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AFLATOXINS IN THE TISSUES OF PIGS FED A CONTAMINATED RATION

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ABSTRACT

AFLATOXINS IN THE TISSUES OF PIGS FED
A CONTAMINATED RATION

By

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Four control and four experimental pigs were fed on a basal diet consisting of corn and soybean oil meal supplied with vitamins and minerals. The feed of the experimental pigs was spiked with 662, 273, 300 and 285 ppb of aflatoxins B_1, B_2, G_1 and G_2, respectively. The basal diet was free of aflatoxins.

After 21 days on experiment, the pigs were slaughtered. Inspection of the tissues of all pigs showed no observable gross or pathological lesions. The pigs fed aflatoxins, however, had 36% heavier livers, gained 25% less weight with an 18% reduction in feed intake. Feed efficiency was not affected.

All pigs fed aflatoxins showed B_1, B_2, M_1 and B_{2a} residues in the tissues but G_1 and G_2 residues were not detected. Organs and tissues of the control pigs were free from aflatoxins.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Aflatoxins are a group of secondary metabolites produced by certain strains of the genus Aspergillus. These metabolites have been found as food and feed contaminants and are of great health and economic significance throughout the world.

Studies have shown that ingested aflatoxins may be deposited in the tissues as the original aflatoxin, or as one of its metabolites (Purchase, 1972). There is also some epidemiological evidence of aflatoxin carcinogenity in man (Shank et al., 1972; Campbell and Stoloff, 1973).

Conventional methods for analysis of aflatoxins in feed and foods of animal origin are based on their extraction with suitable solvents. Following purification of the extracts, the separation and quantitation of the aflatoxins are carried out on thin layer chromatographic plates using standards for visual comparison or for fluorodensitometric analysis.

Earlier investigations on the transmission of aflatoxins from feed to animal tissues using biological assays (Allcroft and Carnagham, 1963; Platonow, 1975) or methods primarily designed for assaying aflatoxins in agricultural products (Kratzer et al., 1969; Keyl and Booth, 1971) failed to detect aflatoxins in the tissues of any of the animals examined, even in those with confirmed signs of aflatoxicosis. More recent methods for analysis of aflatoxins in tissues of animals have

been shown to be more sensitive than the earlier procedures (Brown et al., 1973; Jemmali and Murthy, 1976), but they still lack sensitivity. Thus, information available on aflatoxins in animal tissues is inconclusive and further data are needed.

It was purpose of this study to select a sensitive and simple method for analysis of aflatoxins in tissues of animal origin and to study the carry over of aflatoxins and their metabolites in the tissues of pigs fed aflatoxin-contaminated ration.

REVIEW OF LITERATURE

Occurrence of Aflatoxins

Allcroft and Carnaghan (1963) have reviewed the occurrence of aflatoxin poisoning in poultry and farm animals. They stated that there were many reports of groundnut (peanut) poisoning on record prior to 1960. They reviewed the losses of young turkeys on poultry farms in Great Britain. In 1960, they reported that the mortality was highest among 3-6 week old birds and that total losses amounted to over 100,000 turkeys. Generally the affected birds died within a week, with loss of appetite, lethargic signs, and weakness of the wings. In most cases, postmortem examination showed hemorrages or pale necrotic lesions in the livers and frequently engorged kidneys. Brazilian groundnut meal in feed given to birds was suspected to be the poisoning agent by 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, although no fatal cases were observed on a ration from which the groundnut was removed (Asplin et al., 1961).

The possibility of poisoning due to Brazilian groundnut meal was confirmed by Sargeant et al. (1961a), who gathered samples of groundnut from 13 countries. They detected toxicity in the samples from India, Uganda, Tanganyika, French West Africa, Gambia, and Ghana.

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

Lancaster et al. (1961) fed rats with 20% 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.

Sargeant et al. (1961a) extracted the toxic principle from Brazilian groundnut meal, and concentrated it 250 times. They then examined its effect on feeding it to ducklings and turkey poults. Since the pathological findings of liver damage were similar to those reported for "turkey X disease", the toxic principle was further purified with alumina chromatography. This step yielded colorless crystals. Although the preparation was not yet pure, it fatally affected 1-day-old ducklings within 24 hours using a dosage of 20 μg. On further purification by Whatman No. 1 paper chromatography with 5% acetic acid in n-butanol, the toxic substance gave a single spot at R_f = 0.7, and emitted a bright-blue fluorescence under UV light. They suspected that the substance might be a fungal metabolite, since the highly toxic sample of nuts from Uganda was heavily contaminated with fungi. Therefore, Sargeant et al. (1961a) used paper chromatography and detected fluorescent material at R_f = 0.7 and sucessfully separated the toxic substance from the samples. The fungus producing the toxin was identified as Aspergillus flavus, and the toxic fluroescent metabolite was referred to as aflatoxin.

De Iongh et al. (1964) examining peanuts samples produced in the Argentine in 1922 showed that the contamination of peanuts is not a new development. Thus, aflatoxins have probably been in the feed or food supply for many years.

The first evidence that more than one toxin may be responsible came from two differents groups working independently (De Iongh et al., 1962; Nesbitt et al., 1962). De Iongh et al. (1962) applied the toxic principle on a silica gel column and eluted a fluorescent band with chloroform. They then transferred it to glass plates coated with Kieselgel G and developed it in chloroform containing 2% methanol. This resulted in several spots, which exhibited different fluorescent colors under UV light. They named the differents 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.

About the same time Nesbitt et al. (1962), using alumina chromatoplates with 1-5% methanol-chloroform, were able to resolve the toxic principle into two fluorescent spots under UV light. One had an R_f 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.

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 extracted from sterilized groundnut meal, which had been previously inoculated with

a toxin producing strain of A. flavus, was resolved into several fluorescent spots by these authors. They used silica Gel G and chloroform with 2% methanol as the solvent. The four aflatoxins were identified on the chromatoplates, and futher 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., 1963) were identical to aflatoxings G_1 and B_1, respectively. Hartley et al. (1963) also demonstrated that the material originally called aflatoxin B (Nesbitt et al., 1962) was a mixture of aflatoxin B_1 and B_2 and identified aflatoxin G_1 and G_2, which had not been previously described. Hartley et al. (1963) also reported the molecular weight, chemical formula, melting point and other chemical and physical characteristics of the four toxins isolated by using infra-red, ultraviolet and mass spectrometry.

Soon after Hartley et al. (1963) reported the isolation of the four aflatoxins, the structural formulas of aflatoxin B_1 and B_2 were determined by Asao et al. (1963). The acute toxicity of each aflatoxin was determined by Carnagham et al. (1963) using 1-day-old ducklings. They also determined the fluorescent characteristics of the aflatoxins in methanol.

The first indication of the occurrence of aflatoxins other than B_1, B_2, G_1, and G_2 was reported by Allcroft and Carnagham (1963). They demonstrated that extracts of milk from cows fed aflatoxin containing groundnut meal induced liver lesions in ducklings identical to those caused by aflatoxin B_1. TLC examination showed that there was no flatoxin B_1 present. The milk toxin was shown to be identical to a

blue-violet fluorescent component also present in toxic groundnut meal (De Iongh et al., 1964). Allcroft and Carnagham (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 afatoxin M for the milk toxin.

Later, Holzapfel et al. (1966) isolated aflatoxin M from sheep urine and separated two components, which they designated as M_1 and M_2. They determined their structures and concluded that M_1 was hydroxyaflatoxin B_1 and M_2 was dehydroxyaflatoxin B_1 or hydroxyaflatoxin B_2. Dutton and Heathcote (1966) reported the isolation of two metabolites from cultures of A. Flavus and concluded that they were hydroxy derivatives of aflatoxin B_2 and G_2, and designated them as B_{2a} and G_{2a}. Later, they elucidated their structure 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, development of thin layer chromatography with suitable solvent systems and the use of high pressure liquid chromatography (Ashoor et al., 1975; Swenson et al., 1975; Croy et al., 1978) resulted in identification of many other metabolites, which have been reviewed by Campbell and Hayes (1976).

General Analytical Methods for Aflatoxins

Extraction of Aflatoxin

Aflatoxins were first extracted from mold contaminated peanut meal according the technique introduced by Sargeant et al. (1961). They used a long and complex method that basically consisted of exhaustive Soxhlet extraction of the sample with methanol, followed by further extraction with chloroform and defatting of the final extract with petroleum ether. The same technique was used by De Iongh et al. (1962) and was later modified by Coomes and Sanders (1963), who limited the extracting solvent to methanol which greatly reduced the time of extraction.

