THESIS

This is to certify that the

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TISSUE DEPOSITION AND CLEARANCE OF AFLATOXINS FROM BROILER CHICKENS

presented by

CHEN, CHIHOUNG

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of the requirements for

M. S. degree in FOOD SCIENCE

A. M. Pearson
Major professor

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TISSUE DEPOSITION AND CLEARANCE OF AFLATOXINS FROM BROILER CHICKENS

by

Chihoung Chen

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ABSTRACT

TISSUE DEPOSITION AND CLEARANCE OF
AFLATOXINS FROM BROILER CHICKENS

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A trial was conducted to determine the tissue levels of aflatoxins and the amount of time necessary for tissue clearance from broiler chickens fed an aflatoxin-contaminated diet containing 2057 and 1323 ppb of aflatoxins B_1 and B_2, respectively, for 35 days. The aflatoxins caused depressed growth and enlarged kidneys, livers and gall bladders. Hemorrhagic spots were present on the surfaces of both the muscles and livers of the experimental birds. Livers of some of the experimental chicks were pale and infiltrated with lipid. Analysis of the tissues at 0 day after withdrawal from the aflatoxin-spiked ration showed that aflatoxins were deposited in all tissues, either as the original compounds or as their metabolites. The highest levels were present in the livers and kidneys. Mean values for the combined aflatoxins in all tissues at 0 day were less than 3 ppb. The withdrawal trial showed that broiler chickens rapidly metabolized and removed aflatoxins from their tissues. Four days after placing the chickens on an aflatoxin-free diet, there were no detectable levels in any of the tissues.

To my dear parents

Mr. and Mrs. Pao-Shiang and Char-Shun Chen

and to my lovely wife Yeong-Tsuey

ACKNOWLEDGEMENT

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INTRODUCTION

Aflatoxins, which are the most significant and hazardous of the mycotoxins of economic and public health concern, are a group of secondary metabolites produced by certain strains of *Aspergilli*, principally, *A. flavus* and *A. parasiticus*. Aflatoxins have been found to occur in many agricultural products, particularly those held at high temperatures and high relative humidities. Thus, they become unavoidable natural contaminants in animal feeds, as well as in some foods used for human consumption.

Aflatoxins have been shown to be toxicogenic, carcinogenic, mutagenic and teratogenic. Epidemiological studies (Shank *et al.*, 1972; Peers and Linsell, 1973; Wilson, 1978) have indicated that aflatoxins possess acute toxicity in addition to being hepatocarcinogens to humans. Biologically, the effects of aflatoxins may vary with dose, duration of exposure, species, sex, age and the nutritional status of the animal affected.

In vivo, aflatoxins, like many other xenobiotics, may be biotransformed to increase their polarity and their excretability. During the biotransformation process, some of the metabolites of aflatoxins may cause acute or chronic toxicity. There is evidence that the residues of aflatoxins and their biotransformed metabolites that are found in animal

tissues may become an indirect source of aflatoxin contamination in human foods.

The present study was designed to investigate: (i) the levels of aflatoxins and their metabolites deposited in the tissues of broiler chickens, and (ii) the length of time required to obtain tissue clearance after removal of aflatoxins from the diet of broiler chickens.

REVIEW OF LITERATURE

DISCOVERY OF AFLATOXINS

Aflatoxins are a group of secondary metabolites produced mostly by certain strains of Aspergillus flavus and Aspergillus parasiticus (Wilson et al., 1968). Raper and Fennell (1965) classified the genus Aspergillus into 18 groups with both Aspergillus flavus and Aspergillus parasiticus being placed into the A. flavus group. Members of this group are broadly identified by the production of yellow-green conidia. A. flavus and A. parasiticus can be differentiated from each other primarily by the color of their colonies, and the morphology of their conidial structures. The sterigmata of A. flavus are typically biserrate, whereas, those of A. parasiticus are uniserrate (Raper and Fennell, 1965).

The recognition in the United Kingdom of an unusual disease of turkeys characterized by striking hepatic lesions was announced by Blount (1961). This condition, which was called "Turkey X" disease, accounted for the loss of more than 100,000 birds. At the same time, a similar incidence occurred in a farm in East Anglia, where over 14,000 ducklings died during a 4 to 5 week period (Asplin and Carnaghan, 1961). A similar condition was soon discovered in pigs (Loosmore and Harding, 1961) and calves (Loosmore and Markson, 1961). Investigations showed that the disease

in all these species was caused by a toxic factor contained in imported Brazilian groundnut meal (Blount, 1961; Asplin and Carnaghan, 1961; Loosmore and Harding, 1961; Loosmore and Markson, 1961).

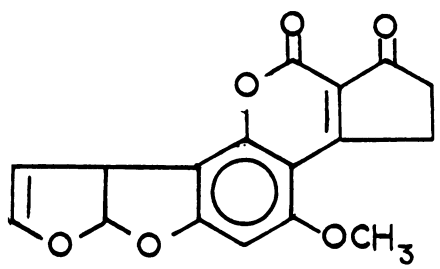
Asplin and Carnaghan (1961) fed chickens and ducklings with Brazilian groundnut meal and found that ducklings are highly susceptible to this toxic meal. Sargeant et al. (1961b) used paper chromatography to separate a crude extract of Brazilian groundnut meal with a mixture of n-butanol and 5 per cent acetic acid as the developing solvent. They found a blue fluorescing spot under UV light with a R_f of 0.7. Later it was shown that groundnut meal other than that from Brazil contained this component (Wannop, 1961). Tests on groundnuts from Kenya (Carnaghan and Sargeant, 1961), Uganda (Sargeant et al., 1961c), West Africa (Sargeant et al., 1961a) and India (Hornby et al., 1962) showed the presence of a toxic component in all samples.

Sargeant et al. (1961c) isolated a fungus from toxic Ugandan groundnuts and identified it as being A. flavus Link ex Fries. They extracted this fungus with chloroform, and the extract gave positive tests for the toxin when fed to ducklings (Sargeant et al., 1961b). In 1962, a British "Interdepartmental Working Party on Groundnut Toxicity Research" proposed the trivial name aflatoxin (A. flavus toxin). Nesbitt et al. (1962) showed that the single blue fluorescing spot of the toxin-containing extracts observed by Sargeant et al. (1961b) could be split into two components by using alumina chromatoplates developed in a chloroform-methanol (98.5:1.5)

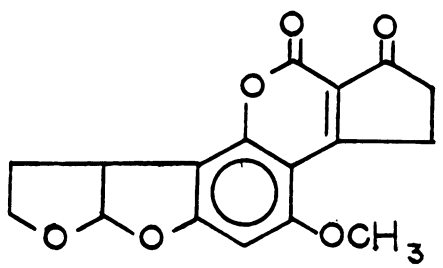
mixture. Hartley et al. (1963) further separated them into four components by using silica gel G plates developed in a mixture of a chloroform-methanol (98:2). Thus, they discovered the four major natural occurring aflatoxins, B_1, B_2, G_1 and G_2. The specific designation was due to their blue (B) and green (G) fluorescence under UV light (Nesbitt et al., 1962; Hartley et al., 1963). Their chemical structures are shown in Fig. 1.

Allcroft and Carnaghan (1963) reported that extracts of milk from cows fed with toxic groundnut meal contained a toxic substance, which was capable of producing the typical aflatoxin-poisoning in day-old ducklings. DeIongh et al. (1964) found that the toxic substance exhibited blue-violet fluorescence under UV light on a silica gel plate developed with chloroform-methanol (97:3), and had a R_f value well below that of aflatoxin B_1. The toxic substance was also found in the extract from the dried A. flavus culture grown on crushed groundnuts. At the same time, they fed lactating rats with pure aflatoxin B_1, and the rats produced the same "milk toxin" in their milk. Therefore, they concluded that the milk toxin is a biotransformation product instead of coming through direct ingestion (DeIongh et al., 1964).

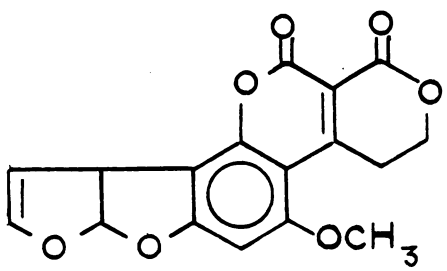
Later, Butler and Clifford (1965) found the so called "milk toxin" in the livers of rats given pure aflatoxin B_1, either orally or intraperitoneally. Allcroft et al. (1966) also isolated the milk toxin from the urine of sheep fed aflatoxin B_1, and confirmed its chromatographic equivalence. They further proposed the trivial name aflatoxin M for the



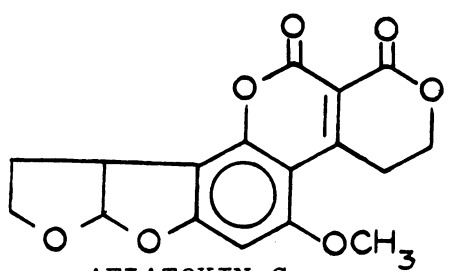
AFLATOXIN B_1



AFLATOXIN B_2



AFLATOXIN G_1



AFLATOXIN G_2

Figure 1 -- Chemical structures of the four principal aflatoxins produced by mold.

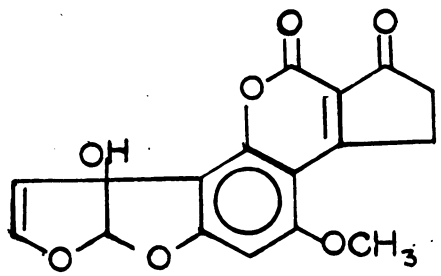
toxin found in milk. Holzapfel et al. (1966) repeated the experiment of Allcroft et al. (1966) and isolated aflatoxin M from the urine of aflatoxin-dosed sheep. They than separated it into two compounds by using paper chromatography. The blue-violet component was named aflatoxin M_1, and the violet spot was named aflatoxin 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 they named them aflatoxin B_{2a} and G_{2a}, respectively. The chemical structures of the four minor natually occurring aflatoxins, M_1, M_2, B_{2a} and G_{2a} are shown in Fig. 2.

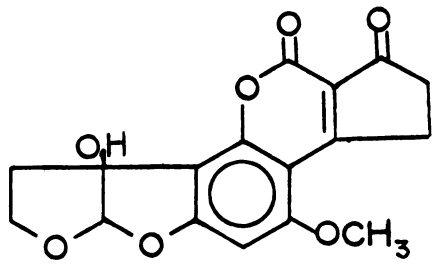
METABOLISM OF AFLATOXINS

Man and animals are exposed to a great variety of organic chemicals from the environment (Maugh, 1978) which cannot be used for energy production or as building blocks for their own body constituents. These foreign compounds are referred to as xenobiotics (Reeves, 1981). Once these compounds have gained access to the body they tend to persist as they distribute themselves in the fat and other tissues, and are difficult to excrete (Klaassen, 1980). They may interact with biologically important molecules and can give rise in this way to a number of pharmacological or toxicological responses (Reeves, 1981).

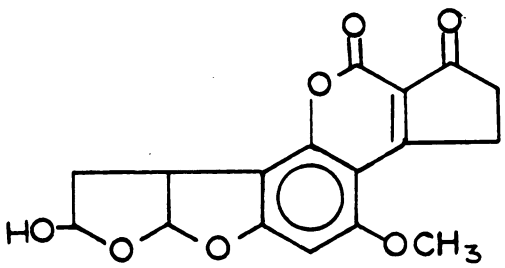
Nutrients and xenobiotics may be metabolized by identical or analogous routes. While the purpose of nutrient



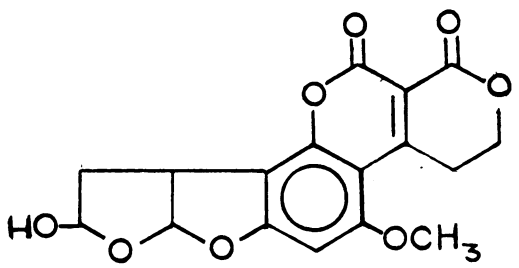
AFLATOXIN M_1



AFLATOXIN M_2



AFLATOXIN B_{2a}



AFLATOXIN G_{2a}

Figure 2 -- Structures of the minor mold-derived aflatoxins.

metabolism is the release of caloric energy and incorporation of essential components into vital structures, the purpose of metabolism of xenobiotics is the reduction of their toxicity and/or the facilitation of their excretion (Reeves, 1981). However, the use of the same metabolic pathway may increase toxicity instead of resulting in detoxification (Hayes, 1975). For this reason, Hayes (1975) referred to all of these metabolic reactions as biotransformations instead of by the popular synonym for foreign compound metabolism, detoxication.

A number of enzymes in animal organisms are capable of metabolizing lipid-soluble xenobiotics so as to render them more water soluble, and thus make them easier to excrete (Hayes, 1975; Reeves, 1981). Generally, biotransformation of xenobiotics makes them more easily excreted by increasing their polarity and ionization (Reeves, 1981). Williams (1959) proposed that these enzymatic reactions are two types: phase I reactions, whcih involve oxidation, reduction and hydrolysis; and phase II reactions, which consist of conjugation. Phase I reactions generally convert foreign compounds to derivatives, which can then undergo phase II reactions.

Microsomal mixed-function oxidases (MFO) are perhaps the most important enzymes involved in phase I reactions. These enzymes are localized in the endoplasmic reticulum, a complex network of membranes within the cell that is continuous with the outer nuclear membrane (Neal, 1980). When a cell is homogenized, the endoplasmic reticulum is degraded to form small vesicles known as microsomes (Palade and Siekevitz, 1956). The enzyme systems, which are sometimes referred to

as cytochrome P-450-containing mono-oxygenases, are composed of two enzymes, NADPH-cytochrome P-450 reductase, and a heme-containing enzyme, cytochrome P-450 (White and Coon, 1980).

Cytochrome P-450 was first demonstrated independently by Klingenberg (1958) and by Garfinkel (1958), and shown to be a carbon monoxide binding pigment, which was located in the microsomal fraction of mammalian liver cells. Its name was derived from the soret peak at 450 nm, which occurs when it is reduced to form complexes with carbon monoxide (Neal, 1980).

The general types of reactions catalyzed by cytochrome P-450 containing monooxygenases include aromatic hydroxylation, aliphatic hydroxylation, epoxidation, desulfuration, sulfoxidation, N-hydroxylation and N-, O- or S-dealkylation (Neal, 1980). The microsomal system contains mutiple forms of cytochrome P-450 (Hodgson, 1979). This system is found predominately in liver. In addition to liver, the cytochrome P-450 dependent activity has been found in vertebrate skin, sebaceous glands, the alveolar epithelium of lung, the intestines, the placenta and the kidney (Ahmad, 1979).

