DEPOSITION AND CLEARANCE OF AFLATOXINS IN THE EGGS AND TISSUES OF LAYING HENS FED A CONTAMINATED DIET

By

Arlene Wolzak-Kappes

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ABSTRACT

DEPOSITION AND CLEARANCE OF AFLATOXINS
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A trial was conducted to determine the levels of aflatoxins deposited in the eggs and tissues of laying hens fed an aflatoxin-spiked diet and the time necessary to achieve clearance upon removal of the contaminated diet. Sixty-four hens were fed a ration containing 3,310 and 1,680 ug/kg of aflatoxins B_1 and B_2, respectively, for 4 weeks, while 16 control hens received an aflatoxin free ration. At the end of aflatoxin feeding, 16 treated and 8 control hens were sacrificed. The remaining treated and control hens were fed the unspiked ration for 32 days. Eight treated hens were sacrificed at 2, 4, 8, 16 and 24 days after withdrawal. All remaining hens were slaughtered at 32 days.

Aflatoxins caused a significant decrease in egg production and egg weights by the third and fourth weeks of feeding, respectively. Both returned to normal by the second week of withdrawal. At the end of aflatoxin feeding, livers, kidneys and spleens of treated hens were significantly larger, while ovaries and hearts were significantly smaller than controls. Livers were pale and hemorrhagic and ovaries had

only small ova (< 25 mm). After 32 days withdrawal all organ weights, except hearts, were not different from controls.

Transfer of aflatoxins to the eggs occurred rapidly with levels reaching a maximum after 4-5 days and remaining relatively constant throughout aflatoxin feeding. Combined residue levels in whole eggs were < 0.5 ug/kg. Levels of aflatoxins B_2, M_1 and M_2 were similar in yolk and albumen, while levels of B_1 and B_{2a} were higher in the yolk.

Aflatoxin residues were widely distributed in tissues, but comprised only a small fraction of total consumption. Aflatoxin B_{2a} was tentatively identified as the most abundant residue. Highest residue levels were detected in gizzard, kidneys and liver and amounted to combined levels of < 3 ug/kg in each.

Upon removal of the spiked diet, residues in eggs and tissues decreased rapidly. There were differences between tissues and between hens in the time required for clearance. AFB_2 was still detected in the liver of one hen 32 days after withdrawal. However, no detectable aflatoxins were present in eggs and most tissues after 4 and 8 days on the unspiked diet, respectively.

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INTRODUCTION

Aflatoxins are secondary metabolites of certain strains of Aspergillus flavus and A. parasiticus. A. flavus, a common food spoilage fungus, can grow in a variety of foods and feeds either before harvest or during storage. The environmental requirements for aflatoxin production are relatively non-specific and almost any natural substrate sustaining the growth of this mold is also suitable for aflatoxin production.

Experimentally, aflatoxins have been shown to be potent hepatocarcinogens in different animal species (Lancaster et al., 1961; Wogan, 1973), as well as mutagens (Lilly, 1965) and teratogens (Di Paolo et al., 1967). Epidemiological studies in Africa and Southeast Asia have shown a strong correlation between aflatoxin ingestion and primary liver cell cancer (Peers et al., 1976; Shank et al., 1972).

The biological effects of aflatoxins vary with dose, duration of exposure, species, age and nutritional status of the affected animal. Aflatoxins are metabolized by cytoplasmic or microsomal enzymes in the liver to more polar derivatives which can undergo conjugation with endogenous compounds. The increased polarity and water solubility of the conjugated aflatoxin metabolites facilitates the excretion of the ingested nonpolar aflatoxins.

Consumption of aflatoxin contaminated feeds is responsible for aflatoxicosis in farm animals. Exposure to dietary levels which fail to induce overt aflatoxicosis in food producing animals may constitute a special hazard to humans (Armbrecht, 1971). It has been demonstrated that part of the ingested aflatoxins may be retained as the original aflatoxin or as one or several metabolites, some of which possess toxic properties (Rodricks and Stoloff, 1977; Furtado et al., 1982). The levels of aflatoxin residues found in products of animals consuming contaminated feed are very low compared to those present in directly contaminated feed and foods. Nevertheless, the potential risk to humans of prolonged exposure to low levels of aflatoxins in animal products can not be overlooked considering that under such conditions several animal species develop liver tumors (Wogan et al., 1974; Sinnhuber et al., 1970).

The determination of aflatoxin residues in animal tissues is important in the evaluation of direct exposure of humans to these extremely toxic compounds. The assessment of low levels of aflatoxin residues deposited in eggs and tissues of hens consuming aflatoxin contaminated feed has been limited by the complex lipid-protein matrix of animal tissues and by the presence of many fluorescent compounds. These factors have also limited the determination of the length of time required to achieve egg and tissue clearance of aflatoxin residues transferred from the feed.

The present study was designed to: 1) Investigate the levels of aflatoxins and their metabolites carried over to eggs and those deposited in the tissues of laying hens fed an aflatoxin contaminated diet; 2) To determine the length of time required to achieve egg and tissue clearance after removal of aflatoxins from the diet of the laying hens; and 3) Finally to evaluate the effects of cooking on the aflatoxin levels in eggs.

REVIEW OF LITERATURE

Discovery of Aflatoxins

Toxic metabolites of fungal origin have affected the health of both man and animals for many centuries (Hesseltine, 1979). During the 1950s, "moldy feed toxicosis" was described as being a serious livestock problem (Forgacs and Carll, 1962).

Scientific interest on mycotoxins and the discovery of aflatoxins occurred in the early 1960s as a result of an acute outbreak of a lethal disease in turkey poults that caused an estimated loss of at least 100,000 birds in England. The disease, called Turkey X disease by Blount (1961), was characterized by anorexia, lethargy and muscular weakness. Postmortem examination indicated that the liver of the affected birds was pale, fatty and showed extensive necrosis, as well as bile duct proliferation. At the same time British farmers suffered losses of partridge and pheasant poults (Asplin and Carnaghan, 1961). In addition several early reports suggested that cattle (Loosmore and Markson, 1961), pigs (Loosmore and Harding, 1961), chickens and ducklings (Asplin and Carnaghan, 1961), and sheep were also affected.

In every case the outbreak occurred when animals had been fed rations containing groundnut meal imported from

Brazil. Although the Brazilian meal was the first implicated (Allcroft et al., 1961), certain batches from other countries including Nigeria, West Africa, Gambia, East Africa and India also showed similar toxic properties (Sargeant et al., 1961a). This distribution pattern suggested that moldy feed was involved. Sargeant et al. (1961b) identified the toxin producing mold in the feed as Aspergillus flavus. The name aflatoxin (from Aspergillus flavus toxin) was given to the toxic chemicals of A. flavus before it was recognized that it comprised a complex mixture of compounds (Nesbitt et al., 1962).

Characterization and Structure Elucidation

Sargeant et al. (1961c) isolated a toxic material that had a blue fluorescence under UV light from groundnut meal, and later described the original bioassay method using 1-day old ducklings (Sargeant et al., 1961b). Nesbitt et al. (1962) resolved the toxic material into two fluorescent spots under UV light by thin layer chromatography (TLC) using alumina plates. These spots differed in the color of their fluorescence. The faster moving spot fluoresced blue, while the slower one exhibited a green fluorescence. For convenience these authors referred to them as aflatoxin B_1 (blue fluorescence), and aflatoxin G_1 (green fluorescence).

The isolation and characterization of the four main aflatoxins was reported by Hartley et al. (1963). A crude mixture

of the aflatoxins was extracted from a sterilized groundnut meal, which had been inoculated with a toxigenic strain of A. flavus, and resolved into several fluorescent spots. The four main aflatoxins were separated on silica gel chromatoplates and named B_1, B_2, G_1 and G_2, based on the color of their fluorescence and on their relative chromatographic mobility.

Further research into the chemical nature of these compounds led to structure elucidation of aflatoxins B_1 and G_1 by Asao et al. (1965), and of B_2 by Chang et al. (1963). Van der Merwe et al. (1963) had shown that B_2 and G_2 were the dihydro-derivatives of B_1 and G_1, respectively. The laboratory synthesis of aflatoxin B_1 (AFB_1) (Büchi et al., 1967) and of B_2 (Roberts et al., 1968) confirmed the structures.

Allcroft and Carnaghan (1962, 1963) found that cows fed "cattle cake" rich in aflatoxins produced milk that was toxic to 1-day old ducklings. Analysis of the milk (de Iongh et al., 1964) showed only traces of AFB_1, but demonstrated the presence of a blue violet fluorescent component which had an R_f value considerably smaller than pure AFB_1. They called this new aflatoxin, "milk toxin".

The "milk toxin" was also found in rat milk (de Iongh et al., 1964) and in the liver of rats (Butler and Clifford, 1965) fed AFB_1, indicating that at least some of the ingested aflatoxin is converted in the liver into the "milk toxin". Allcroft et al. (1966) found "milk toxin" in the urine of

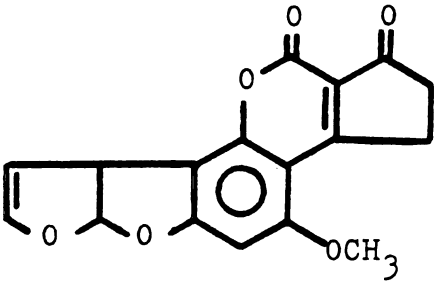
non-lactating sheep given a single dose of mixed aflatoxins (B_1, B_2, G_1 and G_2). As a result of the observation that the "milk toxin" was present in other tissues and fluids of animals ingesting aflatoxins, these authors suggested the trivial name of aflatoxin M for this new compound. Holzapfel et al. (1966) isolated aflatoxin M from the urine of adult sheep given an i.p. dose of mixed aflatoxins, and were able to resolve it into two components, which they designated as aflatoxins M_1 and M_2 on the basis of chromatographic mobility. Chemical and spectroscopic analysis led to structure elucidation of aflatoxin M_1 (AFM_1) as the 4-hydroxy-derivative of AFB_1 and of M_2 as the dihydro-derivative of AFM_1 (Holzapfel et al., 1966).