A simpler and more efficient method was introduced by Nesheim (1964a), who blended ground peanuts with aqueous methanol and hexane, which simultaneoulsy defatted and extracted the aflatoxins. Following centrifugation of the mixture an aliquot of the aqueous methanol phase was used for purification of the extract. Pons and Goldblatt (1965), Pons et al. (1966a), and Stoloff et al. (1966) blended aqueous acetone with the sample to extract aflatoxins from cottonseed, peanuts, and a variety of the other plant products.

Liquid-Liquid Partition Chromatography

Aflatoxin extracts obtained using different extraction procedures, mainly exhaustive solvent extraction, contained large amount of interfering substances, such as lipids, carbohydrates and pigments. Coomes and Sanders (1963) partitioned the extracts between methanol: water: petroleum ether in a separatory funnel in order to eliminate interfering substances from the primary methanol extract. Similar partitioning of

the extracts using methanol: water: chloroform was utilized by Broadbent et al. (1963).

Pons and Goldblatt (1965) removed interfering gossypol pigments in primary aqueous acetone extracts of cottonseed meal as insoluble lead derivatives, by treatment of the extract with lead acetate. Partition extraction with chloroform separated aflatoxins from the residual lead salts and pigments. Pons et al. (1966a) also used lead acetate to precipitate interfering substances from primary aqueous acetone extracts from a variety of aflatoxin contaminated agricultural products.

Column Chromatography

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. (1961). The crude extract was sequentially eluted with chloroform and methanol. Aflatoxins were only detected in the chloroform fraction. Coomes and Sanders (1963), used neutral alumina column chromatography as a final step to eliminate interfering substances from a partially purified extract of peanut meal. Nesheim (1964a, b) used partition column chromatography of an aqueous methanol primary extract of peanut meal on diatomaceous earth (Celite). He eluted interfering lipids and pigments with hexane, and extracted the aflatoxins with a chloroform: hexane mixture (1:1, v/v).

Pons et al. (1966a) chromatographed a partially purified aqueous acetone extract of different contaminated products on a silica gel column. They eluted interfering substances with diethyl ether and the aflatoxins with chloroform: methanol (97:3, v/v).

Partition chromatography on a cellulose column was suggested by Stoloff et al. (1966) for further purification of partially purified aqueous acetone extracts of cottonseed products. Interfering substances were eluted with hexane and the aflatoxins with hexane and chloroform (1:1, v/v). Yin (1969) proposed a rapid method for aflatoxins extraction from peanuts and other comodities, which eliminated all clean up procedures used in previous methods. The method consisted of blending the sample with water: acetonitrile (9:1) and hexane. Following filtration of the extract, an aliquot of the aqueous acetonitrile was evaporated to dryness and dissolved in benzene for further TLC analysis.

TLC and Quantitation of Aflatoxins

The use of TLC for separation of aflatoxins, coupled with their intense fluorescent emission under UV light, has enabled their isolation and quantitation by extremely sensitive analytical methods. The first analytical methods (Sargeant et al., 1961, Coomes and Sanders, 1963) utilized paper chromatography. Sargeant et al. (1961), using Whatman No. 1 filter paper and n-butanol-5 per cent acetic acid for development, obtained a single spot at R_f = 0.7. Coomes and Sanders (1963) also used Whatman No. 1 filter paper and benzene: toluene: cyclohexane: ethanol: water (3:3:5:8:5) for development. This system enabled the resolution of two spots, namely, aflatoxins B and G. The minimum amount of aflatoxin B detectable on filter paper was 2 µg.

Broadbent et al. (1963) used TLC plates coated with active alumina and chloroform: methanol (98.5:1.5) for development. This

system did not detect aflatoxin B_1 and B_2 but as little as 6×10^{-3} µg of aflatoxin B was visible on the plates.

De Iongh et al. (1962) were the first to introduce the use of silica gel (Kiesegel G) for the separation of several fluorescent spots of a prepurified aflatoxin extract from contaminated groundnut meal. They used chloroform with 2% methanol (v/v) as the developing solvents. The same TLC system was used by Hartley et al. (1963) and De Iongh et al. (1964b) for the resolution of aflatoxins B_1, B_2, G_1 and G_2. Subsequently, most procedures adopted the silica gel coated plates with variations in the solvent developing systems.

Many solvents alone or in combination together were proposed in order to eliminate the remaining interfering substances. Heusinkveld et al. (1965) proposed the use of chloroform: methanol: acetic acid (15:4.5:0.5) for the resolution of aflatoxins from a variety of peanut products. Willey (1966) used methyl acetate as the only developing solvent for separation of aflatoxins from a variety of agricultural products. Pons et al. (1966b) proposed the use of silica gel G-HR coated plates and chloroform: acetone (85:15) or chloroform: acetone: 2-propanol (82.5:15:25) as the developing solvents in an unlined and unequilibrated tank, in order to improve resolution and fluorodensitometric measurement of aflatoxins.

For analysis of aflatoxins in animal tissues, Brown et al. (1973) recommended the use of silica gel coated plates and chloroform: acetone: isopropanol (90:6:3). Jemmali and Murthy (1976) recommended the use of ether: methanol: water (96:3:1) as developing solvents in order to eliminate the interference of aflatoxin B_1-like substances

from samples of tissues stored under refrigeration. Two-dimensional TLC in chloroform: acetone: isopropanol (85:10:5), followed by development in ether: methanol: water (96:3:1), was recently utilized by Trucksess and Stoloff (Personal communication, 1978) for better separation of aflatoxins from interfering substances in liver extracts. This method gave better resolution for densitometric analysis.

Because of the strong fluorescence of aflatoxins in solution (Carnagham et al., 1962), attempts were made to utilize preparatory TLC to isolate them for quantitation, either by spectrophotometry (Nabney et al., 1965; Agthe et al., 1968) or by fluorometry in solution (Childs et al., 1970). Early methods for estimation of aflatoxins (Broadbent et al., 1963; Coomes et al., 1963; 1964) used dilution to extinction, that is, the analyst first determined the smallest amount of each aflatoxin that could barely be seen on a TLC plate under UV light, and then determined the amount of dilution required to reach the same point of marginal visibility.

Other authors preferred visual estimation for the determination of aflatoxins as reviewed by Pons and Goldblatt (1969). In this technique, the analyst compared matching fluorescence intensity of spots produced by the samples extract with spots of known amounts of authentic aflatoxin standards. According to Beckwith and Stoloff (1968), the visual estimation of aflatoxins on TLC plates has a precision limit no better than ±20% for a single observation, and under operating conditions, is probably closer to ±28%. Densitometric methods using fluorodensitometers have proven more accurate than the visual methods, with average deviations of about ±2% (Ayres and

Sinnhuber, 1966; Stubblefield et al., 1967). Ayres and Sinnhuber (1966) and Beckwith et al. (1968) found that the ratio of aflatoxin spot density to the emitted light, as measured by the recorder response, followed Beer's law over a fairly broad concentration (0.3-30 ng/spot). In a collaborative study, Pons (1969) determined aflatoxins in cottonseed products, both visually and by fluodensitometry on TLC plates, and showed a good correlation existed between estimates by the two techniques from eight different laboratories.

Assay of Aflatoxins in Foods of Animal Origin

Allcroft and Carnagham (1963) were the first to investigate the presence of aflatoxin residues in products from animals fed toxic ground nut meal. Using ducklings for assay, they failed to demonstrate toxicity in either livers, from chickens fed toxic rations, clotted blood serum and livers from cows also receiving toxic rations, from pig liver taken from an animal with fatal aflatoxicosis or from eggs from pullets fed a highly toxic ration. The sensitivity of the duckling assay was later established by Wogan (1964) as 2 μg during five days of treatment.

Platonow (1965) was unable to demonstrate aflatoxins or their metabolites in extracts of liver or from skeletal muscle of chickens fed a toxic peanut ration (3.1 ppm aflatoxin) for as long as six weeks. He used ferrets for the biological assay. Samples of meat and livers of the chickens fed the toxic peanut 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 aflatoxin was fed to the 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 Willey (1966) used in the chemical assay was a modification of the procedure of Pons and Goldblatt (1965) that was originally proposed for analysis of aflatoxins in cottonseed, peanuts, and a variety of other comodities. 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 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 aflatoxin. 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 aflatoxin 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 aflatoxin 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 method of De Iongh et al. (1964) which is a variant 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 aflatoxin ingestion. The tissues were assayed according the method of Eppley (1966) which was originally suggested for analysis of aflatoxins in peanuts. The method basically consisted of extraction of aflatoxins from the tissues with chloroform, followed by

purification of the extract by silica-gel column chromatography and final identification of the aflatoxins by TLC.

