110 (0.37)	139 (0.47)	744 (2.52)	44 (0.15)
4	66 (0.22)	85 (0.29)	438 (1.48)	66 (0.22)
Mean	97 (0.35)	122 (0.44)	658 (2.36)	49 (0.18)
		Aflatoxin Fed Group		
5	66 (0.39)	97 (0.58)	514 (3.06)	32 (0.19)
6	72 (0.50)	87 (0.61)	493 (3.44)	24 (0.17)
7	94 (0.46)	85 (0.42)	571 (2.79)	35 (0.17)
8	69 (0.38)	85 (0.47)	428 (2.38)	33 (0.18)
Mean	75 (0.43)	89 (0.52)	501 (2.92)	31 (0.18)

TABLE 17 - Performance Data for Six Weeks Feeding Experiment of Pigs from Trial 2 Slaughtered at Zero Day Withdrawal

Pig No.	Starting Weight (kg)	Final Weight (kg)	Weight Gain (kg)
	Control Group		
1	10.2	35.2	25.0
2	9.5	34.5	25.0
3	9.3	33.2	23.9
4	9.5	36.1	26.6
Mean	9.6	34.8	25.2
	Aflatoxin Fed Group 1		
5	10.2	26.1	15.9
6	8.9	26.6	17.7
7	9.3	19.3	10.0
8	9.3	18.0	8.7
9	10.2	27.5	17.3
Mean	9.6	23.5	13.9
	Aflatoxin Fed Group 2		
10	9.8	26.1	16.3
11	10.7	27.3	16.6
12	9.5	17.5	8.0
13	8.9	16.6	7.7
14	10.0	29.3	19.3
Mean	9.8	23.4	13.6
	Aflatoxin Fed Group 3		
15	10.2	23.9	13.7
16	9.1	17.0	7.9
17	10.7	23.9	13.2
18	9.1	17.5	8.9
19	9.3	19.3	10.0
20	8.0	22.0	14.0
Mean	9.4	20.6	11.2

TABLE 18 - Internal Organ Weights in Grams and as Percentages of Body Weight (Values in Parenthesis) of Pigs from Trial 2 Slaughtered at Zero Day Withdrawal

Pig No.	Heart	Kidneys	Liver	Spleen
		Control Group		
1	139 (0.39)	163 (0.46)	792 (2.25)	63 (0.18)
2	140 (0.41)	201 (0.58)	836 (2.42)	66 (0.19)
3	116 (0.35)	142 (0.43)	686 (2.07)	48 (0.14)
4	135 (0.37)	177 (0.49)	663 (1.84)	60 (0.17)
Mean	133 (0.38)	171 (0.49)	744 (2.15)	59 (0.17)
		Aflatoxin Fed Group		
5	61 (0.34)	74 (0.41)	481 (2.67)	38 (0.21)
6	66 (0.40)	91 (0.55)	523 (3.15)	22 (0.13)
7	101 (0.42)	132 (0.55)	699 (2.92)	41 (0.17)
8	67 (0.35)	87 (0.45)	473 (2.45)	37 (0.19)
Mean	74 (0.38)	96 (0.49)	544 (2.80)	35 (0.18)

TABLE 19 - Aflatoxin Levels (μg/kg) Detected in Raw Bellies, Raw-Cooked Bellies, Smoked Bacon and Smoked-Cooked Bacon Expressed as Dry Weight Basis.

Pig No.[a]	Raw		Raw-Cooked[b]		Smoked Bacon		Smoked-Cooked Bacon[b]	
	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
1	0.31	0.44	0.33	0.47	0.34	0.40	0.27	0.36
2	0.38	0.53	0.50	0.63	0.47	0.67	0.53	0.68
3	0.75	0.57	0.77	0.51	0.66	0.50	0.34	0.23
4	0.35	0.25	0.39	0.26	0.35	0.32	0.27	0.20
Mean	0.45	0.45	0.50	0.47	0.46	0.47	0.35	0.37

[a] Each sample represents a bacon from a different pig fed the aflatoxin-spiked diet at zero days withdrawal time.

[b] The sample was processed as described in the experimental section.