Dutton and Heathcote (1966) isolated two additional aflatoxins from cultures of A. flavus, one of which fluoresced blue and the other green. These compounds were identified by spectroscopic analysis and selected chemical reactions as the 2-hydroxy-derivatives of aflatoxins B_2 and G_2, and were named aflatoxins B_{2a} and G_{2a}, respectively. These two aflatoxins are relatively non toxic to ducklings (Dutton and Heathcote, 1968).

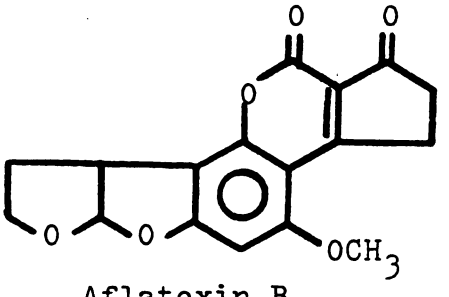
Chemistry of Aflatoxins

The chemical structures of the above mentioned aflatoxins are shown in Figures 1 and 2. Aflatoxins are highly substituted coumarins containing a fused dihydrofuran moiety. In addition this basic nucleus is attached to a pentenone ring

as in aflatoxins B_1, B_2, M_1 and M_2, or to a 6-membered lactone as in the G series (Jones, 1978). In addition, the presence of other substituents, particularly hydroxyl groups, the reduction of the ketone function in the cyclopentanone ring and the reduction of the 2,3-double bond, give rise to the different forms of aflatoxin. As many as 17 different compounds, all designated as aflatoxins have been isolated (WHO, 1979). These are mainly products of aflatoxin metabolism by animals consuming contaminated feed and will be discussed later herein.



Aflatoxin B_1



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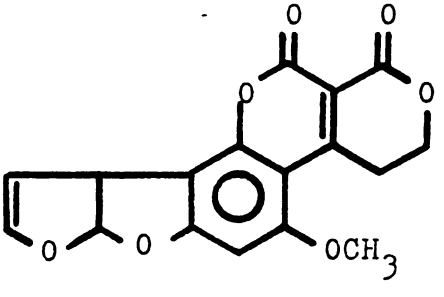
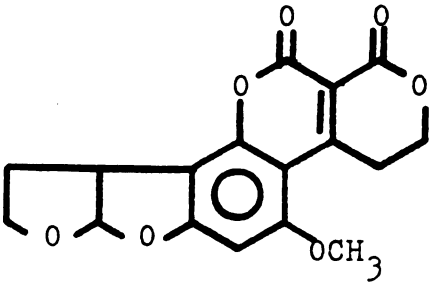


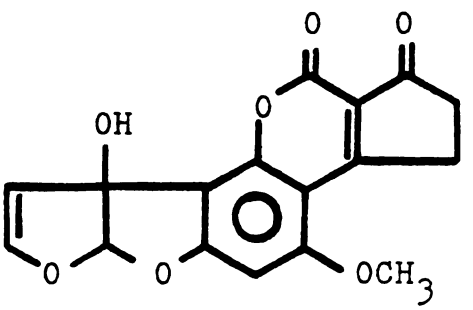
Aflatoxin G_1



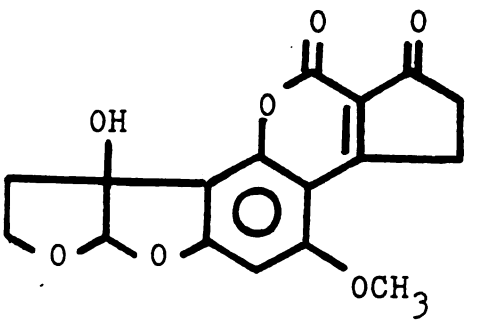
Aflatoxin G_2

Figure 1 - Structures of the major aflatoxins produced by Aspergillus fungi.

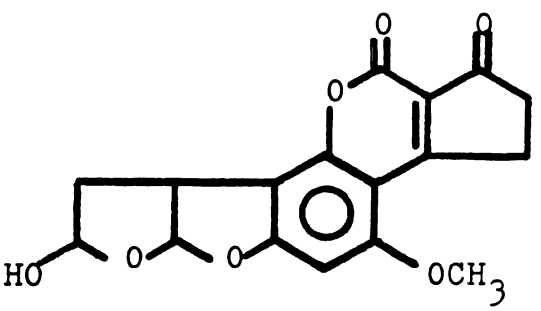
Aflatoxins are soluble in moderately polar solvents like methanol and chloroform, but only slightly soluble in water (WHO, 1979). The toxins absorb UV light (362-363 nm) with extinction coefficients varying from 16,100 for aflatoxins B_2 and G_1 to 21,800 for AFB_1. Fluorescence emission is at 425 nm for aflatoxins B_1 and B_2, and at 450 nm for aflatoxins G_1 and G_2 (Wilson and Hayes, 1973).



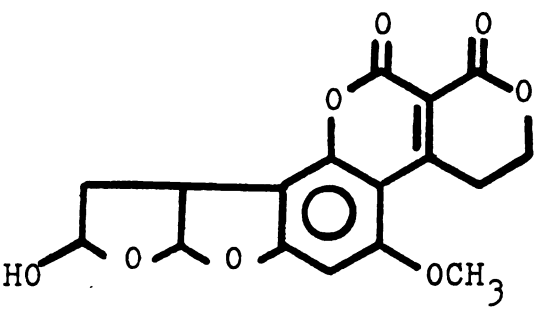
Aflatoxin M_1



Aflatoxin M_2



Aflatoxin B_{2a}



Aflatoxin G_{2a}

Figure 2 - Structures of minor aflatoxins produced by <u>Aspergillus</u> fungi.

Occurrence of Aflatoxins

The presence of mycotoxins in foodstuffs can result from either direct or indirect contamination (Jarvis, 1975). Direct contamination occurs from the synthesis of mycotoxins by specific strains of fungi and is influenced by environmental factors such as humidity and temperature. Consequently, mycotoxin contamination of foodstuffs varies with geographical location, production and storage practices, as well as with the type of food since some food commodities are more suitable substrates for fungal growth than others (Ciegler, 1978; Frazier and Westhoff, 1978). Indirect routes of contamination are the use of aflatoxin contaminated food additives or the transfer of mycotoxin residues to animal products resulting from the use of mycotoxin contaminated feedstuffs (Jarvis, 1975).

Aflatoxins are produced by some strains of *Aspergillus flavus* and *A. parasiticus* on many feed or food ingredients whenever the moisture content reaches 14 to 25% in a temperature range of 25 to 45°C, if an adequate level of zinc is present (Edds, 1979). The water content of the substrate influences aflatoxin production mainly by its effect on the growth of *A. flavus* (Davis and Diener, 1970). Davis and Diener (1970) have shown that the lower moisture limit for the growth and production of aflatoxins by *A. flavus* is such that the moisture content is in equilibrium with a relative humidity of 85%.

Most of the toxigenic strains of A. flavus which contaminate plant products mainly synthesize AFB_1, followed by AFG_1, while AFB_2 and AFG_2 occur in lower concentrations (Diener and Davis, 1966). The ratio of AFB_1 to AFG_1 varies directly with the storage temperature and with the strain (Schroeder and Ashworth, 1966). Other aflatoxins may be produced in minor amounts by cultures of A. flavus and A. parasiticus, including aflatoxins M_1, M_2, B_{2a}, G_{2a}, GM_1 and aflatoxicol.

Stoloff (1977) has reviewed the occurrence of aflatoxins in foods and feedstuffs. On the basis of the surveys carried out, he concluded that corn is the dietary staple in which aflatoxins are most likely to be encountered. Groundnuts and other oilseeds, such as cottonseed, Brazil nuts and pistachio nuts, bear a considerable risk of contamination. In the United States, the southeast has the greatest risk of aflatoxin contamination of corn, while in some underdeveloped countries a major exposure can occur through unrefrigerated prepared foods at the household level (Stoloff, 1977).

Biological Activity of Aflatoxins

Hayes (1978) has classified the effects of aflatoxins into two general groups: 1) short term effects including acute toxicity, and 2) long term effects, which include chronic toxicity.

The suscpetibility of animals to both acute and chronic toxicity from aflatoxins depends on a number of factors. These include host factors, such as species (Ciegler, 1975),

sex (Purchase et al., 1973), age (Newberne and Butler, 1969), nutritional status (Hamilton, 1977), and a number of external factors including dose (Wogan and Newberne, 1967), pre-xenobiotic exposure (Wong et al., 1981; McGrew et al., 1982), duration of exposure (Newberne and Butler, 1969), route of administration (Butler, 1964) and environmental factors (Wyatt et al., 1977).

Up to the present, no animal species has been found to be resistant to the lethal effects of aflatoxins (Wogan, 1977). Busby and Wogan (1981) have stated that acute toxicity of AFB_1 to mammals and birds may vary over two orders of magnitude between a sensitive species like the rabbit (LD_{50} = 0.3 mg/kg) and an insensitive species like the mouse (LD_{50}, approximately 40 mg/kg).