Mabee and Chipley (1973) administered low levels of ^{14}C-labeled aflatoxins to broiler chickens by crop intubation. The radioactivity detected in the liver, heart, gizzard, breast meat, and leg meat accounted for 7.85% 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 their analysis, 81.2% 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% 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% 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 Carnaghan (1962) observed that extracts of milk from cows fed aflatoxin-contaminated rations induced identical lesions to aflatoxins administered directly to ducklings. The milk toxin, which

was later named aflatoxin M_1, was first isolated by Allcroft and Carnaghan (1963) and De Iongh et al. (1964a) from the milk of cows fed aflatoxin-contaminated rations. Keyl and Booth (1971) reported that dairy cows fed a ration containing a daily dosage between 67 to 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.

Allcroft and Roberts (1968) measured the amount of aflatoxin M_1 in milk from cows given diets containing various levels of the aflatoxin. The daily intake of aflatoxin B_1 ranged from 0.875 to 24.5 mg, and excretion of aflatoxin M_1 was proportional to the intake. Keyl et al. (1968) also reported a linear relationship between aflatoxin intake and the concentration of aflatoxin M in the milk.

Working with lactating cows fed a ration containing graded levels of aflatoxin B_1, Polan et al. (1974) established a minimum dosage of 46 ppb of aflatoxin B_1 in the ration before aflatoxin M_1 could be detected in the milk.

Krogh et al. (1973) fed diets containing 300 and 500 ppb of aflatoxins $B_1 + B_2$ to pigs for 120 to 230 days. During the growth period from 20 to 90 kg, the pigs on the aflatoxin-contaminated diets had 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 and severe liver degeneration was observed. 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 of aflatoxin. 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. The extract was placed on the column and the interfering substances were eluted with hexane and ethyl ether. Then the aflatoxins were eluted with chloroform: methanol (97:3, v/v). Quantification was performed according to the IUPAC (1968), using aflatoxins standards for visual comparison. In the same work, Krogh et al. (1973) reported that aflatoxin B_1 added to homogenized liver tissue at a level of 1 ppb showed a recovery of 100 percent. However, at 0.5 ppb the recovery was incomplete.

Brown et al. (1973) developed a method for aflatoxin analysis in meat after testing various procedures of extraction and purification. They obtained a high sensitivity which allowed the detection and quantitation of aflatoxins at 0.05 ppb in spiked samples. The method consisted of methanol extraction and purification of the extract by transfer from aqueous methanol to chloroform using the procedure of Jacobson et al. (1971). Purification was accomplished using a silica gel column prepared by the AOAC method I (1970). Following purification of the extract, it was partitioned on a cellulose-aqueous methanol column according to the method of Pons et al. (1973).

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 in to the total ration. In their study, pigs fed the aflatoxin source separately developed toxic symptoms, and aflatoxins B_1, B_2 and M 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 and was followed by silica gel chromatography as described by Brown et al. (1973). The extracts were further purified by liquid-liquid defatting with hexane, transfer of aflatoxins into the chloroform and column chromatography on acidic alumina and anhydrous sodium sulfate.

Murthy et al. (1975a) reported that there was a diminution in the recovery of aflatoxin B_1 injected into beef with increased periods of storage. The total recovery of injected aflatoxin B_1 dropped from 97.85% after 20 days of storage to 78.85% after 183 days. Although the ether and hexane fractions were expected to remove only the interfering substances, losses of aflatoxins were found to also occur on elution from the silica gel column. The analysis of the samples were carried out by the method of Brown et al. (1973). After purification the aflatoxin extracts were spotted in silica gel MNG-HR TLC plates. The chromatograms were developed in unlined and unequilibrated tanks with chloroform: acetone (9:1) and ethyl ether: methanol: water (96:3:1).

Jemmali and Murthy (1976) proposed a new 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 methanol extract before transfer of 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 according 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 new method detected aflatoxin B_1, B_2 and M_1 in tissues which were negative according to 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 in the ration. Similarly, all the samples except two muscle samples, 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 amounts of residues in the tissues. They also suggested that the liver is the best tissue to use for monitoring and demonstrating the transmission of aflatoxins into tissues. They assayed the tissues for aflatoxins according the procedure of Jacobson et al. (1971) with a modified clean up step as described by Wiseman et al. (1967).

Moregue 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 prothrombim time were not influenced at these aflatoxin levels. Similarly, the enzyme profile 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, Moregue et al. (1977) could not find any residue of aflatoxins in the tissues of pigs fed an aflatoxin-contamined ration. They concluded that 300 ppb of aflatoxins (B_1 equivalent) appeared to be below the minimum toxic dosage under these experimental conditions. The aflatoxin analysis was carried out according the procedure of Brown et al. (1973).

Recently, Trucksess and Stoloff (personal communication, 1978), proposed a new method for aflatoxins in animal tissues, which is a variation of previous methods used for aflatoxin analysis in dairy products (Brown et al., 1973) and eggs (Trucksess et al., 1977). The authors claimed that there was better recovery of any conjugated aflatoxins from the tissues, including those bound to the proteins and nucleic acids. The method employs strong protein and nucleic acid precipitants, such as $(NH_4)_2SO_4$ and lead acetate. It also utilizes chelating and acidulating or competitive reactants, such as citric acid and saturated NaCl solution.

Basically, the method consisted of extraction of the aflatoxins from the tissues by blending them with acetone plus citric acid and a

saturated solution of NaCl. Following precipitation of proteins, nucleic acids and lipids by treating the extract with $(NH_4)_2SO_4$ and lead acetate, a liquid-liquid defatting was accomplished by using petroleum ether (30-60 °C b.p.). The final interfering substances were eliminated by silica gel absorption column chromatography followed by bidimensional thin layer chromatography. The details of this method are given later herein. The method has been submitted for publication (Trucksess and Stoloff, 1978).

Aflatoxin B_1 Metabolism and Toxicity

Of the various chemicals known to induce cancer in experimental animals, strong evidence exists for involvement of aflatoxins in induction of liver cancer. Epidemiologic studies have shown a correlation between the current incidence of primary liver cancer in certain human populations and the ingestion of aflatoxin B_1-contaminated diets (Campbell and Stoloff, 1974; Shank et al., 1972; Phillips et al., 1976).

Experimental animals vary considerably in their toxic carcinogenic responses to aflatoxin B_1 as well as in their abilities to metabolize the aflatoxin. According to Patterson (1973), the diversity of response suggests that variation in metabolism may be an important factor in determining the toxic action of aflatoxin B_1 in different species of animals.

Aflatoxin M_1 was one of the first metabolites to be found in the milk of cows fed aflatoxin contaminated rations (Allcroft and Carnaghan, 1963). It results from the ring hydroxylation of aflatoxin B_1 at the C-4 position (Figure 1 - pathway 1). After Allcroft and

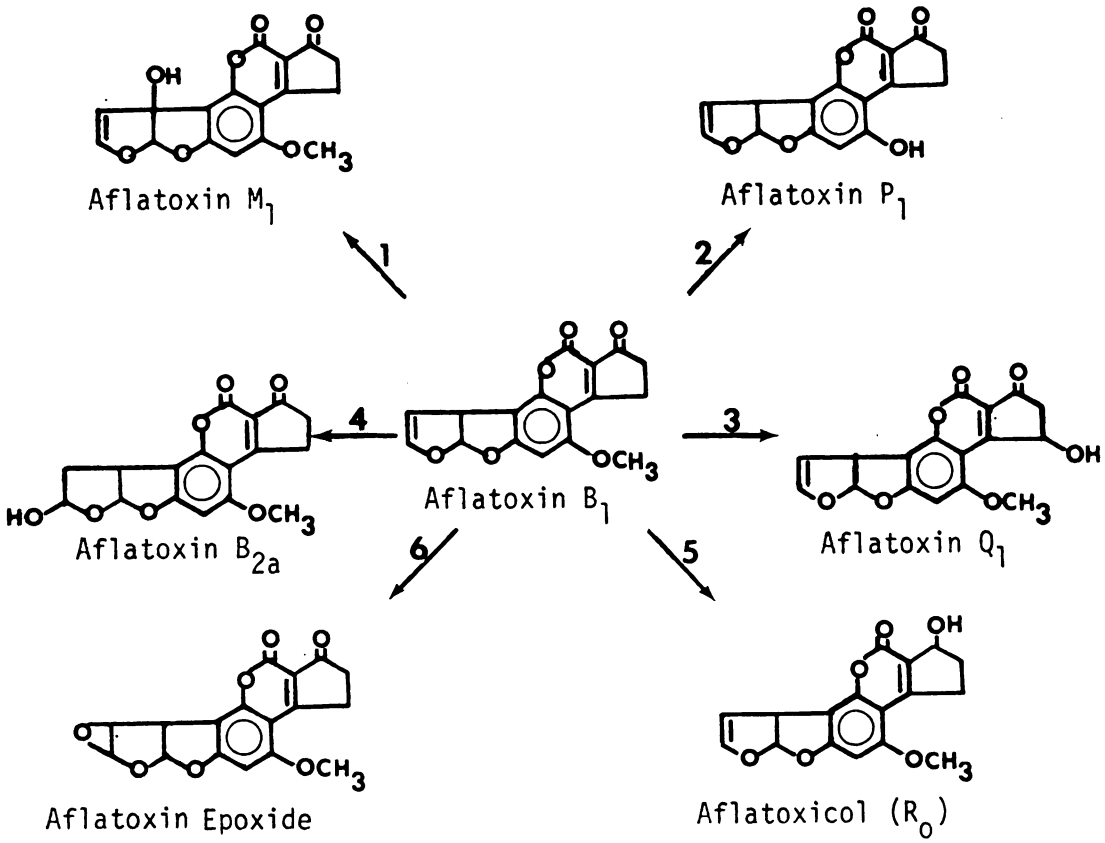

Figure 1.--Structural formulas for aflatoxin B_1 metabolites. Numbers indicate the pathways referred to in the thesis.