Aflatoxin B_1 is metabolized by the hepatic microsomal mixed function oxygenase system to a group of derivatives, such as aflatoxin 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).

In addition to its natural occurrence as a mold metabolite (DeIongh et al., 1964), aflatoxin M_1 is also a mixed-function oxidases mediated liver biotransformation product of aflatoxin B_1 (Schabort and Steyn, 1969). In the presence of fortified NADPH, crude and isolated microsomal preparations from liver of many species have been found to transform aflatoxin B_1 to the 4-hydroxy derivative, aflatoxin M_1, which is shown in Fig. 3-pathway 1 (Schabort and Steyn, 1969). Hsieh et al. (1976) examined extracts of urine derived from rhesus monkeys, which had been previously dosed with aflatoxin B_1. They concluded that aflatoxin M_1 was the predominant oxidative metabolite in this species of primate.

Aflatoxin M_2 is a liver metabolite of aflatoxin B_2 (Fig. 4-pathway 3) and is, presumably, produced by the same liver fractions that convert aflatoxin B_1 to M_1 (Roebuck et al., 1978).

Aflatoxicol (aflatoxin R_o) was independently isolated as the degradation product of aflatoxin B_1 by various microorganisms by Teunisson and Robertson (1967) and by Detroy and Hesseltine (1968). Teunisson and Robertson (1967) found that a culture of Tetrahymena phriformis W degraded aflatoxin B_1 to a bright blue-fluorescing compound with a lower R_f value than B_1 on TLC plates. Detroy and Hesseltine (1968) observed that aflatoxin B_1 was transformed to a blue-fluorescing hydroxylated compound by Dactylium dendroides. They then named it aflatoxin R_o. Later Detroy and Hesseltine (1970) proposed the name aflatoxicol instead of aflatoxin R_o.

Aflatoxicol in animal tissues was not reported until

Patterson and Roberts (1971) produced evidence that aflatoxicol was a biotransformation product of aflatoxin B_1. They found that soluble fractions of liver from chicken, duck, turkey and rabbit were capable of reducing the carbonyl group of the terminal cyclopentenone ring of aflatoxin B_1 and B_2 to the corresponding aflatoxicol (Fig. 3-pathway 2) and dihydroaflatoxicol (Fig. 4-pathway 1). Unlike other metabolites, aflatoxicol biotransformation is catalyzed by a NADP-linked dehydrogenase of the cytosol, which is also known as 17-ketosteroid dehydrogenase (Patterson and Roberts, 1971; 1972). The biotransformation of aflatoxin B_1 to aflatoxicol has been shown to be reversible depending on the $NADPH_2$/NADP ratio in the tissue (Patterson and Roberts, 1972).

Aflatoxicol M_1 is a biotransformation product that can be formed either from aflatoxin M_1 (Fig. 3-pathway 3) or from aflatoxicol (Fig. 3-pathway 4). The cyclopentenone carbonyl of aflatoxin M_1 can undergo reduction by a cytosolic enzyme system, as described for aflatoxicol, to yield aflatoxicol M_1 (Salhab *et al.*, 1977). Aflatoxicol M_1 can also be formed directly by oxidizing aflatoxicol by a mixed function oxidase system (Salhab *et al.*, 1977). Aflatoxicol M_1 can be reoxidized by liver microsomal fractions to yield aflatoxin M_1. By analogy to the conversion of aflatoxicol to aflatoxin B_1, the reoxidation reaction is probably not mediated by the cytochrome P-450 system (Salhab and Edwards, 1977).

Aflatoxin P_1 is a biotransformation product of

aflatoxin B_1 resulting from O-demethylation as shown in Fig. 3-pathway 5 (Shank and Wogan, 1965). Shank and Wogan (1965) prepared ring-labeled and O-methyl-labeled aflatoxin B_1 by submerging cultures of A. flavus in liquid media containing either [1-^{14}C]-acetate or methyl-^{14}C-L-methionine. They then administered the labeled toxin orally to Fisher rats. Some 60 per cent of the administered ^{14}C dose was excreted into the feces through the bile. About 20 per cent was excreted into the urine. A further 27 per cent of O-methyl-labeled ^{14}C appeared in the respiratory CO_2. These results indicated that O-demethylation constitutes a significant metabolic pathway for excretion of aflatoxin B_1 by rats. Aflatoxin P_1 was subsequently identified as the glucuronide or sulfate conjugate in the urine of monkeys given aflatoxin B_1 (Dalezios and Wogan, 1972). Biotransformation of aflatoxin B_1 to P_1 appeared to be a mixed function oxidases dependent product of in vitro liver metabolism (Bassir and Emafo, 1970).

Aflatoxin Q_1 is the product of mixed-function oxidase hydroxylation of aflatoxin B_1 at the β-position to the cyclopentenone carbonyl as demonstrated in Fig. 3-pathway 6 (Masri et al., 1974a). Aflatoxin Q_1 represents the major metabolite produced from aflatoxin B_1 by microsomes of monkey and human liver in vitro (Buchi et al., 1974; Masri et al., 1974a; 1974b). The hydroxylation appears to be carried out by an enzyme system that is different from the one producing aflatoxin M_1 (Krieger et al., 1975).

Salhab and Hsieh (1975) demonstrated that human and monkey liver preparations modified aflatoxin B_1 to form a

derivative with the ketone carbonyl on the cyclopentenone ring being reduced to a secondary alcohol, and a hydroxyl group introduced onto the β-carbon of the alcohol group (Fig. 3-pathway 7). This newly identified metabolite was named aflatoxicol H_1 (Salhab and Hsieh, 1975; Hsieh et al., 1977; Salhab and Edwards, 1977). Aflatoxicol H_1 may also arise by a direct hydroxylation of aflatoxicol (Fig. 3-pathway 8). Because of the rapid conversion of aflatoxicol to aflatoxin B_1, and aflatoxin B_1 to aflatoxin Q_1 in liver homogenates, it is not clear whether direct hydroxylation of aflatoxicol occurs at an appreciable rate (Salhab and Edwards, 1977).

Patterson and Allcroft (1970) suggested that aflatoxin B_1 and M_1 were reduced by chicken, duckling, guinea pig and mouse liver preparations in vitro to their corresponding reduction products, aflatoxin B_2 and M_2, with either NADP or NADPH being required as the cofactors (Fig. 4-pathway 2).

The active mutagenic form of aflatoxin B_1 has been proposed to be the 2,3-epoxide, which is generated metabolically from the 2,3-vinyl ether double bond in the terminal furan ring as illustrated in Fig. 3-pathway 9 (Schoental, 1970). Although this labile molecular species has not been isolated, microsomal enzyme activation is an absolute requirement for mutagenicity of aflatoxin B_1 (Wong and Hsieh, 1976). Support for the existence of the 2,3-epoxide came from the isolation of 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1, a postulated diol detoxification product. The latter was isolated from acid hydrolysates of covalently linked complexes

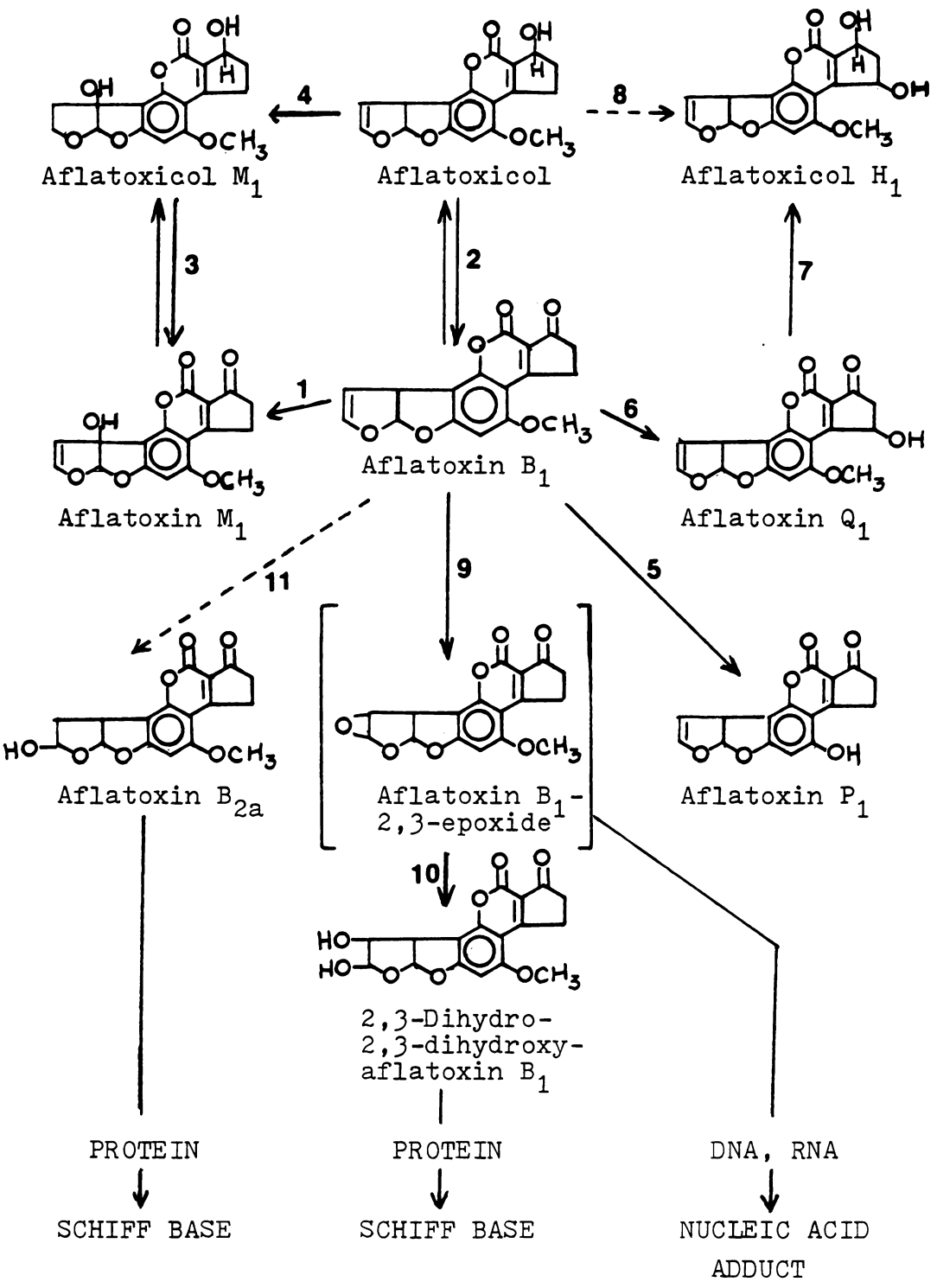


Figure 3 - Metabolism of aflatoxin B_1

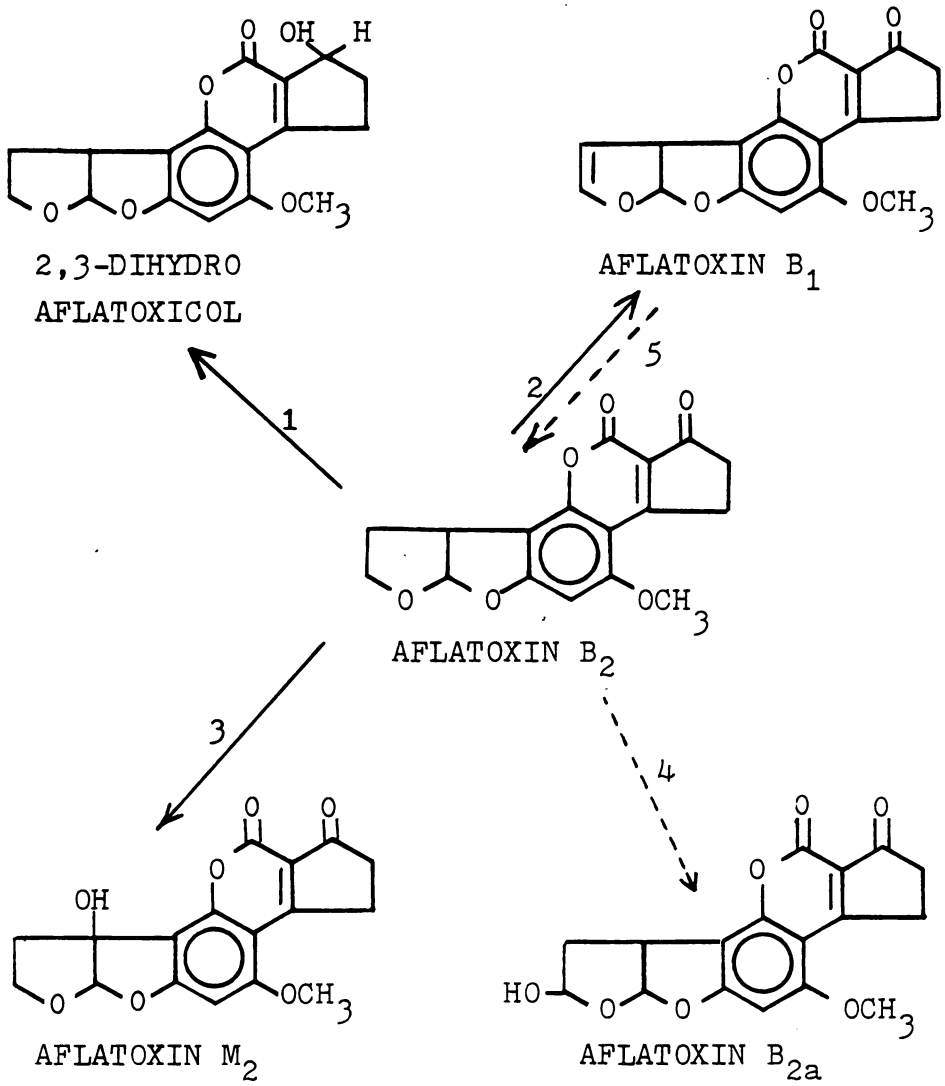


Figure 4 - Metabolism of aflatoxin B_2

of aflatoxin B_1 and nucleic acids generated *in vitro* and *in vivo* (Swenson *et al.*, 1973; 1974; Neal *et al.*, 1981).