AFB_1 is the most potent of the 12 to 15 analogs produced by A. flavus and A. parasiticus (Hayes, 1980). It is also the most commonly occurring member of the group, thus most studies dealing with aflatoxins have concentrated on AFB_1 (Applebaum et al., 1982). Small changes in chemical structure of aflatoxins markedly modify their biological activity. Thus, the LD_{50} for 1-day-old ducklings of aflatoxins B_2 and G_2 (the 2,3-dihydro-derivatives of aflatoxins B_1 and G_1, respectively) were 4 to 5 times higher than that of aflatoxins B_1 and G_1 (Carnaghan et al., 1963).

The liver is the main target organ for aflatoxin poisoning. Hepatic lesions caused by aflatoxins include proliferation of the bile duct (Carnaghan et al., 1966; Krogh et al.,

1973), central lobular necrosis, fatty infiltration (Carnaghan et al., 1966) and primary hepatocarcinoma (Lancaster et al., 1961). Some studies, however, have shown that aflatoxin B_1, and especially AFG_1, can cause necrosis of the kidney tubules and hemorrhagic lesions in other organs such as the lungs and adrenal glands (Butler and Lijinsky, 1970; Epstein et al., 1969; Wogan, 1973).

Experiments by Lancaster et al. (1961) provided the earliest evidence of the carcinogenic properties of aflatoxins. These researchers fed rats a diet containing 20% peanut meal that had been identified as the cause of poisoning in poultry flocks. No signs of acute toxicity were seen in the rats, but after 6 months 9 out of 11 rats developed multiple liver tumors and 2 of them had lung tumors. Early studies of tumor induction in rats using approximately 10 ug of AFB_1 per day led Butler and Clifford (1965) to conclude that AFB_1 is the most potent natural carcinogen. According to Applebaum et al. (1982), this conclusion still holds true.

The other aflatoxins are also considered to be carcinogenic, although they are far less potent (Butler and Clifford, 1965). The variation in potency appears to be related to differences in chemical structure (Wogan et al., 1971). Wong and Hsieh (1976) have concluded that the 2,3-double bond present in aflatoxins B_1 and G_1, but not in aflatoxins B_2 and G_2, is involved in the carcinogenicity and mutagenicity of aflatoxins. The potency of AFM_1 (the 4-hydroxy-derivative of AFB_1) in inducing liver tumors in the rainbow trout was

only one-third of that of AFB_1 (Sinnhuber et al., 1970). This is in contrast to its acute toxicity to 1-day-old ducklings, which was qualitatively and quantitatively similar to that of AFB_1 (Holzapfel et al., 1966; Purchase, 1967).

The mutagenicity of aflatoxins was recognized much later following the development of reliable methods for such tests (Ames et al., 1973). Mc Cann et al. (1975) noted that on a molar basis, AFB_1 was the second most potent mutagen of 300 compounds tested by the Ames test. Later, Wong and Hsieh (1976) screened aflatoxins and their animal biotransformation products for carcinogenic potential. They found that all the metabolites tested were less active than AFB_1. The most mutagenic metabolite tested was aflatoxicol, which was only 22.8% as mutagenic as AFB_1, and it was followed by aflatoxins G_1 and M_1, which had only about 3% of the potency of AFB_1. These workers concluded that the relative in vitro mutagenicity of aflatoxins correlated qualitatively with in vivo carcinogenic data.

Various studies on the metabolism and mode of action of aflatoxins have provided evidence that they require metabolic activation to elicit mutagenesis (Ames et al., 1973; Ong, 1975). Wong and Hsieh (1976) demonstrated that neither aflatoxicol nor aflatoxins M_1, Q_1 or B_{2a} possess mutagenic activity in the absence of the activation factor from rat liver preparations. This indicated that none of these metabolites are the ultimate mutagenic/carcinogenic species.

The cytotoxicity and genotoxicity of aflatoxins B_1 and M_1 in primary cultures of adult rat hepatocytes were compared by Green et al. (1982). AFB_1 was a more potent genotoxic and cytotoxic agent then AFM_1, although these authors pointed out that AFM_1 is still active at relatively low doses. Thus, they concluded that AFM_1 is probably a potent hepatocarcinogen in vivo.

AFB_1 has been found to be a potent teratogen in hamsters (Elis and Di Paolo, 1967), chickens (Bassir and Adekunle, 1970) and Japanese killifish (Llewellyn et al., 1977). AFB_1 has also been shown to have marked suppressive effects on the development of acquired immunity through its action on the cell mediated immune system (Pier, 1981).

Aflatoxicosis in Man

Human exposure to aflatoxins can occur directly by ingestion of aflatoxin contaminated foods like corn and nuts, or indirectly by consuming foods of animal origin (milk, eggs or meat), which contain aflatoxins derived from animals that consume contaminated feed (Hayes, 1980).

A few cases of suspected aflatoxin poisoning in Asia and Africa have been reported (Krishnamachari et al., 1975; Campbell and Stoloff, 1974). Information available is limited since contamination is most frequent in areas where medical services are poor and many cases go unnoticed. In 1974, aflatoxins were implicated in an outbreak of hepatitis in

India (Krishnamachari et al., 1975) during which 106 out of 397 patients died. Corn samples were found to contain between 6.25 and 15.6 mg/kg of aflatoxins.

It has also been suggested that the condition known as Indian childhood cirrhosis might be due in part to aflatoxin poisoning (Amla et al., 1971). Liver biopsies of Indian children, who accidentally ingested large quantities of protein rich peanut meal contaminated with aflatoxins, showed the characteristic bile duct proliferation caused by aflatoxins. The urine of some of these children also contained aflatoxins.

It has been suggested that other human diseases could be related to aflatoxin consumption, including: 1) encephalopathy and fatty degeneration of the viscera (EFDV) also known as Reye's syndrome (Ryan et al., 1979), and 2) primary hepatic carcinoma (Oettle, 1964). EFDV has been recognized as a major cause of morbidity and mortality among infants since it was first described (Reye et al., 1963). It may affect children from only a few months of age up to adolescence. Symptoms progress from a mild viral illness with vomiting and abdominal pain to cerebral involvement with coma (Hayes, 1980).

Reye's syndrome is endemic in northeast Thailand, an area of high rice and low protein consumption (Olson et al., 1970). A study of aflatoxin contamination of Thai foods indicates seasonal and geographical patterns coincident with EFDV (Bourgeouis et al., 1969). Traces of AFB_1 have been found in the body tissues of many Thai children, but in a

series of EFDV patients, 22 had high concentrations in their tissues or gastrointestinal contents (Shank et al., 1971). This suggests that there is probably a chronic, low level of ingestion of the toxin throughout the population in this region. Cases of Reye's syndrome in association with aflatoxin contamination have also been reported in the United States (Chaves-Carballo et al., 1976; Ryan et al., 1979), Czechoslovakia (Dvorackova et al., 1977) and New Zealand (Becroft and Webster, 1972).

The probable involvement of mycotoxins in the genesis of human tumors was first suggested by Oettle (1964) in an extensive review of cancer in Africa. He noted that in South Africa hepatoma was concentrated among the Negroid races and that cirrhosis was a common precursor. A high incidence of human liver cancer occurred in areas of high humidity and temperature and in relatively primitive populations. Fungi require high temperatures and humidity to grow, and will grow in food stored under such primitive conditions (Hayes, 1980). Therefore, the ingestion of food contaminated with mycotoxins could provide a reasonable explanation for the high incidence of liver cancer in certain areas of Africa, the Far East and South America.

A number of studies in various regions of Africa and Asia have related the ingestion of aflatoxins to the incidence of hepatoma in man. Evidence for this association has been suggested in Uganda (Alpert et al., 1971), Taiwan (Tung and Ling, 1968), Kenya (Peers and Linsell, 1973), Swaziland

(Peers et al., 1976), Mozambique (Van Rensburg et al., 1974), the Philippines (Campbell and Salamat, 1971), Southeast Asia (Shank et al., 1972), Senegal (Payet et al., 1966) and India (Amla et al., 1971). Van Rensburg et al. (1974) summarized some epidemiological evidence on the incidence of liver carcinoma and probable aflatoxin contamination of the food supply, which is presented in the following table:

Summary of the Available Data on Aflatoxin Ingestion Levels and the Incidence of Liver Cancer

Country - Area	Estimated aflatoxin intake, ng/kg/day	Incidence of liver cancer cases, $/10^5/yr$[a]
Kenya - high altitude	3.5	0.7
Thailand - Songkhla	5.0	2.0
Kenya-middle altitude	5.8	2.9
Kenya-low altitude	10.0	4.2
Thailand-Ratburi	45.0	6.0
Mozambique-Inhambane	222.4	25.4

a) Cancer rate per 100,000 inhabitants per year

No such studies exist for any United States population, however, livers from 3 of 6 patients who died of primary liver cancer contained detectable levels of AFB_1 (Hayes, 1980). The validity of the apparent link between consumption of aflatoxin contaminated foods and primary liver cancer in humans has

been recently questioned by Stoloff (Anonymous, 1984). He noted that the small excess of liver cancers in white males from the Southeast area of the United States (6-10%), where contamination of foods and feeds with aflatoxins is more common, over white males from the North and West is far from the big difference that would be expected based on the probable greater consumption of aflatoxin contaminated foods by those living in the Southeast. With respect to the evidence gathered in Africa and Asia, Stoloff (Anonymous, 1984) favors the hypothesis that chronic infection by hepatitis B virus is etiologically related to primary liver cancer. It will probably be necessary to look at the data again and continue studies to establish if a real link exists between aflatoxins and human liver carcinoma.