Carnagham (1963) discovered aflatoxin M_1, its presence in milk 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; Allcroft et al., 1966) and humans (Campbell et al., 1970), and in tissues of animals (Allcroft et al., 1966; Purchase and Stein, 1969) ingesting rations containing aflatoxin B_1. Conjugated forms of this metabolite were also found in the bile from rats (Bassir and Osiyemi, 1967) and in tissues from chickens (Mabee and Chipley, 1973) ingesting aflatoxin B_1.

Aflatoxin P_1 is a result of O-demethylation of aflatoxin B_1 (Figure 1 - pathway 2). Dalezios et al. (1971) identified aflatoxin P_1 as the principal urinary metabolite of aflatoxin B_1 in rhesus monkeys. Their experiments indicated that aflatoxin P_1 comprises approximately 60 percent of the urinary aflatoxin derivatives, with 50 percent being a present as glucuronide, 10 percent as sulfate, and 3 percent as the unconjugated phenol. Together, these metabolites accounted for over 20 percent of an injected dose of aflatoxin B_1.

Another hydroxylated derivative, aflatoxin Q_1, is an isomer of aflatoxin M_1, with the hydroxyl on the beta carbon atom of the carbonyl of the cyclopentanone ring (Figure 1 - pathway 3). Aflatoxin Q_1 represented approximately one-third to one-half of the metabolites formed from aflatoxin B_1 by microsomes from human (Masri et al., 1974) and monkey livers (Buchi et al., 1974).

Hydration of the 2-3 vinyl ether double bound in the aflatoxin B_1 molecule results in the formation of aflatoxin B_{2a}

(Figure 1 - pathway 4). Patterson (1973) found that rabbit, duckling, guinea pig, mouse and chick liver microsomes all converted 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 hybrid, which binds to protein by forming Schiff bases with free amino groups (Patterson and Roberts, 1970; 1972; Gurtoo and Campbell, 1974; Ashoor and Chu, 1977). According to Patterson (1973) the metabolic conversion of aflatoxin B_1 to its hemiacetal, B_{2a}, is characteristic in the livers 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 avid protein binding capacity, and thus, its sequestration and further elimination with the desquamated epithelial cells before significant absorption (Patterson, 1973). Acid catalyzed addition of water to the vinyl double bond of aflatoxin B_1 also lead to the formation of the aflatoxin B_1 hemiacetal or aflatoxin B_{2a} (Pohland *et al.*, 1968; Pons *et al.*, 1972).

Aflatoxicol or aflatoxin R_0 results from reduction of the carbonyl group in the cyclopentanone ring of aflatoxin B_1 (Figure 1 - pathway 5). Unlike the previous metabolites, this reduction is not catalysed by the microsomal mixed function oxidases, but by an NADP-linked dehydrogenase of the cytosol, which also has 17-ketosteroid dehydrogenase activity (Patterson, 1973; Patterson and Roberts, 1971; 1972a; 1972b). The transformation of aflatoxin B_1 to aflatoxicol has been shown to be reversible, depending on the $NADPH_2$/NADPH ratio in

the cell (Patterson and Roberts, 1972b). 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). Recently, aflatoxicol was identified as the major metabolite in the plasma of Sprague-Dawley rats, that were dosed orally or intravenously with ^{14}C-aflatoxin B_1 (Wong and Hsieh, 1978). Aflatoxicol, however, was not detected in the plasma of similarly dosed mice and monkeys, which are both resistent to aflatoxin B_1-induced carcinogenisis. These authors suggested that both in vitro and in vivo formation of aflatoxicol may be an indicator of species sensitivity to aflatoxin-induced carcinogeresis and may be useful in the prediction of human susceptibility.

The metabolic epoxidation of the 2-3 vinyl ether double bound of aflatoxin B_1 results in the formation of aflatoxin B_1-2,3- oxide (Figure 1 - pathway 6). Schoental (1970) was the first to suggest aflatoxin B_1-2,3-oxide as the metabolite responsible for aflatoxin B_1 toxicity and carcinogenesis. He developed this concept by analogy with the metabolic activation of polycyclic aromatic hydrocarbons.

Garner et al. (1972) reported that incubation of rat liver microsomes with a NADPH-generating system, in addition to aflatoxins B_1, G_1 and sterigmatocystin, produced a toxic derivative, which was lethal to some bacteria. They also suggested the importance of the 2,3 double bond in the system in order for the compound to a exert its biological toxicity, once aflatoxins B_2, G_2 and B_{2a} become inactive in the same microsome-mediated assay. Swenson et al. (1973) showed

that on incubating aflatoxin B_1 *in vitro* with rat and hamster liver microsomes and RNA, an nucleic acid adduct was formed. On mild acid hydrolysis, the nucleic acid adduct yielded the 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1. Results indirectly suggested that the reactive precursor for the microsomal binding of aflatoxin B_1 to RNA was aflatoxin B_1-2,3-oxide, a strongly electrophilic agent. They also suggested that the aflatoxin B_1-RNA adduct was bound through a covalent linkage between the C-2 of the aflatoxin residue and N-7 or O in the nucleotide residue. This hypothesis has been recently confirmed by Croy *et al.* (1978), who isolated the 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-aflatoxin B_1 as the principal covalent product after hydrolysis of liver DNA of rats dosed with aflatoxin B_1.

Although the epoxide itself has not yet been isolated, presumably because of its great reactivity, 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 synthetic aflatoxin 2,3-dichloride can mimic the biological effects of the aflatoxin B_1-2,3-oxide in several biological and chemical systems. Further indication of the importance of epoxidation of aflatoxin B_1 as an activation reaction is provided by the work of Roebuck *et al.* (1978) and Swenson *et al.* (1977), who showed that aflatoxin B_2 is hepatocarcinogenic via dehydrogenation to aflatoxin B_1. Roebuck *et al.* (1978) also showed that the ratio of nucleic acid adducts formed from the two compounds was very similar to the ratio of their carcinogenic potencies, approximately 1:100 (aflatoxin B_2 : aflatoxin B_1).

EXPERIMENTAL

Feeding Trial

Preparation of the Diet

In order to prepare the spiked ration, the aflatoxins were first extracted with chloroform from the vials and were then diluted to approximately 1-liter. The solution was then slurried with 400 g of finely ground feed, and the chloroform was allowed to evaporate in the dark over night with forced air under a hood. The aflatoxin-spiked feed was divided into two equal lots, and each lot was homogeneously 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 Manufacturing Co.), and homogenously mixed with additional feed to give a final weight of 98 kg. The afatoxin-spiked ration weighed 196 kg and was packed and stored at 0°C until fed. Analysis showed that aflatoxins B_1, B_2, G_1 and G_2 were present in the final ration at levels of 662, 273, 300 and 285 ppb, respectively. The basal ration used for the control group was handled the same, except it did not contain any added nor naturally occurring aflatoxins as confirmed by analysis. Determination of aflatoxins in the feed was carried out according to the AOAC method (1975).

Experimental Animals

Eight crossbread Duroc x Yorkshire barrows from the MSU swine Farm were selected from two different litters and allotted into two groups. The control group was numbered from 1-4, and the experimental group from 5-8. At the time of selection, the animals weighed between 24.5 and 26.3 kg. They were housed and fed individually in confinement, with water and feed being supplied ad libitum. The basal diet consisted of corn-soybean oil meal fortified with vitamins and minerals. The composition of the diet is given on Table 1. The experimental animals were fed the basal diet spiked with the four main aflatoxins - B_1, B_2, G_1 and G_2, which were purchased from CalBiochem. After an adjustment period of one week, the animals were fed the experimental diets for 21 days. The animals were weighed weekly and feed consumption was recorded.