TABLE 22 - Moisture Determination Data for Raw Loin and Cooked Loin Samples

Sample No.	Wet Wt. (g)	Dried Wt. (g)	Moisture (%)
	Raw Loin		
1	4.9382	1.1392	76.9
2	4.8883	1.1483	76.5
3	5.2947	1.2409	76.6
Mean	-	-	76.7
	Cooked Loin		
1	5.9367	2.5931	56.3
2	4.7780	2.2111	53.7
3	4.7032	1.9536	58.5
Mean	-	-	56.2

TABLE 20 - Aflatoxin Levels (μg/kg) Expressed as Dry Weight Basis Found in Raw Loin Tissues and Raw-Cooked Loin

Pig No.[a]	Raw		Raw-Cooked[b]	
	B_1	B_2	B_1	B_2
1	0.85	1.05	0.58	0.75
	0.80	1.12	0.54	0.70
	0.83	1.09	0.56	0.73
2	1.07	1.60	0.85	1.20
	1.18	1.78	0.80	1.25
	1.13	1.69	0.83	1.23
3	1.91	1.61	1.41	0.82
	1.72	1.25	1.33	1.00
	1.82	1.43	1.37	0.91
4	0.77	0.60	0.75	0.58
	0.67	0.44	0.64	0.50
	0.72	0.52	0.70	0.54

[a]Each sample represents a loin from a different pig fed the aflatoxin-spiked diet at zero day withdrawal time.

[b]The sample was cooked by broiling as described in the experimental section.

TABLE 21 - Aflatoxin Levels (μg/kg) Expressed as Dry Weight Basis Found in Hams Processed in Different Ways

Pig No.[a]	Raw		Raw Cooked[b]		Smoked-Cured[b]		Cured-Smoked-Cooked[b]	
	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
1	0.66	0.82	0.54	0.55	0.59	0.60	0.59	o.48
	0.66	0.66	0.66	0.79	0.62	0.80	0.44	0.61
	0.66	0.74	0.60	0.67	0.61	0.70	0.52	0.55
2	1.41	1.41	0.76	1.06	0.75	0.91	1.35	1.22
	0.85	1.24	0.93	1.29	1.10	1.20	0.99	1.51
	1.13	1.33	0.85	1.18	0.93	1.06	1.17	1.37
3	2.05	1.18	0.96	0.84	1.04	0.66	1.70	1.09
	1.75	1.14	1.35	1.01	1.54	1.24	1.25	1.08
	1.90	1.16	1.16	0.93	1.29	0.95	1.48	1.09
4	0.95	0.57	0.55	0.41	0.68	0.43	0.69	0.43
	0.87	0.61	0.63	0.68	0.60	0.36	0.59	0.44
	0.91	0.59	0.59	0.55	0.64	0.40	0.64	0.44

[a] Each sample represents a ham from a different pig fed the aflatoxin-spiked diet at zero days withdrawal time.

[b] The sample was processed as described in the experimental section.

TABLE 23 - Moisture Determination Data for Raw Ham, Raw-Cooked Ham, Cured-Smoked Ham and Cured-Smoked-Cooked Ham

Sample No.	Wet Wt. (g)	Dried Wt. (g)	Moisture (%)
	Raw Ham		
1	7.5300	2.9283	73.6
2	7.5100	3.0171	72.1
3	7.6560	2.9144	74.4
Mean	-	-	73.4
	Raw-Cooked Ham		
1	4.8874	1.8068	63.0
2	5.3250	1.9548	63.3
3	4.9710	1.8396	63.0
Mean	-	-	63.1
	Cured-Smoked Ham		
1	5.0943	1.7117	66.4
2	5.0019	1.6482	66.3
3	4.8911	1.6448	66.4
Mean	-	-	66.4
	Cured-Smoked-Cooked Ham		
1	5.0383	1.7029	66.2
2	5.2595	1.7768	66.2
3	5.1973	1.7614	66.1
Mean	-	-	66.2

TABLE 24 - Moisture and Fat Data for Raw Belly, Fried Belly, Smoked Bacon and Fried-Smoked Bacon

Sample No.	Wet Wt. (g)	Dried Wt. (g)	Moisture (%)	Ether Extract Wt. (g)	Fat (%)
			Belly		
1	5.0514	2.0442	59.5	1.2268	24.3
2	5.3498	1.8944	64.6	0.9618	18.0
3	6.2294	2.3938	61.6	1.4389	23.1
4	5.5829	2.3991	57.0	1.5476	27.7
Mean	-	-	61.0	-	23.3
			Fried Belly		
1	4.9664	2.9494	40.6	1.4650	29.5
2	4.8997	2.9014	40.8	1.4140	28.9
3	5.1469	3.0776	40.2	1.5593	30.3
Mean	-	-	40.4	-	29.6
			Smoked Bacon		
1	5.1034	3.5181	31.1	2.0365	39.9
2	5.2596	3.7322	29.0	2.4485	46.6
3	5.3170	3.6807	30.0	2.2995	43.2
Mean	-	-	30.3	-	43.2
			Smoked-Fried Bacon		
1	4.5630	4.2350	7.2	1.6017	35.1
2	3.5521	3.1853	10.3	0.9837	27.7
3	4.4505	4.1277	7.3	1.2557	30.5
Mean	-	-	8.3	-	31.1

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