Metabolism and Toxicity of Aflatoxins

Animals and man are exposed to a variety of naturally occurring foreign compounds or xenobiotics (Linsell, 1977). Most of these compounds are nonpolar and difficult to excrete (Klaassen, 1980), and thus require metabolic transformation. The essential effects of xenobiotic metabolism are detoxification and elimination, involving the transformation of lipophilic compounds into more polar, water soluble metabolites that can be more easily excreted via bile or urine (Park, 1982). However, in some cases, metabolic transformation of a foreign compound enhances its reactivity toward important

biological molecules and results in increased toxicity (Swick, 1984). Thus, Hayes (1975) proposed the use of the term biotransformation for all of the metabolic reactions of xenobiotics.

The rate of metabolism of a xenobiotic strongly influences the intensity of its effect (Swick, 1984). Although many tissues possess xenobiotic metabolizing capacity (Blumberg, 1978; Guengerich, 1977), the liver is the most active site for most xenobiotics (Park, 1982). The reactions involved in the metabolism of xenobiotics are catalyzed by relatively non-specific enzymes and lead to a variety of different metabolites (Gillette, 1979). Williams (1959) pointed out that these reactions can be classified into two general groups. Phase I biotransformations include those reactions that convert one functional group into another (e.g., oxidation), those that split neutral compounds to form a fragment having polar groups (e.g., hydrolysis of esters or amides), and those which introduce polar groups into nonpolar compounds (e.g., hydroxylation). Phase II biotransformations involve the conjugation of polar groups of foreign compounds with glucuronate, sulfate, glycine, glutathione, methyl groups and water.

The most important enzymes involved in Phase I reactions are localized in the hepatic endoplasmic reticulum, a network of intracellular tubules which on homogenization disrupts to form particles called microsomes (Gillette, 1979). The microsomal enzymes require both NADPH and oxygen (Gillette, 1966),

thus are named mixed-function oxidases (MFO). The enzyme systems, which are sometimes referred to as cytochrome P-450 monooxygenases, are composed of two enzymes: NADPH-cytochrome P-450 reductase and a heme-containing enzyme, cytochrome P-450 (White and Coon, 1980). There are multiple forms of the terminal enzyme cytochrome P-450 both in animals and man, which have selective but not specific substrate requirements (Park, 1982). The evidence for this has been recently reviewed by Lu and West (1980).

AFB_1 is metabolized by the hepatic microsomal mixed-function oxidase system to form a group of hydroxylated derivatives (Campbell and Hayes, 1976), which possess distinctly different toxic properties (Wong and Hsieh, 1976). AFB_1 can also be reduced by a cytoplasmic reductase to aflatoxicol (Wong and Hsieh, 1978). The qualitative and quantitative composition of the aflatoxin metabolites is apparently species specific (Masri et al., 1974a) and can be correlated with differences in susceptibility among animals (Hsieh et al., 1977). The pathways for formation of the major metabolites of AFB_1 are shown in Figure 3.

Aflatoxin M_1, the 4-hydroxy-derivative of AFB_1, was the first metabolite identified, and was so named because of its presence in milk where it occurred in the protein fraction (Allcroft and Carnaghan, 1963). Secretion of AFM_1 into milk as a percentage of aflatoxin in the diet varies from less than 1% to 3% (Polan et al., 1974). Microsomal enzymes have been suggested as being responsible for AFM_1 production via

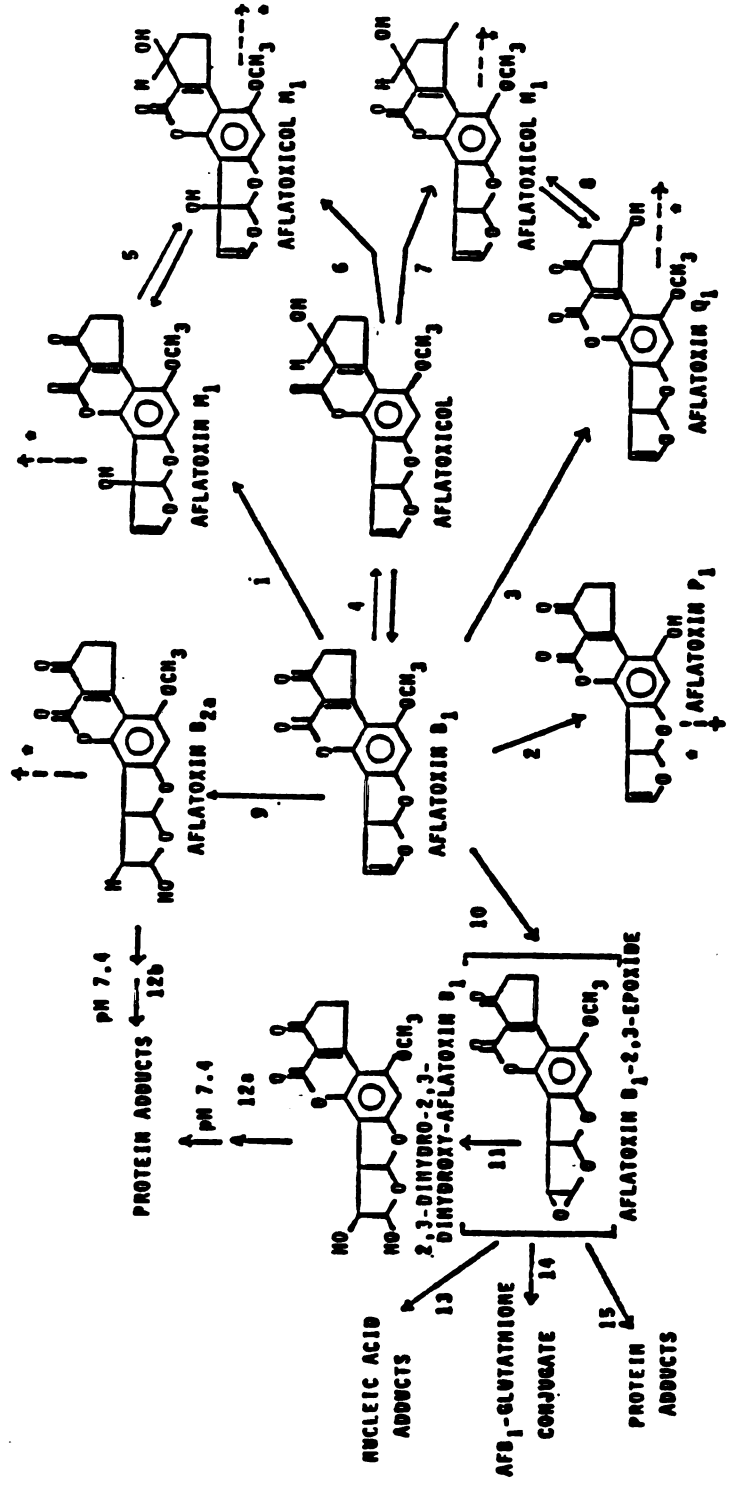


Figure 3 - Transformation of Aflatoxin B_1 to various metabolites

* = known or potential glucuronide or sulfate conjugates.

4-hydroxylation of AFB_1 (Portman et al., 1968) (Figure 3, pathway 1). AFM_1 is produced in vitro from AFB_1 by liver microsomes from a variety of species including human beings (Masri et al., 1974a; Campbell et al., 1970). AFM_1 is excreted into the urine of AFB_1-treated sheep (Allcroft et al., 1966) and monkeys (Dalezios and Wogan, 1972). The glucuronide of AFM_1 was reported in the tissue and excreta of chickens (Mabee and Chipley, 1973a). Several reports suggest that AFM_1 is less toxic (Garner et al., 1972) and less carcinogenic (Sinnhuber et al., 1970) than AFB_1, although on administration to ducklings it induces liver lesions identical to those caused by AFB_1 (Allcroft and Carnaghan, 1963).

Aflatoxin P_1 is produced by O-demethylation of AFB_1 (Figure 3, pathway 2). The formation of this metabolite was suspected by Shank and Wogan (1965) when a significant proportion of the radioactivity administered as ^{14}C-methoxy-labeled AFB_1 to rats appeared in the respired CO_2. Later, Dalezios et al. (1971) found that AFP_1 is the major excretory product in the urine of AFB_1-treated monkeys where it was present as glucuronide or sulfate conjugates. The overall role of this metabolite in aflatoxin toxicity has not been fully elucidated (Swick, 1984). Hydroxylation of the molecule allows conjugation reactions to occur, thus rendering it excretable. However, the molecule retains both the 2,3-unsaturated furan and the lactone portion of the coumarin, generally assumed to be required for aflatoxin toxicity (Wong and Hsieh, 1976). Wong and Hsieh (1980) have found that species, which are relatively

resistant to the carcinogenic effect of AFB_1, are more reactive in the in vivo conversion of AFB_1 to AFP_1 and water soluble conjugates.

Aflatoxin Q_1 is produced by hydroxylation of the carbon atom at the β-position relative to the carbonyl group of the pentenone ring (Masri et al., 1974b). AFQ_1 represents one-third to one-half of the metabolites produced in vivo from AFB_1 by human (Büchi et al., 1974) or monkey liver (Masri et al., 1974b). AFQ_1 appears to be much less toxic than AFB_1 (Hsieh et al., 1974) suggesting that its formation is a mechanism of detoxification in primates (Figure 3, pathway 3).