Aflatoxin B_{2a} was first reported as a natural metabolite in cultures of *A. flavus* by Dutton and Heathcote (1966; 1968). Using *in vitro* assays of aflatoxin-metabolizing activies of different species, Patterson and Roberts (1972) found that the NADPH dependent microsomal enzyme hydrates the vinyl ether at the double bond of aflatoxin B_1 to form its 2-hydroxyl derivative, aflatoxin B_{2a} (Fig 3.--pathway 11). Neal *et al.* (1981) pointed out that previous reports of the production of aflatoxin B_{2a} (Patterson and Roberts, 1972; Gurtoo and Dahms, 1974; Gurtoo and Campbell, 1974) were erroneous. The latter authors identified 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 as B_{2a} due to the similarity of their UV spectral characteristics (Swenson *et al.*, 1973; 1977). Thus, there is doubt whether aflatoxin B_{2a} is a significant microsomal metabolite of aflatoxin B_1.

Aflatoxin B_{2a} was not readily produced from aflatoxin B_2 on incubation with rat liver microsomes (Roebuck *et al.*, 1978). However, it does appear to be a major urinary metabolite of aflatoxin B_2 in rats, accounting for about 8 per cent of the injected dose (Fig. 4--pathway 4) according to Dann *et al.* (1972).

TOXICITY OF AFLATOXINS

Results from many experiments showed that there are a great many factors that can effect the toxicity of aflatoxins.

These include the type of aflatoxin to which one is exposed (Wong and Hsieh, 1976), the dose (Wogan and Newberne, 1967), duration of exposure (Newberne and Butler, 1969), species (Hsieh et al., 1977), age (Newberne and Butler, 1969; Sullman et al., 1970), sex (Purchase et al., 1973), nutritional status (Hamilton, 1977), pre-xenobiotic exposure (Wong et al., 1981), environmental factors (Wyatt et al., 1977) and the route of administration (Butler, 1964).

Aflatoxins B_1, B_2, G_1 and G_2 are the four predominant natural aflatoxins that have been tested for relative toxicity in human embryonic liver cells. Their biological activity was found to decrease in the order of aflatoxin $B_1 > G_1 > G_2 > B_2$ (Zukerman et al., 1968; Sullman et al., 1970). However, when the toxic effects were tested on day-old ducklings (Carnaghan et al., 1963) or chicken embryos (Vesely et al., 1982) and the mutagenetic potency on Salmonella typhimurium (Wong and Hsieh, 1976), the biological effects were decreased in the order of aflatoxin $B_1 > G_1 > B_2 > G_2$.

Wong and Hsieh (1976) have studied the relative mutagenicity of aflatoxins and their animal biotransformation products using the Ames test (Ames et al., 1973). Wong and Hsieh (1976) found that all isolated biotransformation products of aflatoxin B_1 had a decrease in mutagenicity. Aflatoxin M_1 had only about 3 per cent of the potency of aflatoxin B_1. Wong and Hsieh (1976) also reported that the relative mutagenicity in vitro was closely correlated with in vivo carcinogenicity data.

The potency of aflatoxin M_1 in inducing liver tumors in rainbow trout was one-third of that of aflatoxin B_1 at a level of 4 ppb in the diet (Sinnhuber *et al.*, 1970). This is in contrast to its ability to cause acute toxicity as demonstrated by Holzapfel *et al.* (1966) and Purchase (1967). They reported that the LD_{50} of aflatoxin M_1 in ducklings was similar to that of B_1, and that similar liver lesions were produced by the two toxins.

Recently, Green *et al.* (1982) compared the cytotoxicity and genotoxicity of aflatoxin B_1 and M_1 in primary cultures of adult rat hepatocytes. Aflatoxin B_1 stimulated the release of lactate dehydrogenase into the culture mediuum, and the loss of viable cells from the monolayer at a lower dose than aflatoxin M_1. Genotoxicity, which is determined by an assay for stimulation of DNA repair, demonstrated that aflatoxin B_1 is again more potent than aflatoxin M_1. Although aflatoxin M_1 is less potent than aflatoxin B_1 in cytotoxicity and genotoxicity assays using primary cultures of hepatocytes, Green *et al.* (1982) pointed out that aflatoxin M_1 is still active at relatively low doses. Thus, they concluded that it is probably a potent hepatotoxin *in vivo*.

The earliest evidence of the carcinogenetic properties of aflatoxins was provided by the experiments of Lancaster *et al.* (1961), who fed rats a diet containing 20 per cent peanut meal that had earlier been identified as a cause of poisoning in poultry flocks. The rats did not show the signs of acute toxicity seen in turkey poults. After a 6 month feeding period, 9 out of 11 rats developed multiple

liver tumors and 2 of them had lung tumors. The presence of liver tumors in the absence of cirrhosis, cell necrosis or cellular infiltration suggested that the toxic agent acts directly on the hepatic cells.

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. Wong and Hsieh (1976) using the Salmonella typhimurium mutant assay (Ames et al., 1973) demonstrated that none of the aflatoxins, including the biotransformation metabolites, possessed any mutagenic potency in the absence of an activation factor isolated from rat liver preparations. They concluded that none of these aflatoxins or their biotransformation products are the ultimate mutagenic and/or carcinogenic species. Therefore, Wong and Hsieh (1976) concluded that the 2,3-double bond is involved in both the mutagenetic and carcinogenetic activity of aflatoxins.

Garner and Hanson (1971), and Garner et al. (1972) demonstrated that incubation of rat hepatic microsomes with an aflatoxin and a NADPH-generating system produced a metabolite of aflatoxin B_1 that was lethal to certain bacteria. None of the aflatoxins were active in the absence of mixed-function oxidases of liver microsomes, and the bacterial toxicity was reduced on the addition of either DNA or RNA. This suggested that the same metabolite was reacting with nucleic acid (Garner et al., 1972). The idea that DNA is probably the critical target of the aflatoxin metabolite was supported by its lethal effect on certain strains of E. coli, which are very sensitive

to UV light. They apparently lack the gene product necessary for the repair of DNA that has been damaged by light or other mutagens (Garner and Wright, 1973).

Further experimental support for the view that 2,3-epoxide might be an important metabolite of aflatoxin B_1 is found in the work of Swenson *et al.* (1973; 1974; 1975). They isolated an *in vitro* product indistinguishable from synthetic 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 from liver microsomes in the presence of RNA. They injected [^3H]-aflatoxin B_1 into rats and isolated covalently bound derivatives in hepatic DNA, rRNA and protein. Mild acid hydrolysis of the DNA and rRNA adducts formed a derivative indistinguishable from 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1. These observations strongly support the concept that aflatoxin B_1 is biotrnas-formed into aflatoxin-2,3-epoxide, which binds covalently through its high electrophilic C-2 to nucleophilic sites in the DNA and rRNA.

Swenson *et al.* (1975) synthesized aflatoxin B_1-2,3-dichloride as a model of the epoxide. The dichloride would be expected to form a resonance stabilized carbonium ion at carbon 2. These workers showed that the dichloride is highly carcinogenic and mutagenic, and reacts *in vitro* with nucleophiles in the manner expected for the 2,3-epoxide. The compound formed covalent adducts with DNA and RNA, with retention of one half to the chlorine. Polyguanylic acid was the most reactive homopolymer tested towards aflatoxin B_1-2,3-dichloride. This was 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 from rats dosed with aflatoxin B_1.

Wong and Hsieh (1976) had suggested that the 2,3-vinyl ether double bond is not the sole molecular site on the aflatoxin B_1 molecule that determines mutagenic activity. They found that alterations of the cyclopentenone ring, such as its substitution by a terminal lactone ring as in aflatoxin G_1, or by 7-hydroxylation as in aflatoxin Q_1 or by reduction of the keto group as in aflatoxicol, result in a significant lowering of mutagenic potential, despite the presence of an intact 2,3-double bond.

Wong and Hsieh (1978) also suggested that both *in vitro* and *in vivo* formation of aflatoxicol may be an indicator of species sensitivity to aflatoxin induced carcinogenesis. They found that aflatoxicol was the major metabolite in the plasma of Sprague-Dawley rats, dosed orally or intravenously with ^{14}C-aflatoxin B_1. However, aflatoxicol was not detected in the plasma of similarly dosed mice and monkeys, which are resistant to aflatoxin B_1-induced carcinogenesis. Furthermore, the biotransformation of aflatoxin B_1 to aflatoxicol has been shown to be reversible (Patterson and Roberts, 1972; Salhab and Edwards, 1977). The reversibility has been suggested to function as a reservoir for aflatoxin B_1 and its metabolites, which prolonges the cellular exposure to the carcinogens, and hence enhances their carcinogenic effects (Hsieh *et al.*, 1977). It has been proposed that aflatoxin B_1 is converted to aflatoxicol, which is slowly reconverted to aflatoxin B_1, and thus, has the same effect as

chronic administration of aflatoxin B_1 (Swenson, 1981).

Conversion of aflatoxin B_2 to aflatoxin B_1 (Fig. 4-pathway 2) has been demonstrated directly in duck liver homogenates (Roebuck et al., 1978) and indirectly in rat liver in vivo (Swenson et al., 1977). It was estimated by Swenson et al. (1977) that about 1 per cent of the administered aflatoxin B_2 is converted to aflatoxin B_1. This agrees with the relative carcinogenic potencies of these toxins (Wogan et al., 1971).

At physiological pH, both aflatoxin B_{2a} and 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 may bind to proteins (Patterson and Roberts, 1972; Gurtoo and Campbell, 1974; Ashoor and Chu, 1975; Neal et al., 1981). According to Patterson (1977), these two aflatoxins may also bind to various key enzymes and metabolically important liver cell structures, and thus, may be responsible for the acute toxicity of aflatoxin B_1.

CHICKEN AFLATOXICOSIS

Aflatoxicosis is the disease produced when animals ingest aflatoxins, and was first discovered in poultry (Blount, 1961). Asplin and Carnaghan (1961) showed that chickens were more resistant than ducklings to the effects of aflatoxins. Muller et al. (1970) used four different levels of aflatoxins B_1 and G_1, which were equivalent to 500, 1,000, 2,000 and 4,000 ppb of aflatoxin B_1 in the diet, and compared them to a control diet. The diets were fed to chicks, ducklings, goslings, pheasants and turkey poults

for four weeks. Results showed that the order of sensitivity from greatest to least was ducklings, turkey poults, goslings, young pheasants and chickens.

Arafa et al. (1981) determined the relative sensitivity of broiler chickens, White Leghorn hens, quail, goslings and turkey poults to increasing dietary levels of aflatoxins from 1 to 21 days of age. Aflatoxins were included in the diet at three concentrations (700, 1,400 and 2,100 ppb of aflatoxin B_1 equivalents). Turkey poults and goslings were the most sensitive and quail were intermediate. Broiler chickens and White Leghorn hens were the most tolerant to aflatoxins. These findings agree with the earlier work of Muller et al. (1970) and Gumbmann et al. (1970) suggesting that different poultry species show varying degrees of tolerace to aflatoxins.

In a comparative study, Florentin et al. (1969) fed Brahmas, Barred Plymouth Rocks, White Plymouth Rocks, Langshans, Rhode Island Reds, New Hampshires, Single Comb White Leghorns, Delaware x New Hampshires and Cornish x White Plymouth Rock chicks on diets containing 380, 550, 650, 660 and 1,300 ppb of aflatoxin B_1 for eight weeks. In comparison to the controls, all test groups showed depressed growth, higher mortality, enlarged livers and hearts and an increased fat content in the liver. If the growth and mortality effects are considered, the Brahmas were the most susceptible breed and the White Plymouth Rocks the most resistant. Mortality rates for New Hampshires and Rhode Island Reds were similar, but the Rhode Island Reds exhibited more growth

depression. Performance of the broiler cross chicks was similar to that obtained with White Plymouth Rocks.

Smith and Hamilton (1970) determined the sensitivity of 6 different strains of unsexed White Leghorn chicks to aflatoxins by finding the LD_{50} dose. Results showed that the acute LD_{50} varied from 6,500 to 16,500 ppb. Results indicated that there is a considerable variation between different strains of chickens in regard to their susceptibility to aflatoxins.

Gumbmann et al. (1970) fed an experimental diet with aflatoxins equivalent to 800 ppb of pure aflatoxin B_1 to 18 different strains of cross-bred chickens, turkeys and quail for a period of 2 to 6 weeks. They found that New Hampshire chicks and turkey poults were most adversely affected by aflatoxins. When New Hampshire hens were crossed with Leghorn males or vice versa, the sensitivity of the chicks to aflatoxins was no longer detectable. They suggested that the susceptibility to aflatoxins is a genetically controlled factor.

Asplin and Carnaghan (1961) fed Rhode Island Red chickens a diet containing aflatoxin-contaminated peanut meal. The main effect of feeding the aflatoxins was growth retardation. The gross lesions observable showed that young chickens are more susceptible to the toxin than the older ones. In the chickens dying during the first 3 weeks of the feeding experiment, the livers were pale in color but firm in texture, while the kidneys were pale and slightly enlarged. In the birds which were killed during the following 3 months,

the liver and kidney changes were similar but more marked. In those chickens killed during the latter phases of the feeding trial, the organs were normal in texture and color, but discrete white pin point lesions were consistently found in the livers.

Smith and Hamilton (1970) also determined the effects of age on the sensitivity of chickens to aflatoxins by finding the LD_{50}. The birds they used were broiler chickens (Arbor Acre 60 x Peterson). In contrast to the findings of Asplin and Carnaghan (1961), day-old broiler chicks were about as sensitive as birds ready for processing when the sensitivity to aflatoxins was measured in terms of acute LD_{50}. However, when sensitivity was measured in terms of growth rate, young chickens were more sensitive. The growth of day-old chicks was depressed more by aflatoxins than that of 10-day old chicks.

Carnaghan et al. (1966) fed aflatoxin B_1 at the rate of 1,500 ppb to Rhode Island Red chickens. They noted that the livers were enlarged relative to body weight, which was lower than normal. The fat content of the liver was higher than normal. Microscopic changes included periportal fatty infiltration, cell necrosis, progressive biliary proliferation and an increase in connective tissue. Within 3.5 days, there was slight proliferation of the bile duct epithelial cells, and the amount of proliferation became prominent within two through six weeks. A similar pattern of bile duct proliferation was been reported for ducklings (Butler, 1964), turkeys (Wannop, 1961; Siller and Ostler, 1961) and swine (Loosmore and Harding, 1961).