Slaughtering and Collection of Samples

At the end of the experimental period, the pigs were taken to the MSU Meat Laboratory, where they were confined off feed overnight. The following morning the pigs were slaughtered. After slaughtering, the tissues were examined for possible gross lesions by a Michigan State Department of Agriculture Meat Inspector. Samples of heart, kidneys, liver, muscle and spleen were collected from both groups. Samples were weighed, frozen and stored at -20 °C for later analysis.

Table 1.--Composition of the diet[a,b]

Ingredients	Percentage
Corn	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%; Meth + Cysteine-0.55%; Tryptophan-0.19%; Ca^{+2}-0.67%, and P-0.505%.

[b]Digestible Energy: 3,436 KCal/Kg.

[c]Composition of mineral mix as percentage of the diet: Limestone-1.00; Dicalcium phosphate-1.00; and the following elements in ppm of the diet: Selenium-0.1; Zinc-74.8; Manganese-37.4; Iodine-2.7; Copper-9.9 and Iron-59.4.

[d]The vitamin premix contained per kg of total diet: Vit. A-3,300 IU; Vit. D-660 IU; Vit. E-5.5 IU; Vit. K compound-2.2 mg; Riboflavin-3.3 mg; Nicotinic acid-17.6 mg; D-pantothenic acid-13.2 mg, Choline-110.0 mg and Vit. B_{12}-19.8 µg.

METHODS

Extraction of Aflatoxins from Tissues

Attempts were made to use the method by Jemmali and Murthy (1976), which appeared to be simpler and more sensitive than other published methods for analysis of aflatoxins in tissues of animal origin. On gross visual evaluation, the method showed good recovery from fresh pig tissues spiked with aflatoxins. However, liver extracts from aflatoxin-treated animals had too many interfering substances for TLC separation. The final liver extracts were always oily and resulted in an unresolved streak without any spot differentiation on TLC plates. Furthermore, aflatoxin standards superimposed on the spots from liver were not resolved. Attempts were made to eliminate the oil-like substance from the sample extract, but results were not satisfactory. The method of Brown et al. (1973) was also tried, but without any improvement in results, in addition to being complicated and time consuming.

Extraction and analysis of aflatoxins from the tissues of the pigs were finally carried out according to a modification of the procedure of Trucksess and Stoloff (Personal Communication, 1978), who obtained a high sensitivity for aflatoxins at low levels in spiked tissues. For samples spiked with 9.2 ppb, they reported recoveries of 91 and 82% of aflatoxins B_1 and M_1, respectively. At 0.1 ppb, they recovered 81% of B_1 and 66% of M_1. The method was successfully used

in the present study for all samples, although it was specifically designed for analysis of aflatoxins B_1 and M_1 from liver tissues. Details of the method are given below.

100 g of frozen tissue were cut in cubes, without thawing, and blended for three minutes in a Waring blendor at moderate speed with 50 ml of saturated NaCl solution (40 g NaCl/100 ml H_2O) and 3 g of citric acid. Then 250 ml of acetone were added to the homogenate while washing the sides of the blendor jar. The tissue was blended for an additional 5 minutes at moderate speed. After that, the material was filtered through fast filtering prefolded filter paper (Whatman 114 v) and the filtrate was collected in a 1-liter Erlenmeyer flask. The meat residue and the blendor jar were throughly washed with 250 ml of acetone dispensed in small quantities. After complete draining of the acetone from the residue on the filter paper, an additional washing was carried out using 50 ml of acetone.

After filtration was completed, the meat residue was discarded. Then 150 ml of water, 8.5 g of $(NH_4)_2\ SO_4$ and 35 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 made to volume of one liter with H_2O) were added to the filtrate and the solution was stirred for 1/2 minute with a magnetic stirring device. Then, 10 g of diatomaceous earth were added to the solution and stirring was continued for an additional 1/2 minute. The solution was allowed to stand for about 5 minutes before filtering through fast filtering folded filter paper. The flask and residues on the filter paper were washed with 150 ml of acetone. The final filtrate was collected in 1-liter Erlenmeyer flask.

Purification of Aflatoxin Extract

Liquid-Liquid Partition

The filtrate was transferred to a 1-liter 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 1-liter separatory funnel. The petroleum ether layer was then discarded. Then 150 ml of chloroform were added to the aqueous-acetone solution and the separatory funnel was shaken vigorously as before. After the layers separated, the lower chloroform layer was collected in a 1-liter flask. Aflatoxin extraction from the aqueous-acetone layer was repeated two more times using 100 ml of chloroform: acetone (1:1). The aqueous layer remaining after the chloroform extraction was discarded.

The chloroform-acetone extract was then evaporated to dryness in a Rotavapor R (Buchi, Switzerland), using a water bath setting at 40°C. Generally the extraction procedure resulted in a considerable amount of water entrapped within the chloroform-acetone extracts. A soap-like emulsion after the evaporation of the chloroform and acetone indicated the presence of water. The water was removed by transferring the watery extract to a 60 ml separatory funnel. The lower layer was drained into a 1-liter flask. The entrapped water was then washed three times with 50 ml of chloroform: acetone (1:1). The combined chloroform-acetone extracts were then evaporated to dryness as described earlier.

Silica Gel Column Chromatography

A 400 x 25 mm glass column (Labcrest Scientific Glass Co.) was half filled with chloroform. A 2 cm bed of anhydrous granular Na_2SO_4 was added, followed by 10 g of silica gel 60 (70-230 mesh ASTM - EM Laboratories Inc.) which had been previously slurried in 50 ml of chloroform. The chloroform was drained to about 10 cm above the top of the silica gel by forced vacuum generated by a vacuum trap adapted to the tip of the column. Another 3 cm layer of anhydrous Na_2SO_4 was added on top of the silica gel. The excess of chloroform was drained by gravity to the top of the upper Na_2SO_4 layer.

The aflatoxin extract was dissolved in about 10 ml of chloroform and then transfered to the column with a disposable glass pipet. The sides of flask were washed three more times with 5 ml of chloroform, and the washings were added to the column. After each addition of chloroform extract, the column was drained to the top of the packing and the eluate was discarded. Interfering substances were then eluted from the column with 150 ml of hexane followed by 100 ml of anhydrous diethyl ether and discarded.

The aflatoxins were eluted from the column with 250 ml of chloroform: methanol (97:3). The eluate was collected in a 1-liter flask and evaporated to near dryness in a rotary evaporator as described above. Then the sample extract was dissolved in chloroform and quantitatively transferred to 15 ml vials using a disposable glass pipet. The chloroform was evaporated to near dryness on a steam bath under a gentle stream of nitrogen. Special care was taken to avoid overheating of the dry extract. The extract was transferred with

chloroform to a 3 ml vial and then evaporated to dryness using the same procedure as above. Finally 100 µl of chloroform were added to the dry sample, and the vial was sealed with a teflon lined screw cap and shaken vigorously for about one minute on a vortex shaker.

Thin Layer Chromatography

TLC glass plates (10 x 10 cm) were coated with a 0.5 mm wet layer of Adsorbil - 1 (Applied Science Laboratories). These plates were used for TLC and densitometric analysis.

The plates were scored and spotted as shown in Figure 2. A 20 µl sample of the aflatoxin extract was applied to the plate with a 10 µl syringe (Hamilton Co.). Standards of 3.75, 3.75 and 5.00 µg of aflatoxin B_1, B_2 and M_1, respectively, were spotted on the TLC plates about 1 cm from the edge (Figure 2).

The plates were developed in the first direction with chloroform: acetone: 2-propanol (85:10:5) in a sealed and unequilibrated tank. After the development in the first dimension was completed, the plates were removed from the tank and dried under a hood for about 5 minutes. After evaporation of the solvents, the plates were developed in the second direction with anhydrous diethyl ether: methanol: water (96:3:1). After development in the second dimension, the plates were dried as before and prepared for densitometric analysis.