Aflatoxicol (AFL) is the secondary alcohol formed by the reduction of the carbonyl group in the cyclopentenone moiety of AFB_1 (Figure 3, pathway 4). This reaction is catalyzed by NADPH-dependent cytoplasmic enzymes, which also reduce the ketone function of aflatoxin B_2 to form the corresponding dihydro-aflatoxicol (Patterson and Roberts, 1971). Patterson and Roberts (1972) observed that rabbit and bird liver homogenates were active aflatoxicol producers, whereas, rodent and sheep preparations were inactive. The susceptibility of different species to the carcinogenic effects of AFB_1 has been associated with their ability to produce aflatoxicol (Hsieh et al., 1977). Aflatoxicol is less toxic and less mutagenic than AFB_1 (Wong and Hsieh, 1976; Campbell and Hayes, 1976), although it is the most mutagenic of the AFB_1 metabolites (Wong and Hsieh, 1976).

Aflatoxicol is readily converted to AFB_1 by a microsomal

enzyme system that does not appear to involve cytochrome P-450 (Salhab and Edwards, 1977). The reversibility of this enzymatic reaction led to the theory that aflatoxicol may serve as an intracellular reservoir of AFB_1, prolonging cellular exposure to AFB_1 and thereby enhancing its carcinogenic effect (Wong and Hsieh, 1978). Recently, Kumagai et al. (1983) found that the interconversion of AFB_1 and aflatoxicol occurred in red blood cell suspensions, with resistant species having the highest rate of conversion. This finding needs further clarification.

Aflatoxicol M_1 can be formed through reduction of the carbonyl group in the pentenone ring of AFM_1 by a cytosolic enzyme (Figure 3, pathway 5), or by the microsomal mixed-function oxidation of aflatoxicol (Figure 3, pathway 6) (Salhab et al., 1977).

Aflatoxicol H_1 is a dihydroxyl-derivative of AFB_1 with substitutions at the cyclopentenone carbonyl and at the β-carbon (Figure 3, pathway 7). Salhab and Hsieh (1975) reported this compound was formed from AFB_1 when both the microsomes and a soluble enzyme preparation of either human or monkey liver were used. This compound may arise from the reduction of AFQ_1 (Figure 3, pathway 8), or by oxidation of aflatoxicol (Figure 3, pathway 7). However, the exact mechanism operating in the formation of aflatoxicol H_1 is not known (Salhab and Edwards, 1977).

Aflatoxin B_{2a} is the hemiacetal derivative of AFB_1 (Figure 3, pathway 9). Pohland et al. (1968) reported formation

of this compound by the acid catalyzed addition of water to the vinyl double bond in the terminal furan ring of AFB_1. AFB_{2a} is considerably less toxic (Pohland et al., 1968) and less mutagenic (Wong and Hsieh, 1976) than AFB_1. Patterson and Roberts (1970) found that liver microsomal preparations of several mammalian and avian species metabolized AFB_1 into a metabolite with spectral characteristics similar to those of AFB_{2a}. This compound was found to be unstable under physiological conditions and was assumed to bind to protein (Figure 3, pathway 12b). These conditions favor the dialdehyde-phenolate conversion. The nature of the reaction was presumed to be Schiff base formation between the aldehyde groups of ring-opened AFB_{2a} and the free amino groups of protein and amino acids (Gurtoo and Dahms, 1974).

Considerable controversy has arisen recently regarding AFB_{2a}. Lin et al. (1978) were not able to demonstrate the formation of AFB_{2a} after incubation of AFB_1 with rat or hamster liver microsomes. They suggested that formation of the AFB_1-dihydrodiol (Figure 3, pathways 10 and 11), which has identical spectral properties to AFB_{2a} and reacts similarly with proteins, may be the same protein binding metabolite previously reported. Furthermore, Neal et al. (1981) pointed out that earlier reports on the production of AFB_{2a} (Patterson and Roberts, 1970; Gurtoo and Dahms, 1974) were erroneous, being based on the wrong identification of AFB_1-dihydrodiol as AFB_{2a} due to the similarity of their ultraviolet spectral data (Swenson et al., 1973). The dihydrodiol of AFB_1

(2,3-dihydro-2,3-dihydroxy-AFB_1) showed little or no toxic, carcinogenic or mutagenic activity (Swenson et al., 1975; Coles et al., 1980).

Bioactivation of AFB_1 to the highly reactive and labile intermediate, aflatoxin B_1-2,3-epoxide (Figure 3, pathway 10) has been proposed as the ultimate mechanism by which mutagenesis and carcinogenesis are manifested (Garner et al., 1972). Schoental (1970) first postulated that formation of an epoxide intermediate at the 2,3-double bond might account for the toxicity of AFB_1. Later, Garner and Hanson (1971) and Garner et al. (1972) demonstrated that incubation of rat liver microsomes with AFB_1 and a NADPH-generating system produced a metabolite that was lethal to certain bacteria. No lethal effect was observed in the absence of mixed-function oxidases and toxicity was reduced on addition of either DNA or RNA. This suggested that the same toxic metabolite reacted with nucleic acids.

Strong evidence for the formation of aflatoxin B_1-2,3-epoxide was presented by Swenson et al. (1973), who isolated the dihydrodiol after mild hydrolysis of the RNA-aflatoxin B_1 adduct formed by microsomal oxidation of AFB_1 in the presence of RNA. Later, Swenson et al. (1974) found that acid hydrolysis of the DNA- and RNA-bound derivatives of AFB_1 formed in vivo in rat liver, released a major share of bound aflatoxin as the dihydrodiol (Figure 3, pathway 12a). Although binding of AFB_1 to liver proteins was observed, the level was only 4 to 7% of that observed with nucleic acids.

Swenson et al. (1975) suggested that aflatoxin B_1-2,3-epoxide formed an electrophilic carbonium ion at carbon 2, which could react with nucleophilic nitrogen and oxygen atoms in the nucleic acids to yield glycoside-like linkages susceptible to acid hydrolysis. Since the extreme reactivity of the AFB_1-2,3-epoxide precluded its isolation and synthesis, Swenson et al. (1975) synthesized the more stable AFB_1-2,3-dichloride as a model of the epoxide. These workers showed that the dichloride was a more potent mutagen and carcinogen than AFB_1, and that it reacted in vitro with nucleophiles in the same manner as was expected for the 2,3-epoxide. The model compound formed covalent adducts with DNA, RNA, protein and amino acids by reaction with their nucleophilic centers. It reacted more readily with polynucleotides than with mononucleotides and especially with polyguanylic acid. Further evidence of the greater reactivity of the epoxide toward the guanine residues in nucleic acids was reported by Essigman et al. (1977), who isolated and identified 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 as the major adduct formed from AFB_1 in vitro, accounting for approximately 90% of the aflatoxin bound to DNA (Figure 3, pathway 13). Lin et al. (1977) and Croy et al. (1978) have established that this compound is also the major adduct formed from AFB_1 by rat liver in vivo.

The relative biological hazard posed by the formation of aflatoxin adducts with different classes of cellular macromolecules is unknown (Busby and Wogan, 1981). However, in studies comparing in vivo macromolecular binding of the toxic

AFB_1 and the relatively nontoxic AFB_2 to rat liver DNA, rRNA and protein, Swenson et al. (1977) noted that AFB_2 bound to nucleic acids at approximately 1% the level of AFB_1. On the other hand, protein binding of AFB_2 was 35 to 70% of that observed with AFB_1. This suggested that aflatoxin-protein adducts are relatively unimportant in the manifestation of toxic and carcinogenic effects.

Raj et al. (1975) detected a polar fluorescent ninhydrin-positive compound on in vitro incubation of AFB_1 with rat liver microsomes and reduced glutathione (GSH). Later, Degen and Neumann (1978) isolated the glutathione conjugate as the major component in the bile of rats dosed with ^{14}C-AFB_1, and identified it as 2,3-dihydro-2-(S-glutathionyl)-3-hydroxy-aflatoxin B_1. They also reported its formation in vitro on incubation of rat liver postmitochondrial supernatant with AFB_1 and ^{3}H-GSH. These results provided further evidence for the existence of AFB_1-2,3-epoxide and also suggested an important metabolic role for GSH in protection from aflatoxin toxicity. Earlier, Mgbodile et al. (1975) had shown that depletion of the GSH levels in the liver of rats made them more susceptible to the toxic effects of AFB_1. Similarly, Allen-Hoffmann and Campbell (1977) demonstrated that binding of AFB_1 metabolites to DNA is inversely related to the hepatic GSH levels.

Metabolism plays a prominent role in determining the toxicity of AFB_1 (Campbell and Hayes, 1976). Various pathways are known to occur in the hepatocyte. Hsieh et al. (1977) indicated that the ultimate or net toxicity is determined by

the partitioning of the toxin among the various pathways. Using hepatic enzyme preparations in the study of in vitro metabolism of AFB_1, they revealed correlations between: 1) the susceptibility of a species to acute effects of AFB_1 and both their ability to reduce AFB_1 to aflatoxicol and their overall rate of metabolism, and, 2) the susceptibility of a species to the carcinogenic effects of AFB_1 and their relative activities in forming aflatoxicol, AFQ_1 and conjugated metabolites. Later, Wong and Hsieh (1980) compared in vivo metabolism and toxicokinetics of AFB_1 in monkey, rat and mouse. They noted that species susceptibility to the acute and carcinogenic effects of AFB_1 is closely related to the rate and extent of tissue penetration, distribution, metabolism and elimination. Species relatively sensitive to the acute effects of AFB_1 showed a higher volume of distribution, a higher equilibrium transfer rate constant, higher levels of total aflatoxins in liver and plasma, and a longer plasma biological half-life of AFB_1. Species relatively resistant to the carcinogenic effect of AFB_1 were more active in the in vivo conversion of AFB_1 to AFP_1 and water soluble metabolites.