The first case of aflatoxicosis in chickens was recognized by Asplin and Carnaghan (1961), who observed that about 30 per cent of a group of 100 Rhode Island Reds were stunted at 2 weeks of age. It was later discovered that the commercial mash being fed contained 6.25 per cent of Brazilian groundnut meal. A number of field cases were also diagnosed in the United Kingdom (Asplin and Carnaghan, 1961), and outbreaks were later reported in Canada (Archibald et al., 1962), Australia (Gardiner and Oldroyd, 1965; Hart, 1965) and the United States (Hamilton, 1971). Mortality reached 50 per cent in a flock of laying hens which died within 48 hours after being fed highly toxic maize containing 83,000 and 101,000 ppb of aflatoxin B_1. According to Hamilton (1971), a 90 per cent drop in egg production simultaneously occurred.

Broiler type chickens are considered to be relatively resistant to aflatoxin toxicity (Archibald et al., 1962; Arafa et al., 1981). In broiler chickens, aflatoxins limit both the performance and health of the birds. There is an increase in susceptibility to bruising (Tung et al., 1971b), impairment of immunity (Thaxton et al., 1974), impairment of coagulation (Doerr et al., 1976), a decrease in pigmentation (Tung and Hamilton, 1973), an interaction with vitamin deficiencies (Hamilton, 1977), a decrease in bone strength (Huff et al., 1977), a decrease in the concentration of the digestive enzymes (Osborne et al., 1976) and a decrease in hatchability of the eggs (Cottier et al., 1969).

A field case of broiler aflatoxicosis was reported by Smith and Hamilton (1970) that was characterized by a three-fold

increase in mortality, a doubling of the normal carcass condemnation rate, an increase in the feed conversion ratio from the normal 2.0 - 2.1 to 2.3 - 2.4 and the final body weight was 0.25 lbs less than normal. Examination of the affected birds revealed a serious infiltration of the pericardium, a pale comb, shank and bone marrow, an enlarged and pale liver, an enlarged spleen, an enlarged pancreas, a regressed bursa of Fabricius, and a below normal amount of depot fat. When the feed was fed to young chickens, they showed the same symptoms as birds fed an experimentally contaminated diet. Analysis showed that the ingredients in the suspected feed were contaminated with aflatoxins ranging from 100 to 10,000 ppb. Corn was the primary source of the aflatoxin. When an alternate corn supply free of aflatoxin was fed, there were no symptoms of aflatoxicosis in the chickens.

Archibald et al. (1962) fed broiler chickens a diet containing peanut meal contaminated with unknown amounts of aflatoxins. They observed no effects for up to two weeks, thereafter uneven growth was shown. On necropsy, pale livers with lesions were observed, but the livers did not appear to be enlarged.

Kratzer et al. (1969) fed broiler chickens for 8 to 9 weeks with diets containing 0, 400, 800 and 1,600 ppb of mixed aflatoxins. Growth retardation was observed in the groups at the two higher levels. Smith and Hamilton (1970) fed day-old male broiler chickens for three weeks on diets containing aflatoxins at levels of 0, 625, 1,250, 2,500 and

10,000 ppb. The effects of different levels of aflatoxins upon body weight of the experimental birds indicated that there was a threshold dose of 1,250 ppb, which had to be exceeded before any growth rate depression was observed. Smith and Hamilton (1970) also indicated that the threshold dose became larger with longer periods of administration. At aflatoxins levels of 2,500 ppb and above, enlargement of the spleen and pancreas was apparent, and the size of the bursa of Fabricius was decreased. The lipid content in liver was almost doubled at the higher doses. There was a significant increase in relative liver weight with increasing levels of aflatoxins up to 2,500 ppb.

The possibility that aflatoxicosis is a predisposing factor in bruising was investigated by Tung et al. (1971b) They determined the minimal amount of energy required to produce bruises on the breast muscle of broiler chickens receiving graded levels of dietary aflatoxins. Doses of aflatoxins as small as 625 ppb, which is insufficient to affect the growth rate of most commercial strains, required 18 per cent less energy to produce bruising when compared to that for control chicks. At levels higher than 2,500 ppb, the amount of energy needed to produce bruising was decreased by about 50 per cent. These authors considered that the bruising effect might be caused by lowered muscle strength or by increased capillary fragility. They found that doses of 625 and 1,250 ppb had no effect on body weight but significantly increased capillary fragility.

Tung et al. (1971b) fed a diet containing 5,000 ppb

of aflatoxins to 2-week old chicks and found that the capillary fragility increased significantly within 24 hours. They further measured the lateral shear strength of the breast muscle and found that the strength of the tissues was decreased at doses as low as 625 ppb of aflatoxin. These findings suggest that dietary aflatoxins produce a generalized loss of tissue strength and integrity, and result in an increased susceptibility to bruising.

Aflatoxins have been shown to impair blood coagulation in a number of species (Bababunmin and Bassir, 1972). Doerr et al. (1976) fed 3-week old broiler chickens with graded levels of dietary aflatoxins (0, 625, 1,250, 2,500, 5,000 and 10,000 ppb) to determine the specific coagulation responses. Tissue thromboplastin was found to posses two active components, one of which was lost during aflatoxicosis. The thromboplastin protein content was unaltered but the lipid content of the blood was depressed at 10,000 ppb of aflatoxins. The activities of coagulation factors VII and V and of fibrinogen were reduced at levels above 2,500 ppb of aflatoxins. The activity of coagulation factor X was depressed at a level of 5,000 ppb of aflatoxins. Prothrombin activity, the most sensitive factor tested, was reduced by feeding aflatoxins at a level of 625 ppb. These data demonstrated that severe impairment of clotting pathway functions occur during aflatoxicosis in chickens, and that prothrombin is primarily affected.

The anemia associated with severe aflatoxicosis was initially characterized as aplastic anemia by Forgacs and

Carll (1962), and has subsequently been reported as hemolytic anemia by Tung et al. (1975). Early studies by Forgacs and Carll (1962) reported that aflatoxins consumed in moldy feeds resulted in a hemorrhagic anemia syndrome characterized by spontaneous hemorrhages in the musculature and internal organs. They concluded that it was caused by aplastic anemia.

Wannop (1961) reported that erythrocyte numbers were decreased in a field outbreak of aflatoxicosis in turkeys. Tung et al. (1975) found that red blood cell counts, packed red cell volumes and hemoglobin levels were depressed by feeding aflatoxins to chickens. These results are consistent with the presence of aplastic anemia in the birds. However, microscopic examination of the bone marrow revealed a general hyperactivity. A possible explaination for these results may be that aflatoxins result in production of erythrocytes with an impaired integrity. Tung et al. (1975) confirmed the occurrence of anemia in chicks during aflatoxicosis and suggested that it was hemolytic in nature.

Osborne and Hamilton (1981) fed graded levels of aflatoxins to broiler chicks and found that at 1,250 ppb or above there was a significant decrease in the specific activities of the pancreatic digestive enzymes. The enzymes included pancreatic amylase, trypsin, lipase, RNase and DNase. At 10,000 ppb, the specific activities of amylase, trypsin, lipase, RNase and DNase were reduced to 41, 61, 42, 71 and 74 per cent of their control values, respectively. Osborne et al. (1982) observed that feeding aflatoxins at doses as small as 1,250 ppb, which does not inhibit the growth rate

of broiler chicks, produced a malabsorption syndrome characterized by steatorrhea, hypocarotenoidemia, and a decrease in the concentration of bile salts and of all the pancreatic digestive enzymes.

OCCURRENCE OF AFLATOXINS IN FOODS OF ANIMAL ORIGIN

According to Jarvis (1975) foods may become contaminated by mycotoxins through various routes. These routes were summarized as being by direct and indirect contamination. The direct routes of contamination result from mycotoxin production associated with mold growth on food materials of all types. These may include the following: (1) stored natural products intended for direct consumption or for use as food ingredients in manufactured products; (2) mold-ripened foods; and possibly, (3) mold-spoiled manufactured foods. The indirect routes of contamination may result from the use of mycotoxin-contaminated food additives, such as fermentation products of fungal origin, or from mycotoxin residues in animal products resulting from the use of mycotoxin contaminated animal feedingstuffs (Jarvis, 1975).

Allcroft and Carnaghan (1963) first postulated that residues of aflatoxins or their metabolites might be present in the milk, meat or eggs of animals receiving aflatoxin contaminated feeds. They fed ducklings with extracts of milk from cows fed aflatoxin contaminated rations, and found that the ducklings had lesions identical to those produced by administration of aflatoxin B_1 directly. The milk toxin was later named aflatoxin M_1 and M_2 by Holzapfel *et al.* (1966).

Several reviews deal with the excretion of aflatoxins in the milk of farm animals (Allcroft, 1969; Kiermeier, 1973; 1977; Patterson, 1977; Rodricks and Stoloff, 1977; Stoloff, 1980). When cattle (Allcroft et al., 1968) or sheep (Nabney et al., 1967) were fed rations contaminated with aflatoxin B_1, their milk contained aflatoxin M_1. Allcroft and Roberts (1968) measured the amount of aflatoxin M_1 in milk from cows given diets containing various levels of aflatoxin B_1. The daily intake of aflatoxin B_1 ranged from 0.857 to 24 mg. The excretion of aflatoxin M_1 was proportional to the intake. There was a linear relationship between the amount of aflatoxin B_1 ingested and the level of aflatoxin M_1 in the milk.

In order to determine the minimum intake of aflatoxin B_1 which produces detectable levels of aflatoxin M_1 in cow milk, Polan et al. (1974) fed graded levels (10, 50, 250 or 1,250 ppb) of aflatoxin B_1 to 4 lactating cows for 7 days. On day 8, they substituted [^3H]aflatoxin B_1 for a portion of the aflatoxin dose, and fed it an additional 7 days. The milk, blood, urine and feces samples were collected beginning at day 8. Regression analysis indicated that the 15 ppb of aflatoxin B_1 was required for aflatoxin M_1 to be detectable in the milk. The results generally agreed with those of Allcroft and Roberts (1968), who concluded that the level of aflatoxin B_1 fed to milking cows should not exceed 50 ppb in order to insure negligible amounts of aflatoxins in the milk.

Patterson et al. (1980) fed 6 dairy cows a diet contaminated with 10 ppb of aflatoxin B_1. They found that the amount of aflatoxin M_1 in the milk varied from 0.01 to

0.33 µg/liter with a mean value of 0.19 µg/liter. Approximately 2.2 per cent of the aflatoxin B_1 ingested appeared in the milk as aflatoxin M_1. Rodricks and Stoloff (1977) concluded from literature values that the ratio of aflatoxin B_1 in the feed to aflatoxin M_1 in the milk of cows was 300. Patterson et al. (1980) stated that this ratio varies considerably from animal to animal and from one set of data to another, and the overall range for the ratio of aflatoxins in feed to milk varied from 34 to 1,600.

Allcroft and Carnaghan (1963) on assaying for aflatoxins with day-old ducklings failed to demonstrated toxicity from the livers and eggs of hens fed a ration containing 15 per cent of toxic peanut meal. They also were unable to find any toxic factor in the clotted blood serum and livers from cows fed a concentrate ration containing 20 per cent of toxic peanut meal, or from a pig liver taken from an animal with fatal aflatoxicosis. Allcroft and Raymond (1966) using a chemical assay found that the toxic peanut meal was contaminated with 10,000 and 200 ppb of aflatoxin B_1 and B_2, respectively.

Platonow and Beauregard (1965) fed White Leghorn chickens for 2, 4 or 6 weeks on a diet containing 3,100 ppb of aflatoxin B_1. The meat and livers from these chickens were fed separately to ferrets for 4 weeks, with each ferret receiving 100 g/day of tissue. No histological or chemical signs of toxicity were observed. A similar study by Allcroft and Lancaster (1966) using tissues from pigs and cattle fed aflatoxin contaminated peanut meal also provided negative results using ferret. Results from these studies were limited

by the lack of sensitivity of the ferrets to aflatoxins. Butler (1969) demonstrated that ferrets receiving a diet containing 300 ppb of aflatoxins required 28 months for tumor development. Platonow and Beauregard (1965) showed that ferrets receiving 1,000 ppb of aflatoxins died within 21 days.

In addition to feeding the meat and liver from White Leghorn chickens fed aflatoxins to ferrets, Platonow and Beauregard (1965) assayed these tissues chemically by the method of Heusinkveld et al. (1965). The technique used was originally developed for determination of aflatoxin levels in peanuts. No detectable amounts of aflatoxins or their fluorescent metabolites were observed in the samples using this method.

Abram (1965) fed ducklings and chickens a diet containing the meat from birds fed 0.75 ppb of aflatoxins. No histological signs of toxicity were observed. Negative results indicated that the aflatoxin levels in the tissues of the birds were probably below 40 ppb. Similar negative results were obtained for eggs from birds fed the same diet.

Butler and Clifford (1965) found that aflatoxin B_1 and M (at that time aflatoxin M_1 and M_2 had not been separated) were present in the livers of rats that were given pure aflatoxin B_1 by gastric intubation of by intraperitoneal injection. These compounds were also present in both the systemic and the portal blood. The authors suggested that conversion of aflatoxin B_1 to M took place in the livers of the rats.

Allcroft et al. (1966) gave sheep 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 and G_2, respectively, by either gastric intubation or intraperitoneal injection. All sheep were killed two hours later. All liver and kidney samples were removed, and chemically analyzed by the method described by DeIongh et al. (1964). It was found that the livers of all sheep had aflatoxins B_1, G_1 and M_2. The M_2 spot was less intense than those of B_1 and G_1. The kidney extracts showed fluorescent spots similar to those seen in liver extracts, but the intensity of aflatoxin M_2 was greater than that of B_1 and G_1.

Kratzer et al. (1969) fed graded levels of dietary aflatoxins to White Rock chicks and found that at higher levels (800 and 1,600 ppb) adverse biochemical effects were detected in the liver. They analyzed the meat, liver and blood samples by using the method of Wiley (1966). There was no detectable amount of aflatoxin ir the tissues of broilers fed 1,600 ppb of aflatoxins for 60 days prior to slaughter. Similarly, Kratzer et al. (1969) observed no aflatoxins in the eggs, meat, liver or blood of hens fed a ration containing 2,700 ppb of aflatoxins for a period of 48 days. According to Kratzer et al. (1969) as little as 305 ppb of aflatoxin B_1 could be detected by the method of Wiley (1966).

Van Zytveld et al. (1970) fed White Rock chicks for 6 weeks with daily dosages ranging from 0.4 to 1.7 g of mixed aflatoxins/kg of body weight. In 15 of 45 test birds, aflatoxins or their metabolites were isolated from the livers and skeletal muscles. Aflatoxins were found mainly

in chicks that died or grew poorly during the test and only one bird that reached market weight was shown to have residues of aflatoxins in its tissues.