Densitometric Analysis of Aflatoxins

A double beam scanning-recording-integrating spectrodensitometer SD 3000-4 (Schoeffel Instrument) was used for quantifying the TLC plates. The plates were scored prior to spotting as shown in Figure 2 (Schoeffer

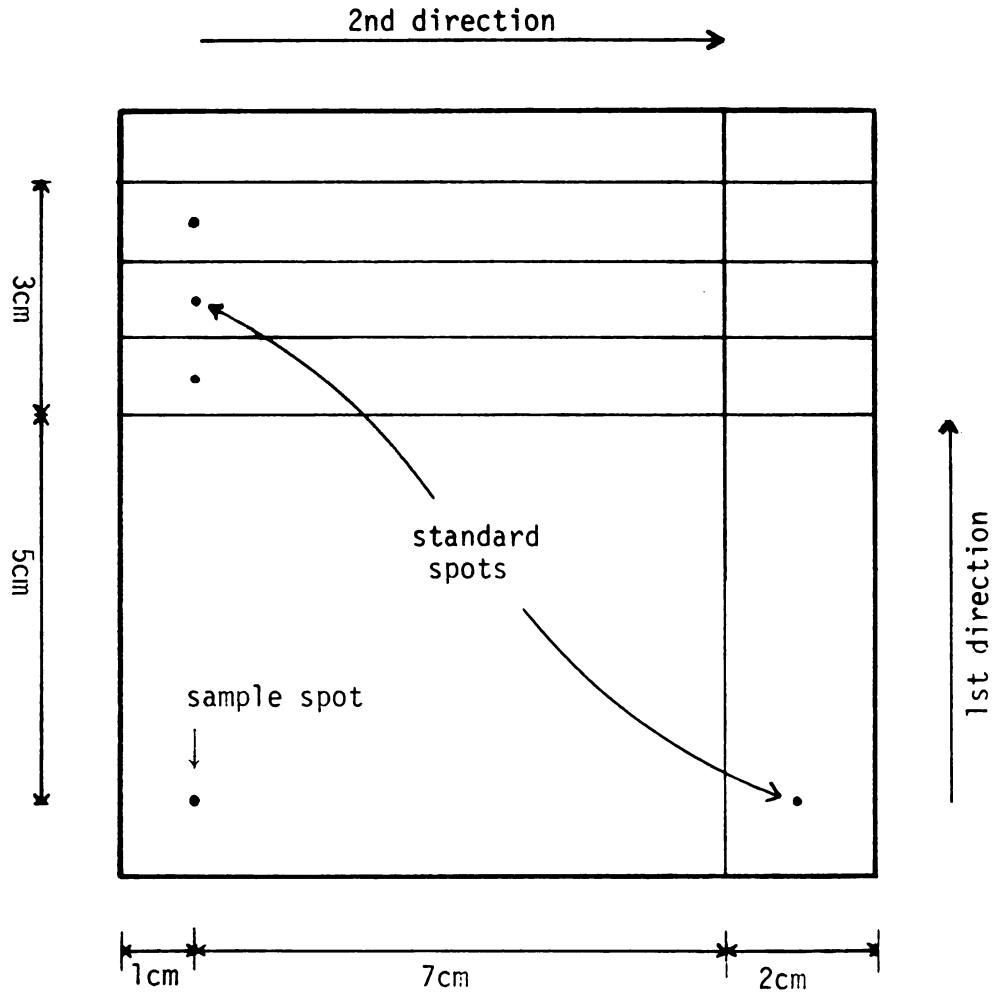

Figure 2.--Spotting and scoring pattern for 2-dimensional TLC plate.

Scoring Device SDA 303), providing 10 mm strips. The average of three readings of the aflatoxin standards (spotted within the three strips parallel to the 2nd direction of development) was used for densitometric comparison in claculating the concentration of the sample. For analysis of the standards, the scanning head was placed within each of the three strips and parallel scanning occurred. In scanning the sample spots, the plates were viewed under UV light and each two spots were localized within a imaginary strip identified through four pencil marks made on the silica gel layer. The marks were about 1 cm apart, parallel to each other and long enough to contain each of the two sample spots. Then the plates were placed on the plate carrier in such a way as to be driven and scanned parallel to the direction of the imaginary strip lines.

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

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

where:

B = Area of 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.

W = Grams of sample in the final extract.

Confirmatory Tests for Aflatoxins

Aflatoxin B_1

20 µl of sample extract were applied about 4 cm from both edges in the left corner of a precoated TLC plate (20 x 20 cm Sil-G-HR-25, Brinkman Instruments, Inc.). Approximately 5, 2 and 5 ng of aflatoxins B_1, B_2 and M_1 standards, respectively, were spotted in the same location on right corner of the plates. Then the plate was developed in a closed, unlined and unequilibrated tank with anhydrous diethyl ether: methanol: water (96:3:1). After development, the plate was air dried under a hood for about 1 minute, and then dried in a chromatographic oven under a stream of nitrogen for about 3 minutes, at 45°C.

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 3 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 a forced-draft chromatographic oven for 10 minutes, at 45°C. After cooling the plate in the dark at room temperature another 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 in 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 confirmatory test for the identity of 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 B_{2a} spot. The second mark was used as a guide in applying about 3 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 3 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 (85:10:7) 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 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 B_{2a} from blue to yellow after the H_2SO_4 treatment was used as an additional confirmatory test for the presence of aflatoxins.

Two-dimensional Chromatography

The chromatographic equivalence of aflatoxins in the sample with the aflatoxin standards which were applied to the same spot after two-dimensional chromatography and using two different solvent systems, was also used as a confirmatory test for aflatoxins.

Aflatoxin B_{2a} was synthesized on the plate as follows: 3 ng of aflatoxin B_1 was applied about 4 cm from both edges in the left corner of a precoated TLC plate (20 x 20 cm Sil-G-HR-25-Brinkman Instruments, Inc.), followed by superimposing 2 µl of TFA on the spot. The plate was allowed to stand for 5 minutes in the dark at room temperature. Then the plate was dried in a chromatographic oven for 10 minutes at 45°C. After that the plate was cooled at room temperature in the dark. Then 5, 2 and 5 ng of aflatoxins B_1, B_2 and M_1 respectively, were

applied to the same spot followed by 20 µl of the sample extract. The plate was developed in the same way as used for quantitation of aflatoxins by densitometry as described earlier herein. Another TLC plate was prepared and developed in the same manner, except it contained only the sample extract. The chromatographic equivalence of the aflatoxin spots on both plates was used as a confirmatory test for the aflatoxins.

Preparation of Aflatoxin Reference Standards

Aflatoxin reference standards were prepared according to the AOAC method (1975) and contained 0.5 µg/ml of aflatoxins B_1, G_1 and M_1 and 0.1 µg/ml of aflatoxins B_2 and G_2. A solvent mixture of benzene: acetonitrile (98:2) was used as the solvent for aflatoxins B_1, B_2 and G_1, while benzene: acetonitrile (90:10) was utilized as the solvent for aflatoxin M_1.

RESULTS AND DISCUSSION

Feeding Trial

The response of the animals to aflatoxins was determined by growth, feed consumption and feed efficiency (feed consumed/weight gain). The data are presented in Table 2. The aflatoxin intake by the pigs was expressed as either the daily dosage or as the daily dosage ratio (Table 3).

The results showed that the pigs fed aflatoxins exhibited depressed growth, and on average gained 25% less weight than the control animals over the 21 day trial. The pigs fed aflatoxins also showed an average of 18% reduction in feed intake as compared to the controls. However, the aflatoxin level used in the trial (Table 3) did not adversely affect feed efficiency. There was no significant difference between feed efficiency for the pigs fed aflatoxins and the control animals (Table 2).

The pigs on the aflatoxin treatment had on average a daily intake of 2.64 mg of aflatoxins, with an average daily dosage ration of 85 µg/kg. This level of intake is toxic and has been reported to kill pigs during a 16-week feeding period according to Armbrecht et al. (1971), who established the differential response of pigs to various graded levels of aflatoxins in the ration during a 26 week feeding trial.

Table 2.--Performance Data for Three Week Feeding Trial[a]

Pig #	Starting Weight (kg)	Final Weight (kg)	Weight Gain (kg)	Total Feed Intake (kg)	Feed Efficiency (Feed/Weight Gain)
Control Group					
1	24.5	44.1	19.6	57.7	2.9
2	26.4	45.0	18.6	47.0	2.5
3	25.9	45.2	19.3	51.4	2.7
4	26.4	41.4	15.0	42.0	2.8
Means	25.8[b]	43.9	18.1[b]	49.5[b]	2.73[b]
Aflatoxin Fed Group					
5	24.8	39.1	14.3	32.7	2.3
6	23.4	35.9	12.5	33.4	2.7
7	24.5	39.1	14.6	44.3	3.0
8	24.5	36.8	12.3	35.7	2.9
Means	24.3[b]	37.7	13.4[c]	36.5[c]	2.73[b]

[a]Mean values with same superscript letters were not significantly different at P = < .01.

Table 3.--Average Daily Intake (mg), Total Average Daily Intake (mg) and Daily Dosage Ratio (µg/kg) of Aflatoxins.[a]

Pig	Average Daily Intake				Total Daily Intake	Daily Dosage Ratio (DR)[b] (µg/kg)
	B_1	B_2	G_1	G_2		
Aflatoxin Fed Group						
5	1.03	0.43	0.47	0.44	2.37	74
6	1.05	0.43	0.48	0.45	2.41	82
7	1.40	0.58	0.63	0.60	3.21	101
8	1.13	0.46	0.51	0.48	2.58	84
Means	1.15	0.48	0.52	0.49	2.64	85

[a]There was no measurable aflatoxin intake in the control group.