Wei et al. (1978) compared the mutagenic activities of water soluble AFB_1 conjugates before and after β-glucuronidase and sulfatase hydrolysis. The conjugates were obtained from urine of rhesus monkeys and primary hepatocyte cultures prepared from adult rats and mice. The water soluble conjugates were found to have no or very low mutagenic activity as measured by the Ames test. After enzymatic hydrolysis, however, the

activity increased several fold. It was also suggested that the intestinal microflora, which can cleave these conjugates, may play an important role determining the toxic effect of aflatoxins. The genotoxicity of various aflatoxin residue fractions in the livers of rats fed ^{14}C-AFB_1 was evaluated by Jaggi et al. (1980). They isolated the macromolecules and water soluble conjugates from the liver, administered these fractions orally to other rats and determined the amount of radioactivity incorporated into liver DNA of the second group of rats. They concluded that macromolecule-bound AFB_1 derivatives are at least 4,000 times less active than AFB_1 in terms of covalent binding to rat liver DNA, and that the water soluble conjugates are at least 100 times less potent than AFB_1 itself.

Aflatoxin B_2, the dihydro-derivative of AFB_1, occurs naturally as a mold metabolite (Hartley et al., 1963). Much less is known about the metabolism of AFB_2, although some metabolites have been identified (Figure 4). Roebuck et al. (1978) compared the in vitro metabolism of AFB_2 by postmitochondrial supernatant fractions of duck, rat, mouse and human livers. Duck liver had the highest activity and produced AFB_1, aflatoxicol 1, aflatoxicol 2, AFM_1 and AFM_2. Rat, mouse and human hepatic preparations produced no detectable AFB_1, and only produced small amounts of compounds thought to be aflatoxins Q_2 and P_2 (the 2,3-dihydro-derivatives of aflatoxins Q_1 and P_1, respectively). Aflatoxin B_{2a} was not produced readily from AFB_2 on incubation with rat liver microsomes (Schabort and Steyn, 1969). However, it appears that AFB_{2a}

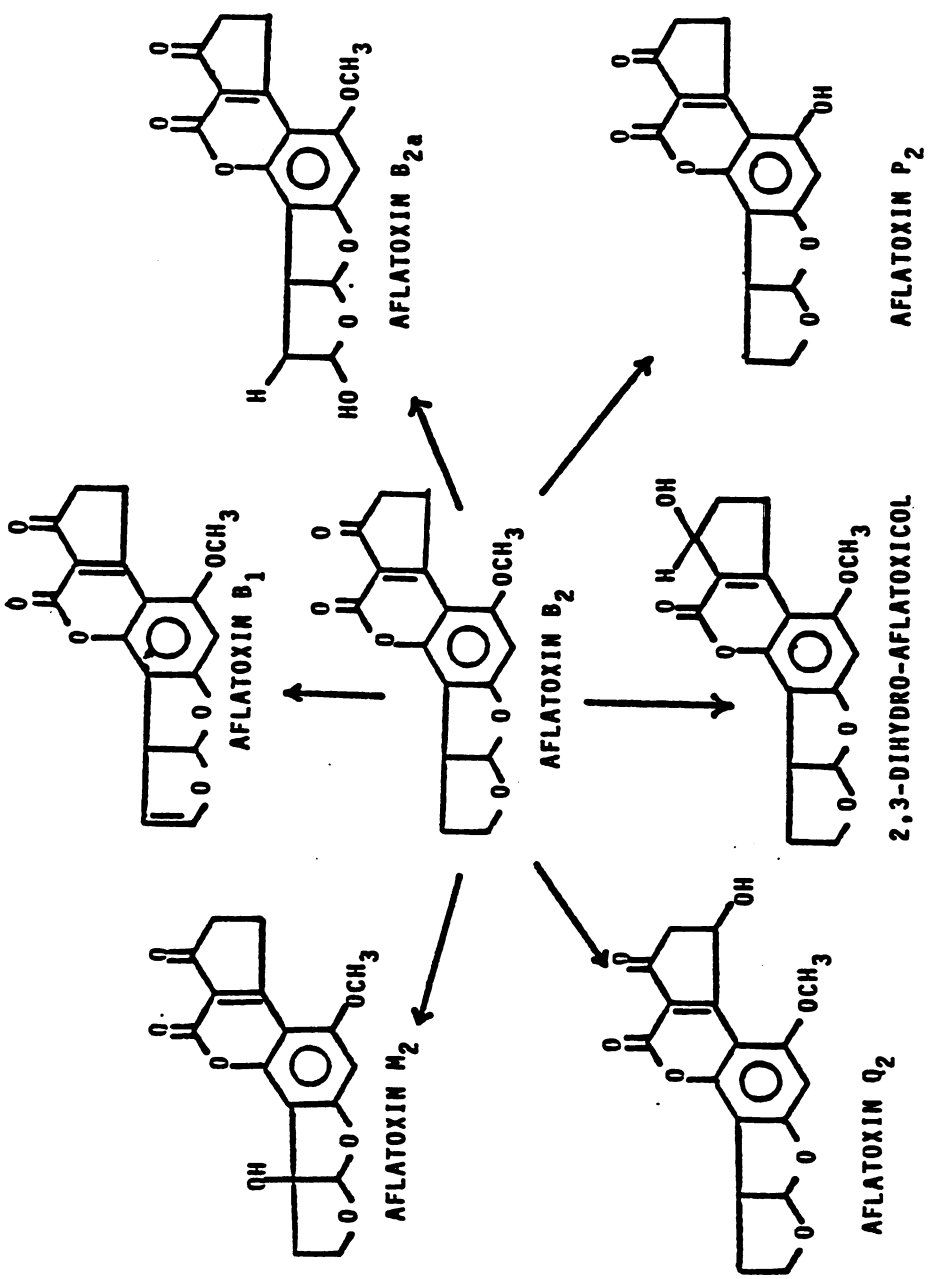


Figure 4 - Metabolic transformations of Aflatoxin B_2

is a major urinary metabolite of AFB_2 in rats, where it accounted for about 8% of the injected dose of AFB_2 (Dann et al., 1972).

AFB_2 is less toxic (Carnaghan et al., 1963) and less mutagenic (Wong and Hsieh, 1976) than AFB_1. The weak toxicity of AFB_2 in certain species appears to be related to the ability of certain animals to reduce AFB_2 to the more potent AFB_1 (Swenson et al., 1975; Roebuck et al., 1978).

The comparative data on the biological activity of aflatoxins and their metabolites has been useful in identifying the structural features which are important determinants of aflatoxin potency (Busby and Wogan, 1981). Wong and Hsieh (1976) concluded that AFB_1 possesses the optimal structure for both mutagenicity and carcinogenicity. On comparison of the mutagenic potential of the aflatoxin metabolites, they postulated that the 2,3-double bond is involved in both their carcinogenic and mutagenic effects. They also noted that this bond is not the only molecular site on the AFB_1 molecule that determines mutagenic activity. They found that alterations occurring elsewhere in the molecule invariably resulted in reduction of mutagenicity. Alterations of the cyclopentanone ring, such as its substitution by a terminal lactone as in AFG_1, 7-hydroxylation as in AFQ_1 or reduction of the keto group as in aflatoxicol, resulted in a significant lowering of the mutagenic potential in spite of the presence of an intact 2,3-double bond.

Aflatoxin M_1 presents an interesting case in structure

activity relationships. Hydroxylation at C-4 to give AFM_1 greatly reduced mutagenicity (Wong and Hsieh, 1976) and carcinogenicity (Wogan and Paglialunga, 1974) but did not severely affect animal toxicity (Pong and Wogan, 1971) or its ability to inhibit RNA synthesis (Pong and Wogan, 1971) or mitochondrial function (Obidoa and Siddiqui, 1978). Thus, AFM_1 may be an especially useful compound for studying the mechanisms responsible for acute toxicity and, those governing such chronic effects as carcinogenicity (Busby and Wogan, 1981)

Specific Effects of Aflatoxin on Laying Hens

Cottier et al. (1968, 1969) fed four levels of AFB_1 to 1-day-old White Rock chickens. When the chickens were 27 weeks old, eggs were collected and incubated. Hatchability of the eggs laid by the hens fed 610 ug/kg of feed decreased, while all of the eggs from the group fed 1,834 ug/kg failed to hatch due to embryo mortality during the first six days of incubation. After feeding the control diet for 6 days, hatchability returned to normal. Conversely, when hens previously fed a control diet were placed on the diet containing 1,834 ug/kg of aflatoxins, the eggs laid on the fifth and sixth days of feeding had reduced hatchability. On returning the same hens to the control diet, hatchability returned to the normal level by the seventh day of clearance (Cottier et al., 1969). The authors concluded that there was not a deleterious carry over of aflatoxin from hens to chicks, since broiler size and mortality

at 9 weeks were similar regardless of the level of aflatoxins in the feed of the parents.

Kratzer et al. (1969) fed White Leghorn hens with a laying mash containing 2,700 ug of aflatoxins/kg of feed. The hens were conditioned to the experimental rations for 2 weeks and the eggs were collected for the following 34 days and studied for hatchability and aflatoxin residues. Feed intake and body weights of the aflatoxin-fed hens were comparable to those of the control group. The aflatoxin ration did not reduce the number of eggs laid, although there was a decrease in the percent hatchability (89 vs. 74%). Histological examination of liver sections, however, revealed minimal to mild lesions. Liver functions were found to be altered as indicated by: reduced liver nitrogen, increased liver lipids, a decrease in liver nucleic acid concentration, a decrease in serum proteins, and an increased incorporation of radioactive leucine and uridine into the protein and RNA by liver slices (Keyl and Booth, 1971). These biochemical effects are a result of the dramatic alterations caused by AFB_1 in the synthesis of nucleic acids and proteins by the liver (Wogan, 1968).