Mabee and Chipley (1973a) intubated 6-week old Vantress-Arbor Acre broiler chickens with ^{14}C-labeled aflatoxin B_1. The daily dose was 0.1 mg/kg of body weight. No toxic effect was observed in broiler chickens during the 14 day experiment. The feces samples were collected every 48 hours during the feeding trial. Five hours after the final dose of aflatoxin was administered, all birds were killed by exsanguination. Samples of leg and breast muscle, gizzard, liver, heart and blood were collected. All the samples were lyophilyzed and extracted with sodium acetate buffer. The supernatant fluid was treated with NaCl and then was further extracted with ethyl acetate. According to the analysis, the broiler chickens excreted 90.64 per cent of the ^{14}C administered. Of the ^{14}C retained, 11.04, 9.83, 4.03, 12.52, 31.66 and 30.63 per cent was found in the blood, liver, heart, gizzard, breast and leg, respectively. In the combined sample, 81.2 per cent of the radioactivity was confined to the sodium acetate buffer extract. Mabee and Chipley (1973b) further treated the sodium acetate buffer with β-glucuronidase to determine the conjugated aflatoxins, and found that 31.5 per cent of the total radioactivity originally present in the buffered extract was transferred to the chloroform extract. The presence of aflatoxin M_1 was confirmed by TLC of the chloroform extract. The authors concluded that broiler chickens can metabolize the majority of aflatoxin B_1, if it

is administered at relatively low levels. Aflatoxin conjugates were the predominant forms of the metabolites produced. Aflatoxin B_1 was metabolized to aflatoxin M_1. Aflatoxin M_1 was shown to be soluble in aqueous extracts and was present mainly as conjugated glucuronides.

Mabee and Chipley (1973b) also used the same methods to study aflatoxin retention and excretion with White Leghorn pullets. They found that the average levels of radioactivity detected in the blood, liver, heart, gizzard, breast and leg muscles were 19.5, 16.1, 3.9, 7.2, 26.4 and 26.9 per cent of the total amount retained by the laying hens, respectively. The ^{14}C detected in liver, heart, gizzard, breast and leg samples accounted for 7.85 per cent of the total dosage. Aflatoxin M_1 glucuronides constituted 38.9 per cent of the total conjugates. The authors proposed that in addition to glucuronide conjugates, other metabolites of afltoxin B_1 were present, probably as sulfate conjugates. They further treated all the samples with carboxypeptidase A, leucine amino peptidase, pepsin or trypsin. Results revealed that an average of 50 per cent of the ^{14}C detected in the ethyl acetate extracts was liberated as the amino acid conjugates of ^{14}C aflatoxin B_{2a} (Chipley et al., 1974).

Mintzlaff et al. (1977) divided 120 HNL-hybrid broilers into 8 experimental groups, and fed aflatoxin B_1 at levels of 25, 100, 500, 1,000, 2,000, 5,000, 10,000 or 15,000 ppb for 8 weeks. They also fed 5 times as much G_1. All birds were killed at the end of the experiment. The livers and muscle tissues were lyophilyzed and analyzed

quantitatively for aflatoxin B_1 by using the A.O.A.C. method. With this method, about 0.1 ppb of aflatoxin B_1 is detectable in the liver and muscle of broiler chicks. The authors found that on increasing the level of aflatoxin B_1 in the feed, the tissue residues in the liver and muscle increased. Liver contained more aflatoxin B_1 than muscle, but the amounts in the thigh and breast muscles were not significantly different. The critical aflatoxin B_1 concentration which resulted in residues in the tissues was 100 ppb. Results showed that on the average liver contained 0.28 per cent, thigh muscle 0.07 per cent, and breast muscles 0.12 per cent of the total administered aflatoxin B_1. Aflatoxin G_1 and M_1 were also detected in the liver and muscles.

Stoloff and Trucksess (1979) analyzed the livers from 4 calves that had been fed with mixed aflatoxins and from 4 pigs that had died after consuming aflatoxin contaminated feed. There were no statistically significant differences in the levels of aflatoxin B_1 or M_1 found in different sections of the livers. The levels of aflatoxins in the pig livers were lower than that found in the calf livers, with only one out of the four pig livers containing detectable amounts of aflatoxin B_1. Since the pigs died from exposure to aflatoxins, and were not sacrificed as were the calves, these authors suggested that the low level and incidence of aflatoxins in the pig livers may be attributed to poor feed consumption, although postmortem changes could not be ruled out.

The withdrawal time required for clearance of aflatoxins from pig tissues was studied by Furtado et al. (1982).

Two feeding trials involving 20 pigs in each were used. In both trials spiked diets were fed, which contained 551 and 355 ppb of aflatoxins B_1 and B_2, respectively. After feeding 42 days, all pigs were fed the unspiked control diet. There was a significant reduction of aflatoxins in all organs and tissues one day after placing the pigs on an aflatoxin-free ration. After two days, only one pig contained trace amounts of afltoxins in tissues. After four days, there were no detectable levels of aflatoxins in any of the tissues. It was also found that a naturally contaminated diet containing only 20 and 31 ppb of aflatoxins B_1 and B_2, respectively, resulted in trace amounts of aflatoxins B_1, B_2, M_1 and M_2 in the kidneys and livers after a 13-14 hours withdrawal period.

Miller et al. (1982) also studied the clearance time of aflatoxin residues in swine tissue by performing an acute test. In the acute study, 8 pigs were given a single dose of 1.2 mg of aflatoxins/kg of body weight. Liver, kidney and muscle tissues from these pigs were analyzed for aflatoxin residues. Residue levels were correlated with post-dosage time. Residues were present in the liver and kidney of one pig at 12 hours after dosing, but not in muscle. However, detectable muscle residues were reportedly found in the muscle of pigs killed 24 hours after dosing. Residue levels were c nsistently lower at 72 hours than that at 24 hour post-dosing.

In a chronic study, Miller et al. (1982) fed 30 mixed breed pigs (10 animals/diet) a ration containing 0, 400 or 800 ppb of aflatoxins for 10 weeks. Aflatoxin residues in

the liver, kidney and muscle tissues were detected immediately after slaughter using a HPLC method. No detectable residues were found in the muscles from pigs fed 400 ppb of aflatoxins in the diet, but were present in the muscle of pigs fed 800 ppb of aflatoxins. Aflatoxin residues in the kidney and liver were higher than in the muscles of pigs fed 800 ppb.

Luthy et al. (1980) administered ring-labeled [^{14}C]-aflatoxin B_1 to 2 pigs over a 9-day period. They found that the major excretory route was feces (accounting for 51 and 65 per cent of the dose) and that less than 20 per cent of the dose was excreted in the urine. Only about 4 per cent of the dose recovered in the feces and urine was in identifiable forms, most of it being aflatoxin M_1 and B_1 with traces of aflatoxicol.

Jacobson and Wiseman (1974) fed laying hens with an aflatoxin-contaminated laying ration. The hens were fed as follows: (1) 10 days-100 ppb, (2) for the following 12 days-200 ppb, and (3) the next 15 days-400 ppb. The eggs from hens at all three feeding periods contained measurable amounts of aflatoxin B_1. The relationship between the amounts of aflatoxin B_1 fed and the average amounts found in the eggs appeared to be approximately linear. The average level for aflatoxin B_1 in the eggs collected during period one was 0.23 ppb, from period two was 0.78 ppb and from period three was 1.4 ppb.

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 TISSUE 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	B_1	1,200

A summary of the data from various studies presenting the ratios for the level of aflatoxin B_1 in feeds in relationship to the level likely to be encountered in selected animal products is presented in Table 1 (Rodricks and Stoloff, 1977). Assuming that the experimental data reflect reality, meat from swine is more likely to be contaminated than poultry meat or beef, since the observed feed/tissue ratios appear to be lower for swine than for broilers or beef cattle.

EXPERIMENTAL

FEEDING TRIAL

Preparation of the Diet

During the entire experimental period, the basal diet used was a broiler starter ration, formula CBS 711 prepared at the MSU Swine Research Center. The composition of the diet is given on Table 2.

In order to prepare the spiked ration, the crystalline aflatoxin B_1 and B_2 (Calbiochem-Behring Co. Saw Diego, CA.) were first solubilized with chloroform, and then were diluted to 2 liters in a volumetric flask. The chloroform solution was then divided into two equal fractions and each of the was slurried with 1.5 kg of the pre-dried basal diet that had been held overnight in an oven at 100°C. The chloroform was allowed to evaporate in the dark overnight using forced air under a hood.

These two fractions of aflatoxin-spiked feed were mixed homogenously together in a 4-speed Reynolds Mixer (Reynolds Electric Co.), and were then divided into three equal fractions by weight. Each of the aflatoxin premixed feed samples was separately transferred to a stainless steel Wenger horizontal mixer (Model No. 61129, Wenger Mixer Manufacturing Co.), and mixed with additional feed until homogenous to give a final weight of 100 lb. Each 100 lb lot was divided

Table 2 -- Chick, broiler starter diet (formula CBS 711)

Ingredients	Amt./1000
Corn, #2 yellow	482.2
Soybean meal, 48%	365
Alfalfa, 17%	68
Corn oil, stabl.[a]	42
Methionine, dl	0.8
Dicalcium phosphate	22
Limestone	9
Choline chloride, 50%	3.5
Salt	3.5
Vitamin mix	3
Mineral mix (c.c.c.)	0.5
Selenium mix (c.c.c.)	0.5

[a] Ethoxyquin added to corn oil at 125 mg/kg diet.

Specifications:

Protein, crude	23.4%
Metabolizable energy	3.05 kcal/g
Fat	6.6%
Fiber	3.7%
Calcium	1.07%
Phosphorus, avail	0.55%
Methionine	2% of protein
Lysine	5.5% of protein

into two 50 lb lots, and was cross mixed with another 50 lb lot during preparation. Finally, the total of 300 lb of aflatoxin-spiked feed was evenly packed into twelve 5-gallon plastic pails, and stored at -20°C. One day before being used, the feed was transferred to a refrigerator and held at approximately 4-8°C until fed.

Experimental Animals

A total of 48 day-old male Hubbard White Mountain broiler chicks (White Cornish x White Plymouth Rock) ordered from Commercial chicks, Inc. (Thorntown, Inc.). All chickens were housed in a wire floored electrically heated battery (75 x 27 x 7.5 cm) at the MSU Poultry Research Center. All birds were kept in 4 cages under continuous dim illumination with feed and water available ad libitum. After an adjustment period of one week, all chicks were weighed and labeled with a Zip wing band (size 2.75). They were randomly divided into a control and an experimental group. The chicks in the control group were kept in one cage on the upper layer of the battery, while those in the experimental group were randomly placed in three cages on the lower layer of the battery, with 12 chickens per cage. This gave all groups the same floor, feeder and watering space.

The experimental group was fed the basal diet spiked with aflatoxins B_1 and B_2 at levels of 2057 and 1323 ppb, respectively. The chicks in the control group were fed the

unspiked control diet. The feeding period was for 6 weeks. The chicks were weighed weekly, and feed and water consumption were recorded daily. At 4 weeks of age, all the chicks were moved into wire floored growing batteries (48 x 31 x 4 inch).

At the end of the experimental feeding period, 6 birds from both the control and the experimental groups were slaughtered. The remaining 30 experimental group birds and 6 controls were all fed the uncontaminated basal ration, and held for 1, 2, 4, 8 and 16 days. At the end of each period, 6 experimental chickens were killed. After 16 days, the last 12 birds, 6 from the experimental group and 6 from the control group were killed.

Slaughtering and Collection of Samples

On the day proceding slaughter at 12 noon the feed and water were removed. Body weights, feed consumption and water consumption data were recorded. The chickens were then taken to the MSU Poultry Science Laboratory. At approximately 3 pm (3 hours after removal of the feed), the chickens were slaughtered. Carcasses of all birds were inspected for gross lesions. Samples of blood, heart, liver, spleen, gizzard, kidney, intestine and of breast and leg muscles were collected and weighed. All samples were frozen and stored at -20°C for later analysis.

ANALYSIS OF AFLATOXINS

Sample Preparation

The leg and breast muscle samples were deboned, and the collagenous tissues and excess fat were removed from the lean portions. Then the muscle samples were passed through a meat grinder (Hobart Manufacturing Co.), using a plate having 3/16-inch diameter holes. The gizzards were opened with scissors and any undigested feed was discarded on removal of the lining. The tissues were stored at -20°C until analysis. The internal organs (gizzard, heart, liver and spleen) were cut into fine pieces before extraction.

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 25 g of 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 200 ml of acetone was added to the homogenate while washing the sides of the blender jar. The tissue was blended for 3 minutes at moderate speed followed by 2 minutes at high speed. Another 100 ml of acetone were added to wash the blender jar again, and the tissue was blended for an additional 1 minute at high speed. The material was then filtered through fast filtering prefolded filter paper (Whatman 114v), and the filtrate was collected in a 500 ml Erlenmeyer flask. After filtration was completed, the flask was stoppered with

an aluminum foil covered cork stopper, and placed in a freezer at -20°C for 5 minutes, which precipitated the excess fat.

The precipitated fat was removed by filtration, using the same type filter paper. The filtrate was transferred to another 500 ml Erlenmeyer flask. Then 150 ml of distilled water, 7 g of $(NH_4)_2SO_4$ and 20 ml of $Pb(OAc)_2$ solution (200 g $Pb(OAc)_2 \cdot 3H_2O$ in 500 ml H_2O containing 3 ml of acetic acid and made to volume of one liter with H_2O) were added. The solution was stirred for 0.5 minute using a magnetic stirring device. Then 10 g of Celite were added to the solution, and stirring was continued for an additional 0.5 minute. The solution was allowed to stand for about 5 minutes before filtering into a 500 ml graduate cylinder using fast filtering prefolded filter paper.

Purification of the Aflatoxin Extract

Liquid-Liquid Partition

The filtrate was 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 was collected in the same 500 ml flask, and the chloroform extract was discarded. The chloroform-acetone extract was then evaporated to dryness in a rotary evaporator (Buchi, Switzerland) using a water bath setting of 37°C.

Silica Gel Column Chromatography

A 25 x 250 mm chromatographic column (Fisher and Porter) was filled using about 50 ml of chloroform. A slurry of 10 g of silica gel 60 (70-230 mesh ASTM-EM Laboratories, Inc.) in 60 ml of chloroform was added to the column. The silica gel was allowed to settle and then the chloroform was drained to about 10 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 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 15 ml of chloroform-hexane, and the washings were added to the column. After each addition of the chloroform-hexane extract, the column was drained to the top of the packing and the eluate was discarded. Any interfering substances were eluted from the column in 100 ml of ether-hexane (3:1). The eluate was then discarded.