[b]Amount of aflatoxin adjusted according to body weight, defined by DR = Wa/0.5(Ws + We)t (Armbrecht et al., 1971)
Where,
(Wa) µg of aflatoxin ingested during the interval (t) in days, starting weight (Ws) and ending weight (we) in kg.

Results from this experiment agree with those reported by Armbrecht et al. (1971), except for the fact that they found aflatoxins decreased feed efficiency. Similarly, Keyl and Booth (1971) and Krogh et al. (1973) reported decreased growth and feed efficiency for pigs fed diets containing 810 and 500 ppb of aflatoxins, respectively, during a 120 day feeding trial.

According to Murthy et al. (1975) the response of pigs to aflatoxin - contaminated diets depends not only on the aflatoxin level but also on whether the protein and non-protein components are fed as a mixture or separately. They reported that the performance of pigs was not affected when they were fed a mixed diet containing 936 ppb of aflatoxin B_1. However, feeding of naturally contaminated peanut meal (830 ppb of aflatoxin B_1) separately from the remainder of the ration resulted in a marked depression in growth.

The differences found upon comparing the feed efficiency from this experiment with previous reports can be explained on the basis of the short term exposure of the pigs to the aflatoxin contaminated diet. Although the aflatoxin level used in this study was adequate for decreasing growth and feed intake of the animals, the time of exposure was not long enough to alter their 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. Also the weights of the internal organs (hearts, kidneys, livers and spleens) were recorded (Table 4).

Table 4.--Internal Organ Weights in grams and as percentages of body weight[a,b]

Pig #	Organs Liver	Heart	Kidneys	Spleen
Control Group				
1	1,106.3 (2.51)[b]	183.5 (0.42)	196.8 (0.45)	-
2	902.6 (2.01)	143.8 (0.32)	206.8 (0.46)	79.7 (0.18)
3	1,037.5 (2.30)	160.0 (0.35)	195.8 (0.43)	64.5 (0.14)
4	913.8 (2.21)	150.0 (0.36)	179.5 (0.43)	66.8 (0.16)
Means	990.0 (2.26)[c]	159.3 (0.36)[c]	194.7 (0.44)[c]	70.3 (0.16)[c]
Aflatoxin Fed Group				
5	1,007.9 (2.58)	140.0 (0.36)	154.9 (0.40)	56.5 (0.14)
6	1,204.5 (3.36)	161.8 (0.45)	161.5 (0.45)	67.6 (0.19)
7	1,231.7 (3.15)	166.6 (0.43)	163.0 (0.42)	81.0 (0.21)
8	1,164.8 (3.17)	158.7 (0.43)	143.8 (0.39)	53.4 (0.15)
Means	1,152.2 (3.07)[d]	156.8 (0.42)[c]	155.8 (0.42)[c]	64.6 (0.17)[c]

[a]Values in parenthesis give the weight of organs as percent of body weight.

[b]Means followed by the same superscript letters are not significantly different at P = < .01.

Inspection showed that all internal organs, including the livers and all carcasses, were free of any observable macroscopic lesions for both the control and the aflatoxin-treated pigs. Therefore the tissues were not rejected for human consumption by the Meat Inspector. However, the pigs fed aflatoxins exhibted 36% heavier livers on average than the control animals (Table 4). The other internal organs, however, were not affected by the treatment and no significant differences were found between the control and the pigs fed aflatoxins (Table 4).

The results agree with those of Keyl and Booth (1971), who observed liver enlargement in pigs fed rations containing 450 ppb of aflatoxins or higher. Armbrecht et al. (1971) have shown that more than 700 ppb of aflatoxins are required in order to cause enlargement of the internal organs. They also reported that organ enlargement may be accompanied by lesions (particularly in the liver), but in some cases the tissues may be normal in appearance on using light microscopy and histochemical staining techniques for evaluation. Murthy et al. (1975) reported no organ lesions in pigs fed an aflatoxin contaminated ration containing up to 936 ppb, regardless of whether the protein and non-protein protions were fed separately or mixed together. However, severe internal organ lesions (particularly in the liver) were found on pigs fed a separate ration, in which the protein portion contained 2,500 ppb of aflatoxin B_1.

Keyl and Booth (1973) and Armbrecht et al. (1971) have also reported kidney enlargement in some of the animals but usually to a lesser extent than for the livers.

In contrast to other reports, 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 for monitoring the pig's response to aflatoxins (Jacobson et al., 1978).

Analysis of Aflatoxins in Tissues

The internal organs (heart, kidney, liver and spleen) and muscle were extracted and analyzed for aflatoxins according to the method of Trucksess and Stoloff (Personal communication, 1978). The separation and quantitation of the aflatoxins in the tissue extracts were accomplished by two-dimensional chromatography coupled with fluorodensitometry. The results are presented in Table 5.

The results showed no detectable aflatoxin residues in any of the internal organs and muscle samples of the control pigs. However, measurable amounts of aflatoxins B_1, B_2, M_1 and B_{2a} were found in the hearts, kidneys, livers, spleens and muscles of all pigs fed aflatoxins, except for two samples. One of these was muscle and the other spleen, and contained aflatoxins at levels below the limit of detection (< 0.01 ppb).

Table 5.--Aflatoxin Residues Detected in Pig Tissue (μg/Kg)[a]

	B_1	B_2	M_1
Liver 5	0.06	0.03	0.14
Liver 6	0.06	0.06	0.20
Liver 7	0.10	0.05	0.13
Liver 8	0.05	0.01	0.02
Means	0.07	0.04	0.12
Heart 5	1.41	0.14	0.54
Heart 6	0.07	0.02	0.05
Heart 7	0.04	0.01	0.05
Heart 8	0.10	0.12	0.09
Means	0.41	0.07	0.18
Spleen 5	0.01	tr	tr
Spleen 6	tr	0.01	0.02
Spleen 7	0.15	0.02	N.R.
Spleen 8	0.12	0.05	0.02
Means	0.07	0.02	0.01
Muscle 5	0.10	0.02	0.24
Muscle 6	0.10	0.03	0.02
Muscle 7	0.06	0.03	0.02
Muscle 8	tr	tr	N.R.
Means	0.07	0.02	0.07
Kidney 5	0.04	0.02	N.R.
Kidney 6	0.06	0.04	N.R.
Kidney 7	0.24	0.05	N.R.
Kidney 8	0.75	0.55	N.R.
Means	0.27	0.17	N.R.

[a]The mean percentage of the aflatoxin dosage retained by the tissues (liver, spleen, heart, kidney and muscle was calculated to be 0.015% for B_1 and 0.005% for B_2. See Appendix B.

tr - Traces too small to measure quantatively (< 0.01 ppb)

N.R. - Spots not resolved for flourodensitometric determination because of the presence of interfering material.

Aflatoxin B_1, B_2 and M_1 Residues in Tissues

The results of this study agree with those reported by Jacobson et al. (1978), in that they found transmission of aflatoxins B_1 and M_1 to livers, kidneys and muscle of pigs fed a diet containing aflatoxin B_1 at levels of 100, 200 and 400 ppb during a 4-week feeding trial. However, the levels of aflatoxin residues found in the tissues in the present study are lower than those reported by Jacobson et al. (1978), especially for tissues of the animal which they fed 400 ppb.

Other studies have shown that aflatoxins are transmitted to the tissues of animals upon feeding aflatoxin-contaminated rations (Murthy et al., 1975; Jemmaly and Murthy, 1976). However, the data are inconclusive and less consistent as compared to those of this experiment and those of Jacobson et al. (1978). Jemmali and Murthy (1976) developed a new method for analysis of aflatoxins in animal tissues. They reported aflatoxin B_1, B_2 and M_1 in some gall bladder, kidney, liver muscle, and spleen samples of two pigs fed a diet containing 3,484, 923, 199 and 248 ppb of aflatoxins B_1, B_2, G_1 and G_2, respectively, during a 33 day feeding trial. On repeating the tissues analysis using the procedure of Brown et al. (1973), however, they obtained negative results for samples which were positive on assaying with their own procedure, except for two liver and one Kidney samples. Even these values were lower than those obtained by their own method.