Sims et al. (1970) fed hens a ration containing 2, 4 and 8 mg of aflatoxins per kg of feed. Three additional groups were administered comparable amounts per os daily. Hens were killed at different times during the feeding experiment. Egg production decreased, with the decrease being greater in the hens receiving the aflatoxins per os. The hens to which the

aflatoxins were given per os also had a significant ($P < 0.05$) decrease in body weight. No effect was observed on egg weights. Gross pathological changes were observed in the liver, including enlargement (up to 2 to 3 times normal), pale color, petechial and large hemorrhagic areas. Bile duct hyperplasia indicated that significant chronic liver damage had occurred in the two groups receiving the highest levels of aflatoxins.

The fatty liver syndrome in laying hens is a disease of considerable importance to the poultry industry (Abbott and Couch, 1971). It is characterized by a reduction in egg production and by a pale friable liver with lipid content almost double normal (Bossard and Combs, 1970). Because young chickens with aflatoxicosis have a pale, enlarged and friable liver (Carnaghan et al., 1966; Smith and Hamilton, 1970), the possibility that dietary aflatoxins can induce the fatty liver syndrome in laying hens was investigated. Hamilton and Garlich (1971) fed laying hens rations containing 1.25, 2.5, 10 and 20 mg of aflatoxins per kg of diet for 3 weeks. All doses of aflatoxins above 2.5 mg/kg caused a decrease in egg production. The decrease was dose-dependent and approached no production at the highest levels. There was a decrease in egg weight, but no change in the percent of egg shell or shell thickness. Total liver lipids were significantly increased, and the liver was enlarged, but no effect was observed on the relative weights of the spleen and pancreas.

Reed et al. (1968) reported that a mixture of inositol, choline, vitamin B_{12} and vitamin E added to the diet of laying

hens cured the fatty liver syndrome. Since dietary aflatoxin produced a disease similar to the fatty liver syndrome, the efficacy of the vitamin mixture was tested by Hamilton and Garlich (1972). They fed hens with 10 mg of aflatoxins/kg of diet with and without vitamin supplementation. Egg production and egg weight dropped significantly after 3 weeks of aflatoxin consumption. There was a slight but significant increase in percent of egg as shell. Livers were enlarged and had an increased lipid content. After three weeks of feeding the unspiked diet, all parameters returned to control values. The vitamin mixture did not affect development or recovery of the fatty liver syndrome caused by aflatoxins.

Hamilton (1971) reported a natural case of aflatoxicosis in a flock of 1,000 hens. The hens were fed corn that had been mechanically dried to 12-15% moisture, and stored for about 6 months in an unaerated bin. Within 48 hours of consumption, there was high mortality and it was diagnosed as hepatotoxicosis associated with the moldy corn. About 50 hours later, the surviving hens were placed on a commercial diet. Eight days after initial consumption of aflatoxins mortality was 50% and production was down 95%. Analysis of the feed showed one fluorescent spot which cro-chromatographed with AFB_1. The levels in two feed samples were 83 and 101 mg/kg. A. flavus was also isolated identified in the moldy corn.

Garlich et al. (1973) investigated the effects of short term feeding of aflatoxins on egg production and on several

parameters related to egg production. They fed White Leghorn laying hens diets containing 20 mg/kg of aflatoxins for seven days and then continued feeding of the unspiked mash for 3 weeks more. Egg production rate was not affected during the aflatoxin feeding period, but declined significantly ($P < 0.05$) on the first day of recovery and reached a minimum of 35% seven days later. Egg production returned to normal 19 days after consumption of the aflatoxin-free diet. Biochemical parameters showed a faster response to aflatoxin feeding. By the second day of aflatoxin feeding, plasma calcium, protein, cholesterol and triglycerides had decreased significantly and serum alkaline phosphatase had increased. The plasma calcium, protein and alkaline phosphatase values approximated control values by the seventh day of feeding the unspiked diet, while plasma total lipid and cholesterol approximated control values by the tenth day. Garlich et al. (1973) proposed that aflatoxins rapidly cause liver lesions, which are indicated by the rapid increase in serum alkaline phosphatase. These lesions result in impaired lipid transport and depressed synthesis of proteins and fatty acids. This caused an increase in liver lipids and a decrease in plasma lipids and proteins, both of which are produced in the liver and are precursors of yolk lipids and proteins. Egg production is not decreased as rapidly as plasma proteins and lipids, and the hen apparently compensates by producing smaller eggs with a smaller yolk (Huff et al., 1975).

Lötzsch and Leistner (1977) concluded that the decrease in egg production as a result of aflatoxin feeding is an

important natural limiting factor in transmission of aflatoxins to eggs. They found that laying hens fed continously with diets containing more than about 10,000 ug of AFB_1/kg stopped laying after a few days, thus excluding hazardous aflatoxin residues in eggs. An interesting finding of this study was that egg production of quail and chickens fed diets containing 2,000 up to 8,000 ug of AFB_1/kg increased before the toxin containing feed was withdrawn, although recovery in egg production was not complete (Lötzsch and Leistner, 1977). Laying hens fed a diet containing 5,000 ug of AFB_1/kg reached minimum egg production during the sixth and seventh weeks of aflatoxin feedinf, and showed a slight improvement during the two last weeks of aflatoxin feeding (Lötzsch and Leistner, 1976). Sawhney et al. (1973) also reported an improvement in egg production of quail on aflatoxin test diets. Lötzsch and Leistner (1977) proposed that the enhancement of egg laying in aflatoxin-fed poultry may reflect some sort of adaptation of the hens to aflatoxins.

In order to determine if eggs produced during aflatoxicosis had abnormalities other than a smaller size, Huff et al. (1975) induced aflatoxicosis in laying hens by feeding a ration containing graded amounts of aflatoxins (0, 1.25, 2.5, 5.0 and 10 mg/kg) for four weeks. At the end of the feeding trial, the size of the liver and its lipid content were increased, while egg production and egg size were decreased. The liver size increased significantly at doses of 5 and 10

mg/kg, while the liver lipids increased dramatically at a dose of 2.5 mg/kg. Egg production was decreased by about 70% from the controls at a level of 10 mg/kg. A significant decrease in egg size occurred at all doses except at 1.25 mg/kg. Total yolk weight and percentage yolk were lowered by 34 and 24%, respectively, at the 10 mg/kg level. The carotenoid concentration in the yolk and plasma were elevated at 10 mg/kg. The authors concluded that plasma and yolk lipids respond to aflatoxin inhibition of lipid synthesis and transport from the liver, but plasma and yolk carotenoids which are dietary in origin increase when egg production decreases during aflatoxicosis.

Howarth and Wyatt (1976) studied the effect of aflatoxicosis on reproductive performance of broiler hens by evaluating fertility, hatchability and progeny performance. They fed hens for 4 weeks at dose levels of 0, 5 and 10 mg of aflatoxins/kg of diet. Egg production decreased significantly during weeks 3 and 4 of aflatoxin feeding for the groups fed 10 and 5 mg/kg, respectively. Fertility was not affected, but hatchability decreased significantly within one week of toxin feeding. The hatchability of fertile eggs collected during week 1 of treatment was 95.1, 68.9 and 48.5% for the control and the 5 and 10 mg/kg groups, respectively. At the levels used, no effects of the aflatoxins or their metabolites were observed on the performace of the surviving chicks. Hens necropsied at the end of the feeding trial exhibited typical symptoms of aflatoxicosis, including enlarged, fatty and

friable livers and enlarged spleens.

Howarth and Wyatt (1976) proposed that the rapid decrease in hatchability suggested an extremely rapid transfer of ingested aflatoxins or their breakdown products to the egg, or else the rapid changes caused by aflatoxins in the hen's protein, carbohydrate and lipid metabolism may alter the chemical composition of the egg and its subsequent hatchability. The rapid effect of aflatoxins on hatchability poses a special problem to the poultry industry, since the decline in egg production does not become obvious until a serious decrease in hatchability has occurred.

Alterations in calcium metabolism of the laying hen during aflatoxicosis have been reported by Garlich et al. (1973). However, results on the shell characteristics have been conflicting (Hamilton and Garlich, 1971; Hamilton and Garlich, 1972). Washburn and Wyatt (1978) studied the effects of aflatoxins on shell quality of high and low shell strength lines selected from commercial layer strains. They found that there was no adverse effect on shell strength at 5 mg/kg, with groups receiving the aflatoxins having stronger shells than controls. Aflatoxin-fed hens laid smaller eggs than controls, apparently because of a smaller yolk size. No difference, however, occurred in shell weights of aflatoxin-fed and control hens. Thus, a significant increase in the percent of egg shell was noted for the aflatoxin-fed group.

There is considerable concern about the transmission of aflatoxins from animal feeds into the human food chain. The

Food and Drug Administration (FDA) has placed restrictions on animal feeds and feed ingredients contaminated with aflatoxins (Stoloff, 1980). The extreme losses that can occur upon aflatoxin contamination of products such as corn, has prompted the study of ways to detoxify them. One such process is ammoniation of contaminated feeds. Two aspects to consider in all detoxification processes are: 1) the completeness of aflatoxin destruction, and 2) the safety of the decomposition products.