The aflatoxins were eluted from the silica gel column

with 150 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 acetone and quantitatively transferred to 2 dram vials using a disposable glass pipet. The acetone solution was evaporated to dryness (N-Evap Evaporator, Model 106, Organomation Assoc.) under a stream of nitrogen using a water bath setting of 40°C. Special care was taken to avoid overheating of the dry extract in order to avoid decomposition. The aflatoxin extract was then dissolved in 50 µl of benzene-acetonitrile (9:1), and the vial was sealed with a teflon lined screw cap. The samples were held in the vials at -20°C until removed for 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

. Precoated 20 x 20 cm silica gel plates (Sil-G-HR-25, Brinkman Instrument Co.) 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 (Sigma Chemical Co.). Standards of 5.0, 1.5, 5.0, 1.5, 2.0 and 2.0 ng of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2, respectively, were spotted on the plates as standards in both directions of development about 1.5 cm from the edges.

The plates were developed in the first direction with ether-methanol-water (95:4:1) in a sealed and unequilibrated tank. After the development in the first dimension

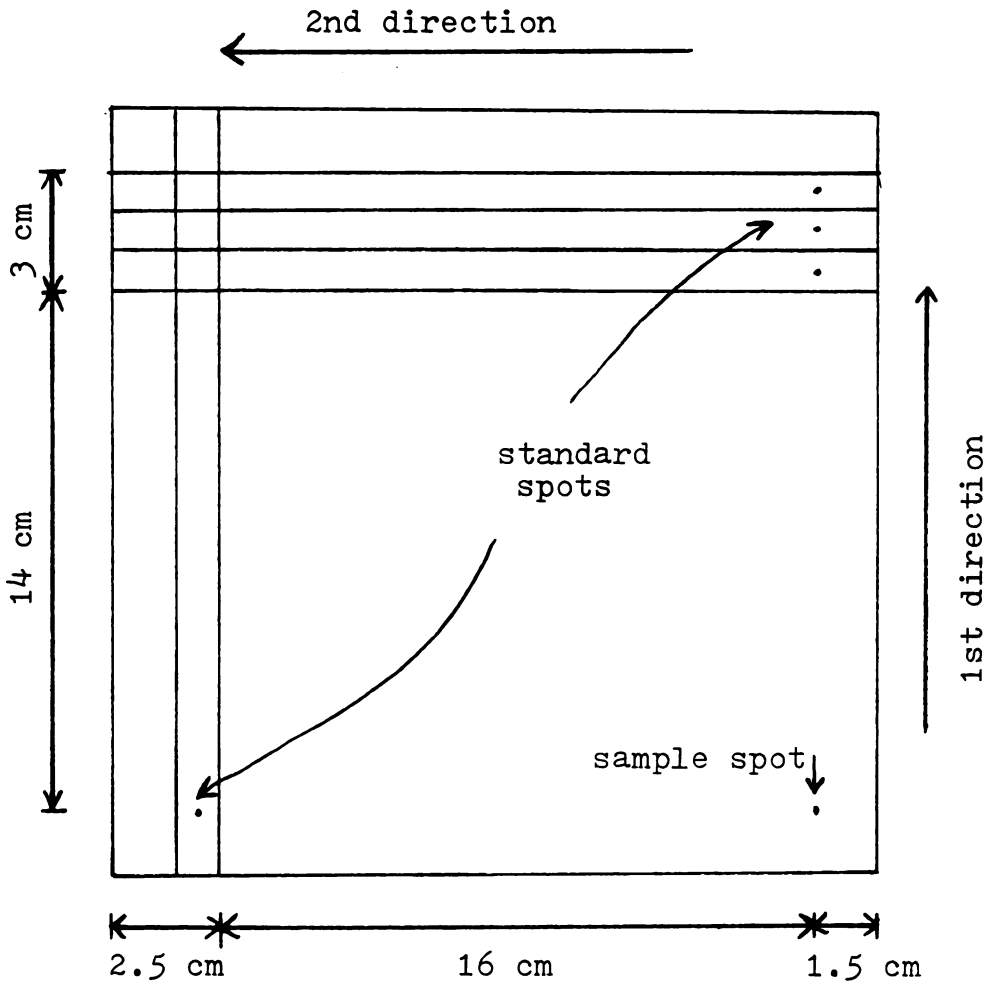


Figure 5 - Spotting and scoring pattern for two-dimensional 20 x 20 cm TLC plates.

was completed, the plates were removed from the tank and dried under a hood for about 2 minutes. The drying was finished by placing in a forced-draft oven for 1 minute and the plates were immediately developed in the second dimension with chloroform-acetone (85:15). After development was completed, the plates were removed and dried as before. After drying, the plates were examined visually under UV light and prepared for densitometric analysis.

Densitometric Analysis of Aflatoxins

Aflatoxins were quantified with a double-beam scanning recording-integrating spectrodesimeter SD 3000-4 (Schoeffel Instruments). The plates were scored prior to spotting as shown in Fig. 5 with a Schoeffel Scoring Device SDA 303, which provides 18 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. Scanning was carried out for the analysis of the standard spots. 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 plates. The marks were about 1 cm apart and located approximately 3 mm ahead of the sample spot along the second dimension of the development. Then the plate was placed on the carrier in such a way as to be driven and scanned parallel to the direction of the imaginary lines.

Aflatoxin concentrations were calculated according

to the following formula:

$$\mu g/kg = (B \times Y \times S \times V)/(Z \times X \times W)$$

where:

B = Area under aflatoxin peak in the sample;

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.

ANALYSIS OF AFLATOXINS IN THE FEED

Analysis for aflatoxins in 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 Erlenmyer flask. Then, 25 ml of water, 25 g of Celite 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 2v) and the first 150 ml of filtrate were collected, dried and transferred to a 25 x 250 mm chromatographic column as described earlier herein. The aflatoxins were eluted from the column, spotted on 20 x 20 cm TLC plates and quantified as described before for analysis of aflatoxins in tissue samples.

CONFIRMATORY TESTS FOR AFLATOXINS

Aflatoxin B_1

A total of 25 µl of sample extract was applied about 1.5 cm from both edges in the lower left corner of a precoated TLC plate (20 x 20 cm Sil-G-HR-25, Brinkman Instruments, Inc.). Approximately 5.0, 1.5, 5.0, 1.5, 2.0 and 2.0 ng of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 standards, respectively, were spotted in the same location in both directions of development on the plates. Then the plates were developed in a closed, unlined and unequilibrated tank using ether-methanol-water (95:4:1) in the first dimension and chloroform-acetone (85:15) in the second dimension. After the development was completed, the plates were examined visually under UV light.

The spot corresponding to aflatoxin B_1 was identified by comparison with the aflatoxin B_1 standard. It was marked in the silica gel on the left with a pencil along the second direction of development. Another pencil mark was made about 1 cm apart to the left of the first one. The second mark was used as a guide to apply about 5.0 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 oven for 5 minutes. After cooling the plate in the dark at room temperature, another 5.0 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}. The chromatographic equivalence of the sample and the aflatoxin standard spots after treatment with TFA was used as confirmatory test for identifying aflatoxin B_1.

Aflatoxin M_1

After second dimension was finished, 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_2 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 forced-draft oven for 5 minutes. After cooling the plate in the dark at room temperature, 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 (85:15). 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 identifying of aflatoxin M_1.

General Confirmatory Test for Aflatoxins

The technique of Prezybylski (1975) was used for additional confirmation of the presence of aflatoxins at low levels. After development of the TLC plate and identification of the spots under UV light, the plate was sprayed with 25 per cent sulfuric acid (v/v). Then the plate was dried in a forced-draft oven for 1 minute. Changes in the characteristic fluorescence of aflatoxins B_1, B_2, M_1 and M_2 from blue to yellow, and aflatoxins G_1 and G_2 from green to yellow after the H_2SO_4 treatment were used as an additional confirmatory test for the presence of aflatoxins.

PREPARATION OF AFLATOXIN REFERENCE STANDARDS

Aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 were obtained from Sigma Chemical Company and used as reference standards. Aflatoxins B_1 and B_2 used for preparation of the spiked diets were purchased from Calbiochem-Behring Corporation.

Aflatoxin reference standards were prepared according to the A.O.A.C. method (1975) and contained 0.5, 0.15, 0.5, 0.15, 0.2 and 0.2 µg/ml of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2, respectively. A solvent mixture of benzene-acetonitrile (98:2) was used as the solvent for aflatoxins B_1, B_2, G_1 and G_1, while benzene-acetonitrile (90:10) 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. (1975) procedure for determining moisture was used. Four 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 an desiccator and weighed to four decimal places. The loss in weight was calculated as percentage moisture. 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. (1975). 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 diethyl ether for approximately 3 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 per cent fat was calculated as grams of fat extracted from each one hundred grams of tissue. Three replicates were run for each sample.

STATISTICAL ANALYSIS

Statistical analysis was carried out using the procedures described by Gill (1978). In testing the difference between the means for the control and for the experimental groups, a preliminary F-test was performed to test the difference between two variances. When there was no difference between the two variances at the 80 per cent confidence level, a t-test was used to determine if the difference was significant. If the variances were different at the 80 per cent confidence level, another preliminary test was performed to determine the difference between the two coefficients of variation. When the coefficients of variation were different at the 80 per cent confidence level, a t-like test (Behrens, 1929) was used to determine the significance between the two means. Otherwise, the direct test developed by Lohrding (1969) was applied to determine the significance between the means.

SAFETY PROCEDURES

All glassware and vials in contact with aflatoxins were soaked with 5-6 per cent NaOCl (household bleach) 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 of work areas were routinely scanned with an UV lamp and any contaminated areas were treated by washing thoroughly with 5-6 per cent 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. The discarded solvents involved in the experiment were removed by the MSU Chemical and Biological Safety Unit. All work involving aflatoxins was done under a hood, this included preparation of silica gel columns, spotting, development and drying of the TLC plates. Similarly, any work involving the use of toxic solvents, such as ammonia, chloroform, benzene, methanol and acetonitrile, was also carried out under a hood.

RESULTS AND DISCUSSION

FEEDING TRIAL

The response of broiler chicks to aflatoxins was determined by growth, feed consumption and feed efficiency data (feed consumed/unit weight gain). Results showing body weights, body weight gains, average feed intake and feed efficiency are presented in Table 3. The amount of aflatoxins consumed and the daily dosage ratio of the experimental group are presented in the Table 4.

Final weights and overall mean body weight gains are presented in Table 3 and demonstrate that growth retardation was significant in the experimental group when compared with the control group. There was no significant difference between the feed efficiency for chicks fed aflatoxins and for those in the control group. These results are generally in agreement with those reported by Kratzer et al. (1969) and by Smith and Hamilton (1970).

The chicks in the experimental group had on average a daily intake of 208 µg of aflatoxin B_1 and 134 µg of aflatoxin B_2. The daily dosage ratio (DR) has been reported as the best measure of the response of the exposed animals to aflatoxins (Armbrecht et al., 1971). The results in Table 4 showed that the DR ratio of aflatoxins B_1 and B_2 was highest in week I and lowest in week V. Thus, the DR ratio

Table 3 -- Performance data showing body weights, body weight gains, average feed intake and average feed efficiency for the 5-week feeding trial (12 birds in the control group and 36 birds in the experimental group).

		Starting Weight (g)	Final Weight (g)	Weight Gain (g)	Avg. Feed Intake Per Chick Per Day (g)	Avg. Feed Efficiency (Feed/Gain)
Control[a] Group	Mean	138.4	1731.5[d]	1593.5[e]	100.3	2.2
	S.D.[c]	4.1	43.2	42.3		
Experimental[b] Group	Mean	141.7	1614.7[d]	1473.1[e]	101.1	2.4
	S.D.[c]	7.3	147.7	144.9		

[a]No aflatoxins were detectable in the control diet.

[b]Fed rations containing aflatoxins B_1 and B_2 at levels of 2057 and 1323 ppb, respectively.

[c]Standard deviations.

[d]The control group was significantly heavier than the experimental group at $P<0.001$.

[e]The control group gained significantly more weight than the experimental group at $P<0.001$.

Table 4 -- Average aflatoxin consumption and daily dosage ratio of the experimental group.

	Avg. Aflatoxin Consumption Per Chick Per Day (μg)		Daily Dosage Ratio[a] (DR)	
	B_1	B_2	B_1	B_2
Week I	129	83	487	313
Week II	138	88	287	185
Week III	213	137	294	189
Week IV	274	176	263	169
Week V	289	186	206	132
Overall	208	134	238	154

[a] Amount of aflatoxin 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.

tended to decrease as the chicks grew older. In other words, the young chicks were exposed to a greater amount of aflatoxins per unit body weight when compared to the older ones. This might explain why Asplin and Carnaghan (1961) found young chicks to be more sensitive to aflatoxins than older ones. However, when sensitivity was measured in terms of the LD_{50}, there was no great difference between young chicks and those large enough for processing (Smith and Hamilton, 1970).

GROSS OBSERVATIONS ON TISSUES

At slaughter, all birds were inspected for gross lesions. On examination of the birds, several distinctive symptoms were observed, which are summarized in Table 5. Inspection showed hemorrhagic spots on the surface of the muscles, especially on the breast muscles of the experimental chicks, but not in the controls. Also in the present study hemorrhagic spots were observed on the livers of three chicks in the experimental group. These results agree with those reported by Tung *et al.* (1971 b), who stated that dietary aflatoxins increase the susceptibility of broiler chickens to bruising. This was because the fragility of the blood vessels increased and the muscle strength decreased. Doerr *et al.* (1976) reported that aflatoxicosis will impair the coagulation of blood.

The pale livers observed in the experimental group of the present study were associated with a higher lipid content (Table 6). Tung *et al.* (1971a; 1972) also indicated that inhibition of lipid transport is a primary lesion during

Table 5 -- Summary of the gross lesions observed upon inspection (6 birds in each group).

	SYMPTOMS			
	Hemorrhages[a] on the Muscle	Pale liver	Gall Bladder Enlarged	Slipped Tendon
0 day after withdrawal from aflatoxin-spiked ration				
Control Group	0	0	0	2
Experimental Group	6	4[b]	4	0
1 day after withdrawal from aflatoxin-spiked ration				
Experimental Group	5	0	0	0
2 days after withdrawal from aflatoxin-spiked ration				
Experimental Group	6	4	1	0
4 days after withdrawal from aflatoxin-spiked ration				
Experimental Group	2	2[b]	0	0
8 days after withdrawal from aflatoxin-spiked ration				
Experimental Group	0	0	0	1
16 days after withdrawal from aflatoxin-spiked ration				
Control Group	0	0	0	1
Experimental Group	0	0	0	2

[a] Most of the hemorrhagic spots were on the breast muscle.