On using the method of Brown et al. (1973), Murthy et al. (1974) detected B_1, B_2 and M_1 aflatoxin residues in the liver, gall bladder, kidney, spleen, heart and muscle samples of one pig on aflatoxin

contaminated peanut meal fed separately from the remainder of the ration during a 19-day feeding trial. However, three other pigs on the same treatment had no aflatoxin residues in the tissues, except one pig which had measurable amounts of B_1 and B_2 in both liver and kidney samples. They also found there were no aflatoxin residues in any tissues of pigs fed the same diet, when the contaminated peanut meal was incorporated into the total mixed ration. Moregue et al. (1977) also failed to find any aflatoxin residues in the tissues of pigs fed a diet containing up to 300 ppb of aflatoxins (B_1 equivalents) on also using the method of Brown et al. (1973).

Earlier investigations on the transmission of aflatoxins from feed to animal tissues using biological assays (Allcroft and Carnagham, 1963; Platonow, 1965) or methods primarily designed for assaying aflatoxins in agricultural products (Keyl et al., 1968; Kratzer et al., 1969; Keyl and Booth, 1971) failed to detect aflatoxins in tissues from any of the animals examined, even in those with confirmed signs or fatal aflatoxicosis.

The conflicting and negative results presented in most of the previous studies on transmission of aflatoxins to tissues of animals ingesting dietary aflatoxins seems more likely to be due to the insensitivity of the analytical procedures than to the absence of aflatoxins. Evidence for this is seen by the fact that the aflatoxins levels ingested were high enough to cause tissue and organ lesions.

In the present study, the animals fed the aflatoxin contaminated ration during the 3-week feeding trial were apparently healthy with no observable gross or pathological lesions of the tissues, except for

livers enlargement, which is not necessarily accompained by microscopic lesions (Armbrech et al., 1971). Thus, the relatively low levels of aflatoxins found in the tissues as compared with the daily dosage ratio ingested may be explained by the fact that the animals were exposed to them for only a short period of time. Longer periods of time on the ration may have produced liver and kidney damage, which is not evident during such short time feeding trials, in which the pigs may be able to metabolize and excrete the aflatoxins without significant deposition in the tissues. Part of the ingested aflatoxins may be excreted in the urine or feces, either as the original aflatoxin, or as one of the various metabolites, or as one of their water soluble conjugates, as has been reported in rats (Wogan et al., 1967; Bassier and Osiyemi, 1967), monkeys (Dalezios et al., 1971) and pigs (Jacobson et al., 1978). These water soluble aflatoxin conjugated forms may also be deposited in the tissues and be unextractable by organic solvents as was found in tissues from chickens by Mabee and Chipley (1973). This is supported by the results of Hayes et al. (1978) who have demonstrated that about 60% of aflatoxin B_1 was transformed into an unidentified water soluble material upon incubation with a liver preparation from cattle.

Aflatoxin G_1 and G_2 Residues in Tissues

In this study, residues of aflatoxins G_1 and G_2 were not detected in any of the tissues samples analyzed. This was not expected since aflatoxins G_1 and G_2 were present in the ration at the relatively high levels of 300 and 285 ppb, respectively. However, the results agree with those reported by Armbrecht et al. (1972), who also did not detect

any residue of aflatoxins G_1 and G_2 in milk or in kidneys and livers from sows fed a high level of aflatoxins G_1 and G_2. Similarly, Murthy et al. (1975) and Jemmali and Murthy (1976) did not find any detectable aflatoxins G_1 and G_2 in tissues of pigs fed a diet containing relatively high levels.

Results suggest that aflatoxins G_1 and G_2 were metabolized and eliminated more efficiently from the tissues than the other aflatoxins. The possibility also exists that aflatoxins G_1 and G_2 may have been metabolized and deposited in the tissues, either as unidentified metabolites or as unextractable aflatoxin conjugates.

Aflatoxin B_{2a} Residues in Tissues

Aflatoxin B_{2a}, a metabolite of the aflatoxin B_1, was also found in measurable amounts in the heart, kidney, liver and muscle samples of all pigs fed the aflatoxin contaminated ration. However, it was not quantitated since standards for analytical comparison were not available.

Aflatoxin B_{2a} has never before been reported as a residue in tissues of animals fed on aflatoxin contaminated rations. However, the metabolic transformation of aflatoxin B_1 to B_{2a} by the liver has been suspected to occur in vivo, since aflatoxin B_{2a} has been shown to be the major metabolite produced upon in vitro incubation of aflatoxin B_1 with hepatic sub-fractions (Patterson and Roberts, 1970; Gurtoo and Campbell, 1974). It has also been reported that aflatoxin B_{2a} rearranges itself to form dialdehydic phenolate resonance hybrid ions at physiological and alkaline pH values. In this form, it reacts with amino acids, peptides and proteins to form Schiff bases (Patterson

and Roberts, 1970; 1972; Gurtoo and Campbell, 1974; Ashoor and Chu, 1975). Thus, the removal of aflatoxin B_{2a} through its transformation into the phenolate form, which rapidily interacts with cellular components and becomes unavailable to the extracting solvents, may explain why aflatoxin B_{2a} has not previously been observed as a residue in tissues of animals ingesting dietary aflatoxins. The use of strong protein and nucleic acid precipitants, such as ammonium sulfate and lead acetate, in combination with acidulating agents and competitive reactants, such as citric acid and concentrated sodium chloride solution, may account for the detection of aflatoxin B_{2a} in the present study. These reagents may cause dissociation of the ligand formed with the cellular components, and thus release aflatoxin B_{2a}. This possibility has been confirmed by Ashoor and Chu (1975), who have shown that the interaction of aflatoxin B_{2a} with amino acids, proteins and liver microsomes *in vitro* are reversible and pH dependent.

CONCLUSIONS

Pigs fed on a diet contaminated with 662, 273, 300 and 285 ppb of aflatoxins B_1, B_2, G_1 and G_2, respectively, during a 21 day feeding trial gained 25% less weight and had an 18% reduction in feed intake as compared to control animals fed the same diet free from aflatoxins. The treatment, however, did not adversely affect feed efficiency. The internal organs were not grossly altered, except for the livers, which weighted 36% more than those of the controls. There was no evidence of any pathological lesions of the internal organs, including the livers.

The diet containing aflatoxins resulted in measurable amounts of B_1, B_2, M_1 and B_{2a} residues in all tissues analysed except for two samples. One sample was muscle and the other spleen, and contained aflatoxins levels below the limits of detection (< 0.01 ppb). The average levels found in the tissues varied from 0.07 to 0.27 ppb for B_1, from 0.02 to 0.17 ppb for B_2 and from 0.02 to 0.18 ppb for M_1.

Aflatoxin B_{2a} levels in the tissues were not determined because standards were not available. Aflatoxins G_1 and G_2 were not detected in any of the tissues examined. This suggests that they may have been more efficiently metabolized and excreted by the pigs than the other aflatoxins.

Although aflatoxin M_1 has been previously identified in the tissues of pigs and other animals, this is the first time that B_{2a} has

been reported as a residue. Aflatoxin B_{2a} has been shown to form strong covalent ligands with tissue macro molecules in vitro. In this study, the use of suitable protein precipitants together with acidulating and dissociating agents may have caused the dissociation of the covalent ligand within the tissues and allowed detection of aflatoxin B_{2a}.

Detection of aflatoxin B_{2a} is important because it may represent a potential health hazard, since the conjugates may be hydrolyzed by acidic conditions or enzymes in the digestive tract and result in subsequent absorption.

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APPENDICES

APPENDIX A

STATISTICAL ANALYSIS

Analysis of Variance

k = 2 treatments n = 4 observations

1. Mean of observation in the ith 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 ith sample.

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

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

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

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

6. Error sum of squares

$$ESS = TSS - TrSS$$

7. Treatment degree of freedom

$$df_1 = K - 1$$

8. Error degree of freedom

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

9. Total degree of freedom

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

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

Calculations of Percent Retention in Tissues[a]

Residual aflatoxins found in the heart, kidney, liver, muscle and spleen of the pigs fed the aflatoxin-contaminated ration were expressed as average percent of aflatoxins B_1 (equivalent) and B_2 in the tissues. The values were computed by calculating the percentage of intake in comparison to retention in the tissues using the data in Table 3 (intake) and Table 5 (retention in the tissues).

Calculations for muscle were based on an estimated lean body mass of 22.6 kg, which was taken from data for similar weight pigs in a report by Hoberg and Zimmerman (1979). Aflatoxin values for muscle, kidney, liver, spleen, and heart were added together and divided by intake.

Aflatoxin M_1 residue was expressed as its B_1 equivalent. Aflatoxin B_1 equivalent, however, does not include aflatoxin B_{2a}, even though it was present in appreciable amount, since it was not quantitated.

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[a]Hogberg, M. G. and Zimmerman, D. R. 1979. Effects of protein nutrition in young pigs on developmental changes in the body and skeletal muscles during growth. J. Animal Sci., submitted.