[b] On 0 day withdrawal from the aflatoxin-spiked ration, 2 out of 4 livers had hemorrhagic spots, and on 4 days withdrawal from the aflatoxin-spiked ration 1 out of 2 livers had hemorrhagic spots.

chicken aflatoxicosis. They reported that aflatoxins can cause a dose-related increase in the lipid content of the livers of young broilers. This was accompanied by a decrease in total serum lipids, serum phospholipids, serum cholesterol and serum triglycerides. These authors also measured the incorporation of radio-labeled acetate into the long-chain fatty acids of the livers from birds receiving aflatoxins. Results indicated that fatty acid biosynthesis decreased in a dose-related fashion. The authors suggested that aflatoxins inhibit lipid synthesis and transport in broiler chickens. However, hepatic lipid biosynthesis increased in some instances as was reported by Chou and Marth (1975).

The weights of the internal organs (hearts, spleens, livers, kidneys, gizzards and intestines) were recorded. The mean for organ weights and a summary of organ weights expressed as the percentage of the live body weight are presented in Tables 7 and 8.

Data from the present experiment (Table 7) showed that the livers and kidneys of the chicks on the experimental diet were significantly heavier at the end of the aflatoxin feeding trial (day 0) than those of the controls ($P < 0.01$). On the average, the livers of the experimental group were 29 per cent heavier than those of the control group. The difference may in part be due to the higher lipid content of the livers for the experimental birds. The kidneys of the experimental group were 46 per cent heavier than those of the control group.

The hearts of the experimental group were slightly

Table 6 --Fat and Moisture content of livers for both the control and the experimental groups at 0 day after withdrawal from the aflatoxin-spiked ration.

Chick No.	Moisture Content (%)	Fat Content[a](%)
Control Group		
C1	71.7	1.4
C2	69.9	1.7
C3	69.8	0.7
C4	70.2	1.0
C5	73.3	0.3
C6	70.0	1.5
Mean	70.8	1.2
Experimental Group		
01	73.1	1.7
02	69.3	2.6[b]
03	67.4	4.5[b]
04	62.7	17.7[b]
05	70.0	3.9
06	65.6	8.8[b]
Mean	68.0	6.5

[a] The fat content was based on the wet basis.

[b] Gross lesion inspection showed that these birds had pale livers.

Table 7 -- Internal organ weights in grams of both the control and the experimental groups (0 day and 16 days after withdrawal from aflatoxin-spiked ration).

	ORGAN WEIGHTS (grams)					
	Heart	Spleen	Liver	Kidney	Gizzard	Intestine
0 day after withdrawal from aflatoxin-spiked ration						
Control Group						
Mean	9.1	1.6	33.2[a]	6.5[b]	28.8	67.1
S.D.	0.8	0.3	6.0	1.9	4.8	10.2
Experimental Group						
Mean	10.2	1.9	42.7[a]	9.4[b]	29.7	74.9
S.D.	1.0	0.5	4.8	1.4	2.3	12.5
16 days after withdrawal from aflatoxin-spiked ration						
Control Group						
Mean	12.9	3.4	38.2	9.5	47.1	69.9
S.D.	2.5	1.2	5.2	2.5	5.9	7.1
Experimental Group						
Mean	11.9	2.3	35.6	9.6	39.5	66.7
S.D.	2.2	0.8	8.1	3.4	6.0	13.0

[a] The livers from the experimental group were significantly heavier than those of the control group ($P < 0.01$).

[b] Kidneys from the experimental group were significantly heavier than those for the control group ($P < 0.01$).

Table 8 -- Summary of organ weights expressed as a percentage of live body weight for both the control and the experimental groups (0 day and 16 days after withdrawal from aflatoxin-spiked ration).

	ORGANS					
	Heart	Spleen	Liver	Kidney	Gizzard	Intestine
0 day after withdrawal from aflatoxin-spiked ration						
Control Group						
Mean	0.57	0.10	2.03	0.37	1.74	4.08
S.D.	0.05	0.01	0.17	0.15	0.14	0.51
Experimental Group						
Mean	0.62	0.12	2.73[a]	0.61[b]	1.82	4.54
S.D.	0.09	0.04	0.61	0.16	0.13	0.42
16 days after withdrawal from aflatoxin-spiked ration						
Control Group						
Mean	0.47	0.12	1.46	0.37	1.85	2.74
S.D.	0.05	0.04	0.12	0.09	0.28	0.18
Experimental Group						
Mean	0.54	0.10	1.51	0.39	1.71	3.18
S.D.	0.04	0.02	0.13	0.10	0.28	0.82

[a] The livers from the experimental group were significantly heavier than those of the control group ($P<0.0005$).

[b] The kidneys from the experimental group were significantly heavier than those of the control group ($P<0.0005$).

enlarged (Table 7) when compared to those of the control group ($P<0.1$). Gall bladder enlargement was also noticed in the inspection for gross lesions. Differences in the weights of the livers, kidneys and hearts between the control and the experimental groups were not significant at 16 days after removal of aflatoxins from the diet, but were significant at all earlier times.

In this study, several birds with slipped tendons were observed in both the control and the experimental groups. The incidence of slipped tendons in the control group (3 out of 12) was higher than that in the experimental group (3 out of 36). However, the occurrence of slipped tendons is not believed to be associated with the treatments. These observations are in agreement with Washburn et al. (1976), who indicated that the relatively high incidence of leg problems is related to the rapid growth rate of broiler chickens, and that birds growing at comparatively high growth rates are more inclined to develop leg problems.

The incidence of all other symptoms of aflatoxicosis gradually decreased with the time elapsing after withdrawal from the aflatoxin-spiked rations. This indicates that the chicks recover from aflatoxicosis after removal of aflatoxins from the ration. Similar results have also been reported by other authors (Blount, 1961; Smith and Hamilton, 1970).

AFLATOXIN RESIDUES IN THE TISSUES OF CHICKS FED A CONTAMINATED DIET

Results of aflatoxin analysis for the tissues of birds slaughtered following removal of the contaminated feed (no withdrawal period) are presented in Table 9. As expected there were no detectable amounts of aflatoxin residues in any of the tissues from the birds in the control group. Measurable amounts of aflatoxins B_1 and B_2 were found in all analyzed tissues of all the birds fed the aflatoxin-spiked ration. The highest levels were found in the gizzards, and the livers contained the second highest levels. Measurable amounts of aflatoxins M_1 and M_2, the metabolites of aflatoxins B_1 and B_2, were also found in the most of the tissues of the chicks in the experimental group.

Although the gizzards had the highest levels of aflatoxins B_1 and B_2 with mean values of 0.26 and 0.19 µg/kg, respectively, it may have been caused by direct contamination. In the slaughtering procedure, the opening of the gizzards with scissors may have caused direct contact of the surfaces of the organ with the undigested feed inside the gizzard and may have led to contamination of the tissues. Further support for contamination is found by the ratio of aflatoxin B_1 to B_2 in the gizzards (1.32), which is similar to the ratio in the aflatoxin-spiked ration (1.55). The relatively small standard deviation of the ratio (0.24) in the gizzards indicated that the individual values were within a rather narrow range. Other tissues did not have B_1 to B_2 ratios similar

Table 9 -- Aflatoxin residues detected in selected chick tissues at 0 day after withdrawal from the aflatoxin-spiked ration (μg/kg).

	Levels of Aflatoxins[a]							
Chick No.	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Leg Muscle				Breast Muscle			
01	0.13	0.02	tr.	0	0.08	0.02	0.02	tr.
02	0.04	0.02	0.02	0	0.02	0.01	0.03	0
03	0.16	0.01	tr.	tr.	0	0.01	0	0
04	0.05	0.02	0.06	0	0.02	0.05	0.05	0
05	0.09	0.02	0.04	0	0.06	0.04	tr.	0.01
06	0.06	0.02	0.03	0	0.08	0.04	0.02	tr.
Mean	0.09	0.02	0.02	tr.	0.04	0.03	0.02	tr.
	Liver				Gizzard			
01	0.13	0.03	0.05	1.50	0.26	0.20	0	0.31
02	0.29	0.13	0.05	3.00	0.11	0.07	tr.	0
03	0.07	0.06	0.04	6.53	0.50	0.33	tr.	0.05
04	0.22	0.13	0.14	0.64	0.16	0.17	0.03	tr.
05	0.06	0.10	0	1.13	0.35	0.24	0	0.12
06	0.22	0.12	0.06	0.87	0.17	0.15	tr.	0.02
Mean	0.17	0.10	0.06	2.27	0.26	0.19	tr.	0.08
	Heart[b]				Kidney[b]			
Mean	0.08	0.06	0	0.19	0.05	0.05	0.10	2.07

[a] tr = trace amounts, visible but too small of an amount to quantitate (< 0.02 μg/kg).

[b] Composite of six samples in one analysis.

to that in the feed and such small standard deviations (Table 11). In contrast to aflatoxins B_1 and B_2, aflatoxins M_1 and M_2 residues in the gizzard were low (Table 10). This may also indicate that the high levels of aflatoxins B_1 and B_2 in the gizzards were caused by direct contact with the contaminated feed.

Table 10 -- The total amounts of aflatoxins B_1 and B_2, M_1 and M_2, and B_1, B_2, M_1 and M_2 in the tissues of chicks slaughtered 0 day after withdrawal from the aflatoxin-spiked ration[a].

TISSUES	AFLATOXINS (μg/kg)		
	$B_1 + B_2$[b]	$M_1 + M_2$[c]	$B_1 + B_2 + M_1 + M_2$
Leg Muscle	0.11	0.03	0.14
Breast Muscle	0.07	0.02	0.09
Liver	0.27	2.33	2.60
Kidney	0.10	2.08	2.18
Heart	0.14	0.19	0.33
Gizzard	0.45	0.08	0.53

[a] Data were calculated from the mean values presented in Table 9.

[b] The sum of aflatoxins B_1 and B_2 represented the carry over of ingested aflatoxins to tissues.

[c] The sum of aflatoxins M_1 and M_2 represented the deposit of aflatoxins metabolites in the tissues.

Table 11 -- The ratio of aflatoxin B_1 to B_2 (B_1/B_2) in selected chick tissues at 0 day after withdrawal from the aflatoxin-spiked ration[a,b].

	TISSUES					
Chick No.	Leg Muscle	Breast Muscle	Liver	Gizzard	Heart	Kidney
01	6.50	4.00	4.33	1.30	-	-
02	2.00	2.00	2.23	1.57	-	-
03	16.00	0	1.17	1.51	-	-
04	2.56	0.40	1.69	0.94	-	-
05	4.50	1.50	0.60	1.46	-	-
06	3.00	2.00	1.83	1.13	-	-
Mean	6.75	1.65	1.98	1.32	1.33	1.00
S.D.[c]	5.28	1.42	1.28	0.24	-	-

[a] Data were calculated from values in Table 9.

[b] The ratio of aflatoxin B_1 to B_2 (B_1/B_2) in the aflatoxin-spiked ration was 1.55.

[c] Standard deviation.

The total amount of aflatoxins (B_1 + B_2 + M_1 + M_2) was highest in the livers and kidneys (Table 10). These two organs also showed the highest residual levels of aflatoxins M_1 and M_2 (Table 10). These results agree with those reported by Furtado et al. (1982), who found the highest amounts of aflatoxins B_1, B_2, M_1 and M_2 in the livers and kidneys of pigs fed an aflatoxin-contaminated ration in two different feeding trials. The capacity of the liver and kidneys to concentrate aflatoxins is probably associated with their important role in the metabolism and elimination of xenobiotics (Klaassen, 1980; Neal et al., 1981).

Except for the livers and kidneys, the levels of aflatoxins M_1 and M_2 were much lower than the levels of B_1 and B_2 (Table 10). This is probably because of the higher polarity and increased water solubility of aflatoxins M_1 and M_2, which are more easily eliminated from the tissues than the unmetabolized aflatoxins, B_1 and B_2. These hydroxylated aflatoxins may be excreted either as the original compound or as aflatoxin conjugates after being bound to various endogenous compounds (Mabee and Chipley, 1973a; 1973b; Neal et al., 1981). These results are consistent with the findings of Furtado et al. (1982) for the tissues of pigs fed aflatoxins.

In the present study, the aflatoxin M_2 levels in the livers and kidneys were 2.27 and 2.07 µg/kg, respectively. These values were significantly higher than the corresponding aflatoxin M_1 level of 0.17 and 0.10 µg/kg for the same organs. The high levels of aflatoxin M_2 in the livers and kidneys may be associated with conversion of aflatoxin B_1 to B_2 and

aflatoxin M_1 to M_2, respectively. Patterson and Allcroft (1970) reported that aflatoxins B_1 and M_1 were reduced to their corresponding reduction products, B_2 and M_2, for in vitro liver preparations from chickens, ducklings, guinea pigs and mice.

Table 12 -- Ratios of aflatoxins B_1 or B_2 levels in the feed in relation to aflatoxins M_1 or M_2 levels in livers and kidneys.

TISSUE	AFLATOXIN	FEED TO TISSUE RATIO[a,b]
Liver	B_1	12,000
	M_1	34,283
	B_2	13,228
	M_2	583
Kidney	B_1	41,140
	M_1	20,570
	B_2	26,456
	M_2	639

[a] Higher values indicate more efficient tissue clearance.

[b] Values for M_1 and M_2 were calculated based on the assumption that B_1 and B_2 were entirely converted to these their respective metabolites.

As indicated in the Table 12, the ratio of aflatoxin B_1 in the feed in relation to aflatoxin B_1 in the livers was 12,100. This value is approximately 10 times higher than that of 1,200 predicted by Rodricks and Stoloff (1977), which was an estimated value taken from the data of several studies (Table 1). The difference may be due to the various parameters reported by Rodricks and Stoloff (1977) to effect the

pattern of tissue residues. These include: (1) the species and breed of animals, (2) the level of exposure, (3) the diet and health status of the animals, and (4) the time interval after cessation of aflatoxin exposure before slaughter and collection of samples for analysis. The difference could also be due to the fact that other aflatoxins in the ingested feed may interfere with the metabolism of aflatoxin B_1. Swenson (1981) indicated that the biotransformations of aflatoxin B_1 to M_1, B_2 to M_2 and G_1 to GM_1 may be catalyzed by the same enzyme system.

Further support for this viewpoint comes from the study of Mintzlaff et al. (1977), in which broilers were fed different levels of aflatoxin B_1 and 5 times as much G_1 for a period of 8 weeks. The birds were killed at the end of the experiment, and the livers and muscle tissues were analyzed for aflatoxin residues. The authors found that the livers of the chickens fed aflatoxin B_1 at a level of 2,000 ppb contained 7.9 ppb of aflatoxin B_1. This value is considerably higher than the level of aflatoxin B_1 found in the livers of the chicks in the present study (0.17 ppb). The difference may be due to the high level of aflatoxin G_1 in the feed used in the study by Mintzlaff et al. (1977). On calculating the total amount of aflatoxins B_1, B_2 and G_1 in the feed of the present study, the level was equivalent to 563 ppb of aflatoxin B_1, which is similar to the level of 500 ppb used in the study of Mintzlaff et al. (1977). On comparing these two values, aflatoxin B_1 residues for the present study (liver, 0.17; leg muscle, 0.09; breast muscle,

0.04 ug/kg of tissue) are fairly comparable to those reported by Mintzlaff et al. (1977) for 500 ppb of aflatoxin B_1 in the feed (liver, 0.38; leg muscle, 0.06; breast muscle, 0.16 ug/kg of tissue).

According to Wogan (1968) and Smith and Hamilton (1970), the LD_{50} of different animals to aflatoxins varies from 500 to 16,5000 ug/kg of body weight. In the present study, the total amounts of aflatoxins deposited in different tissues ranged from 0.09 to 2.60 ppb at 0 day after withdrawal from the aflatoxin-contaminated diet (Table 10), and the highest level was found in the livers. Thus, it appears that there is little likelihood of acute toxicity to man from eating these aflatoxin-contaminated tissues. However, the levels may be high enough to cause chronic toxic effects during prolonged exposure to the contaminated tissues.

WITHDRAWAL TRIAL

Results of the withdrawal trial showed that broiler chickens can metabolize and remove aflatoxins from their tissues. One day after withdrawal from the aflatoxin-contaminated feed, measurable amounts of aflatoxins B_1 and B_2 were still found in the livers, gizzards, leg and breast muscles of most of the chickens (Table 13). Except for one gizzard sample, which had an unusually high level of aflatoxin B_1 (0.39 ppb), the levels of aflatoxins B_1 and B_2 in all the analyzed tissues one day after withdrawal from the contaminated feed were far lower than the levels found in the tissues of the chickens at the time of withdrawal. There were no detectable amounts of

Table 13 -- Aflatoxin residues detected in selected chick tissues at 1 day after withdrawal from the aflatoxin-spiked ration (μg/kg).

	Levels of Aflatoxin[a]							
Chick No.	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Leg Muscle				Breast Muscle			
11	0.07	0	0	0.03	0	0	0	0
12	0.05	0	0	tr.	0	0.05	0	0
13	0.03	0.06	0	0.08	0.06	0	0	0
14	0.03	0	0	0	tr.	tr.	0	0
15	0.04	0	0	0	0.03	0.05	0	0
16	0.03	0.02	0	0	0.03	0	0	0
Mean	0.04	tr.	0	0.02	0.02	0.02	0	0
	Liver				Gizzard			
11	0	tr.	tr.	0.75	0.39	0.03	tr.	0
12	0	0.05	0.02	0.28	tr.	tr.	0	0
13	0.06	0.03	0.08	0.12	0.06	tr.	0.02	0
14	0.19	0.08	0	0.06	0.07	0.06	0	0
15	0.07	0.03	0.13	0.77	tr.	0.02	0	0
16	0.25	0.13	0	0.22	0.07	0.04	tr.	tr.
Mean	0.10	0.05	0.04	0.38	0.09	0.03	tr.	0
	Heart[b]				Kidney[b]			
Mean	0	0	0	0	0	0.03	0	0.16

[a] tr = trace amounts, visible but too small of an amount to quantitate (< 0.02 μg/kg).

[b] Composite of six samples in one analysis.

aflatoxins B_1 and M_1 in the kidneys, but measurable amounts of aflatoxins B_2 and M_2 were found in the same organ. Hearts were completely free of detectable aflatoxins one day after withdrawal from the contaminated feed.

Two days after placing the birds on the aflatoxin-free diet, with the exception of the livers, only a few samples contained detectable levels of aflatoxins (Table 14). Measurable amounts of aflatoxin B_2 were found in only one liver and one breast muscle sample from two different chickens. However, measurable amounts of aflatoxin M_2 were found in the livers (4 out of 6) and breast muscles (2 out of 6) two days after placing the birds on the aflatoxin-free diet.

Although the levels of aflatoxins B_1, B_2 and M_1 in the tissues consistently decreased after removal of the birds from the contaminated diet, the levels of aflatoxin M_2 in the muscle tissues first tended to increase and then declined during withdrawal. Only trace amounts of aflatoxin M_2 were found in the leg muscles of chickens killed without any withdrawal period, but an average of 0.02 ppb of aflatoxin M_2 was found one day after withdrawal from the aflatoxin-spiked diet. Only trace amounts of aflatoxin M_2 were detected in the breast muscle of chickens killed without any withdrawal period, and no aflatoxin M_2 was detected in the same tissue one day after withdrawal from the contaminated feed. However, 2 out of 6 chickens had detectable amounts of aflatoxin M_2 in the breast muscles two days after withdrawal from the contaminated feed. This might indicate that the aflatoxin metabolizing enzyme

Table 14 -- Aflatoxin residues detected in selected chick tissues at 2 days after withdrawal from the aflatoxin-spiked ration (μg/kg).

	Level of Aflatoxin[a]							
Chick No.	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Leg Muscle				Breast Muscle			
21	0	0	0	0	0	0	0	0
22	tr.	0	0	0	0	0.03	0	0
23	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0.02
26	0	0	0	0	0	0	0	0.03
Mean	0	0	0	0	0	tr.	0	tr.
	Liver				Gizzard			
21	0	0	0	0.08	0	0	0	0
22	0	0	0	0.18	0	0	0	0
23	0	tr.	0	0.04	0	0	0	0
24	0	0.03	0	0.11	0	0	0	0
25	0	tr.	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0
Mean	0	tr.	0	0.07	0	0	0	0
	Heart[b]				Kidney[b]			
Mean	0	0	0	0	0	0	0	0

[a] tr = trace amounts, visible but too small of an amount to quantitate (< 0.02 μg/kg).

[b] Composite of six samples in one analysis.

systems were altered during aflatoxicosis, but after withdrawal from the aflatoxins that the enzyme systems may have recovered their ability to metabolize the toxic components again. In studying albino rats, Singh and Clausen (1980) found that aflatoxin B_1 induced aryl hydrocarbon hydroxylase (one form of the cytochrome P-450 mixed function oxidase system) activity in the liver, but depressed the system in kidney and brain.

Results of the withdrawal trial showed that aflatoxins B_1 and M_1 were more efficiently removed from tissues of broiler chickens than aflatoxins B_2 and M_2. This may be due to the fact that aflatoxins B_1 and M_1 can be biotransformed into aflatoxins B_2 and M_2 as mentioned earlier herein (Patterson and Allcroft, 1970). The high excretion rates of aflatoxins B_1 and M_1 may be also due to the fact that the vinyl ether double bond in the 2,3-position can be easily biotransformed into the 2,3-epoxide and bind to the nucleoside, or be further hydrated into the dihydrodiol compounds of aflatoxins B_1 and M_1. *In vitro*, aflatoxin B_1 can be rapidly and almost exclusively biotransformed into the dihydrodiol compound of aflatoxin B_1 (2,3-dihydro-2,3-dihydroxy-aflatoxin B_1) by chicken liver preparations (Neal *et al.*, 1981). The vinyl ether double bond is not present in aflatoxins B_2 and M_2 as shown in Fig. 1 (Brechbuhler *et al.*, 1967), which may also help to explain the lower excretion rate of aflatoxins B_2 and M_2.

Four days after putting the birds on an aflatoxin-free diet, there were no detectable levels of aflatoxins in any of the tissues (Table 15). This suggests that placing

Table 15 -- Aflatoxin residues detected in selected chick tissues at 4 days after withdrawal from the aflatoxin-spiked ration (μg/kg).

	Levels of Aflatoxin[a]							
Chick No.	B_1	B_2	M_1	M_2	B_1	B_2	M_1	M_2
	Leg Muscle				Breast Muscle			
41	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0	0
	Liver				Gizzard			
41	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0	0
	Heart[b]				Kidney[b]			
Mean	0	0	0	0	0	0	0	0

[a] tr = trace amounts, visible but too small of an amount to quantitate (< 0.02 μg/kg).

[b] Composite of six samples in one analysis.

chickens 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 chicks that had previously been fed a highly contaminated diet. This result is consistent with the finding of Furtado et al. (1982), who found that pigs cleared aflatoxins from their tissues within 4 days after removal from the diet.

The present study suggests that only a small fraction of the aflatoxins consumed was deposited in the tissues of the chickens, and that it was readily removed after withdrawal from the aflatoxin-contaminated feed. Part of the ingested aflatoxins may be excreted into feces as the original aflatoxins, as one of the various metabolites, or as one of their water soluble conjugates. Mabee and Chipley (1973a) found that 90.64 per cent of the ^{14}C-activity was excreted in the feces of broiler chickens fed ^{14}C-labeled aflatoxin B_1. The excretion of aflatoxins or their metabolites into the intestine via the bile also seems to be a major pathway by which absorbed aflatoxins are excreted (Sawhney et al., 1973).

Mabee and Chipley (1973a) found that aflatoxin M_1 glucuronide was one of the major metabolites isolated from the tissues of broiler chickens dosed with ^{14}C-aflatoxin B_1. Aflatoxin P_1 has also been found to be a major urinary metabolite of aflatoxin B_1 in rhesus monkeys according to Dalezios and Wogan (1972). It is excreted predominantly in the form of water soluble glucuronides and sulfate conjugates. Hayes et al. (1977) found that upon incubation of ^{14}C-aflatoxin B_1 with bovine liver preparations, about 60 per cent of the

original activity was transformed to water soluble materials, and approximately 10 per cent 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-contaminated feed. Thus, these authors suggested that aflatoxin Q_1 was in conjugated form *in vivo*. All these 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, 1973a). T These conjugates are believed to be excreted faster than free aflatoxins because of their higher water solubility (Klaassen, 1980).

In vivo and *in vitro* studies have provided the evidence that 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 is the major metabolite of aflatoxin B_1. Neal *et al.* (1981) pointed out that previous reports on the production of aflatoxin B_{2a} were erroneous (Patterson and Roberts, 1972; Gurtoo and Campbell, 1974; Gurtoo and Dahms, 1974). The error was due to the fact that aflatoxin B_{2a} and 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 have similar UV spectral characteristics (Swenson *et al.*, 1973; 1977; Neal *et al.*, 1981). Some 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 has been found to be covalently bound to the microsomal proteins (Gurtoo and Campbell, 1974). Transformation of aflatoxin B_1 to 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 was found to be a major pathway when compared to the biotransformation of aflatoxin B_1 to M_1 by liver preparations from rabbits, ducklings, guinea pigs and rats (Patterson and

Allcroft, 1970). Neal et al. (1981) compared the metabolism of aflatoxin B_1 in liver preparations from rats, mice, guinea pigs and chickens. These authors found that chicken liver microsomes metabolized aflatoxin B_1 at the highest rate, and it was almost exclusively transformed into 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1. If chickens behave the same as in the in vitro study (Neal et al., 1981), the formation of the 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 may be a significant metabolic pathway.

Aflatoxin B_{2a} was detected in the urine of rats, which had been injected with aflatoxin B_2 (Dann et al., 1972). Both aflatoxin B_{2a} and 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 may rearrange themselves to form dialdehydic phenolated resonance hydrid ions at physiological and alkaline pH values. In this form, they may react with amino acids, peptites and proteins to form Schiff bases as shown in Fig. 3 (Patterson and Roberts, 1971; 1972; Gurtoo and Campbell, 1974). These aflatoxins will probably remain in the tissues longer than the free or conjugated aflatoxins. This may explain the results of Sawhney et al. (1973), who fed White Leghorn hens a single oral dose of ring-labeled aflatoxins, and still recovered the radio activity in the excrement 7 days after the aflatoxin treatment. Thus, more studies on the toxicity and metabolism of aflatoxin-adducts are needed.

SUMMARY AND CONCLUSIONS

A feeding trial was conducted to determine the amount of time necessary for tissue clearance of aflatoxins from broiler chickens fed an aflatoxin-contaminated diet. There were 48 chicks in the trial, with 12 being fed the control diet and 36 being used to determine the time necessary for tissue clearance after removal from the contaminated diet. The diet used in the experimental group was spiked with aflatoxins B_1 and B_2 to give a concentration of 2057 and 1323 µg of aflatoxins B_1 and B_2/kg of feed, respectively. The control ration was shown to be free of aflatoxins. The initial phase of the feeding trial in which the chicks were fed either the control or the spiked diet lasted for 35 days.

Results demonstrated that there was a significant difference in the final weight and the rate of weight gain between the controls and the group fed the aflatoxin-spiked ration. The chicks fed aflatoxins exhibited depressed growth and on the average gained 8 per cent less weight than the control chicks over the 35 day feeding trial. There was also a 29 per cent increase in the liver weights and 48 per cent increase in the kidney weights of the chicks fed aflatoxins in comparison to the control group.

Tissues and organs from the control group were normal in appearance. On examination for gross lesions in the tissues

of the experimental chicks, several distinctive symptoms were observed, which included: (1) Hemorrhagic spots were present on the surfaces of both the muscles and the livers; (2) the livers were pale and exhibited fatty infiltration; and (3) The gall bladders were enlarged. Although slipped tendons were observed, they were present in both the control and the experimental groups and appeared to be related to the rapid growth rates. All symptoms of aflatoxicosis gradually decreased after withdrawal of the aflatoxin-spiked rations.

Analysis of the tissues of chickens at 0 day withdrawal from the aflatoxin-spiked ration 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 highest levels of aflatoxins were present in the liver and kidneys. The mean value for the combined aflatoxins in all tissues was less than 3 ppb. The leg and breast muscles showed the lowest levels of residual aflatoxins with an average of less than 0.2 ppb. Except for the liver and kidneys, the levels of aflatoxins M_1 and M_2 were much lower than those of B_1 and B_2.

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

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