To investigate the safety of feeding corn ammoniated to inactivate aflatoxins to White Leghorn layer-breeders, Hughes et al. (1979) conducted a two year study using four types of corn: 1) normal corn (control), 2) ammoniated control corn, 3) aflatoxin contaminated corn and 4) ammoniated aflatoxin contaminated corn. Corn made up 66.3% of the diets, resulting in aflatoxin levels of 500 ug/kg in the aflatoxin contaminated diet, and 3 ug/kg in the ammoniated aflatoxin containing diet. They found that aflatoxins in the feed at the 500 ug/kg level did not cause any adverse effects, although some small differences were observed in one of the two trials. During the second trial the effects observed were a reduction in egg production, reduced egg weights, an increased percentage of blood spots and a reduction in feed consumption, and during the first trial, reduced day old chick weight. They did not observe any effects on fertility and hatchability, probably due to the low level of aflatoxins used. The ammoniation treatment showed a trend toward a reduced feed consumption and lower body weight gains. The ammoniation treatments, however, caused no

adverse effects on mortality, egg production, egg quality, reproduction or the economic parameters evaluated. These authors concluded that corn treated by the ammoniation process would be safe in diets at levels of up to 66.3% of the diet.

Aflatoxins have been shown to impair blood coagulation in a number of species (Bababunmin and Bassir, 1972). Doerr et al. (1976) demonstrated that severe impairment of blood clotting pathway functions occurs during aflatoxicosis in chickens and that prothrombin is primarily affected. The effects of aflatoxins on some blood parameters were evaluated by Hughes and Jones (1979) in the hens fed the aflatoxin contaminated and ammoniated corn diets (Hughes et al., 1979). Erythrocite count, hemoglobin percent, hematocrit and mean corpuscular hemoglobin were not affected by feeding aflatoxins at levels of either 2.3 ug/kg (ammoniated aflatoxin containing corn) and 500 ug/kg (contaminated unammoniated corn). Pullets fed ammoniated corn without aflatoxins and those fed the 500 ug/kg ration had reduced mean corpuscular volume. The authors had no explanation for this and suggested that it was not a true response.

Exarchos and Gentry (1982) dosed laying pullets with AFB_1 by oral intubation at levels of 0.7 mg/kg of body weight per day or higher for 14 days. When the dose was 5 mg/kg of body weight, egg production ceased after 3 days. At lower doses (1.0 and 0.7 mg/kg), a delayed effect on production was observed, which occurred four to five weeks after the 14 day dosing period. They suggested that at massive doses the

toxin produced an immediate effect on the reproductive system, while at lower levels, the toxin was apparently taken up by organs and/or tissues, where it was held for several weeks and probably metabolized. They indicated that the mechanism of aflatoxin release, which resulted in the delayed effect on production, is not known.

Trucksess et al. (1983) fed laying hens with a ration containing 8,000 ug of AFB_1/kg for 7 days. At this time half of the hens were killed and the remainder were fed with an aflatoxin free diet for 7 days and then sacrificed. No significant difference in food intake, body weight, egg production or egg weights was observed during the aflatoxin feeding period or upon removal of the aflatoxin contaminated diet. The weights of livers relative to body weights of the hens exposed to AFB_1 for 7 days increased in comparison to the relative liver weights of control hens and continued to increase after withdrawal of the aflatoxin contaminated diet. This was attributed to infiltration of fat as indicated by the color and texture of the livers from the treated hens. The relative ovary weights decreased in the treated hens over the same time periods, which adversely affected the size and number of ova, and indicated a detrimental effect on future egg production.

Occurrence of Aflatoxins in Foods of Animal Origin

Even before the chemistry of aflatoxins was fully established, Allcroft and Carnaghan (1962, 1963) proposed and investigated the possible presence of aflatoxin residues or their metabolites in the milk, meat or eggs of animals receiving aflatoxin contaminated feeds. They found that extracts of milk from cows fed aflatoxin contaminated rations induced lesions identical to those produced by administration of AFB_1 to ducklings. However, on using the 1-day old duckling assay as described by Sargeant et al. (1961b), they failed to demonstrate toxicity from the livers and eggs of hens fed a ration containg 15% of toxic peanut meal (equivalent to 1,500 ug of aflatoxins/kg of diet), or from the clotted blood serum and livers of cows fed a ration containing 20% of toxic peanut (equivalent to 2,000 ug of aflatoxins/kg of diet), or from the liver of a pig which died of aflatoxicosis (Allcroft and Carnaghan, 1963).

Early studies aimed at detecting aflatoxin metabolites in animal products suffered from two major drawbacks: 1) extraction procedures may not recover all the aflatoxins present; and 2) the biological test methods were relatively insensitive (Purchase, 1972). The complex sample matrix, the presence of potentially interfering compounds and the need for high sensitivity are common problems encountered in analysis of animal tissues and products for aflatoxins (Gregory and Manley, 1981). Reviews of the early literature by Armbrecht (1971) and

Purchase (1972), and more recently by Rodricks and Stoloff (1977) indicate that the levels of aflatoxins found in tissues of animals fed aflatoxin contaminated feed are far lower than the levels found in the contaminated feed per se. This is due to the efficient metabolism and excretion of absorbed aflatoxins by most animals. Studies with labeled aflatoxins have shown that most of the administered dose ends up in the excreta of the dosed animals, appearing mainly as water-soluble conjugated aflatoxin metabolites (Mabee and Chipley, 1973a, 1973b; Chipley et al., 1974; Hayes et al., 1977).

Rodricks and Stoloff (1977) summarized the data from various studies by calculating the ratios between the level of AFB_1 in feeds and the level likely to be encountered in selected animal tissues. Their estimates are presented in Table 1. As shown, milk has the lowest feed to tissue ratio, thus it is the most vulnerable animal product to aflatoxin contamination from the diet. On examination of the same data, Patterson et al. (1980) noted considerable variation in the feed to tissue ratio, with great variability occurring from one animal to another and from one set of data to another. Indeed, the overall range of values in milk was from 34 to 1,600.

Applebaum et al. (1982) have concluded that milk is probably the only human food of mammalian origin known to be naturally contaminated with AFM_1. Contamination of milk with AFM_1 occurred in the United States in 1977 in seven southeastern states, in 1978 in Arizona and again in the

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

Animal	Tissue	Aflatoxin in tissue	Feed to tissues Ratio
Beef cattle	Liver	B_1	14,000
Dairy cattle	Milk	M_1	300
Swine	Liver	B_1	800
Layers	Eggs	B_1	2,200
Broilers	Liver	B_1	1,200

Southeast in 1980 (Applebaum et al., 1982). The occasional presence of AFM_1 in milk has also been observed in Holland (Schuller et al., 1977) and West Germany (Polzhofer, 1977).

Several studies have indicated that the amount of AFM_1 excreted in milk is directly proportional to the amount of AFB_1 ingested (Allcroft and Roberts, 1968; Masri et al., 1969). The secretion of AFM_1 into milk as a percentage of AFB_1 consumed varies from less than 1% (van der Linde et al., 1965; Polan et al., 1974) to 3% (Masri et al., 1969). Polan et al. (1974) undertook a study to determine the minimum intake of AFB_1, which would produce a detectable level of AFM_1 in cows' milk. Cows fed a concentrate containing 50 ug of AFB_1/kg had only traces of AFM_1 in their milk and no AFM_1 was detected in milk from cows receiving 10 ug of AFB_1/kg of diet. By regression analysis, they calculated that 15 ug of AFB_1/kg in the total ration would produce detectable levels of AFM_1

in milk. Recently, Patterson et al. (1980) detected AFM_1 in the milk of cows fed 10 ug of AFB_1/kg of diet. They found that the amount of AFM_1 in the milk varied from 0.01 to 0.33 ug/L, with a mean value of 0.19. They could not detect AFB_1 or AFM_1 in blood plasma samples obtained from these cows shortly after milking. Since the concentration in the milk was at least 20 times higher than in the plasma, they suggested that the toxin is excreted by the mammary gland against a concentration gradient, and thus, probably involves an active metabolic process. The highest concentration found in the milk in this study, 0.33 ug/L, is still 15 to 60 times lower than the regulatory levels permitted in several countries for products used in the manufacture of human food.

Allcroft and Roberts (1968) detected AFM_1 in milk within 5 hours of oral administration of purified AFB_1 to dairy cows. When significant quantities of AFB_1 are consumed, AFM_1 will first appear in milk within 12 hours post-feeding, a time corresponding to the next normal milking period (Kiermeier, 1977).

Polan et al. (1974) found that the concentration of AFM_1 in milk usually plateaud by day 4 after feeding and observed no AFM_1 in the milk 2 days after aflatoxin feeding ceased. After removal from the aflatoxin contaminated diet, no aflatoxin residues in milk were detected after 3 to 4 days by Lynch (1972) and after 5 days by Allcroft and Roberts (1968). However, Masri et al. (1969) observed low levels of toxin in the milk at 7 days after removal from the aflatoxin

containing diet. In this study daily intakes of AFB_1 ranged from 20 to 30 mg. Factor that may account for the differences in the results are most likely associated with individual variation among the cows tested, differences in the quantity and purity of the aflatoxins administered and differences in the methods utilized for analysis.

Allcroft and Carnaghan (1962, 1963) were the first to test eggs for aflatoxins using the 1-day old duckling assay. They tested eggs from pullets, which were fed since hatching with a ration containing 15% of toxic groundnut meal. This was later shown to be equivalent to 1,500 ug/kg of feed (Allcroft and Raymond, 1966). Eggs were freeze-dried, extracted and administered orally to ducklings, so that each duckling received an equivalent of 16 eggs in 7 days. Another group of ducklings received crushed whole eggs for 6 weeks. None of the ducklings showed any signs of aflatoxin toxicity. Wogan (1964) found that the minimum quantity of AFB_1 required to produce bile duct proliferation in ducklings was 0.4 ug/duckling/day for 5 days, i.e., a total dose of 2 ug/duckling. Assuming that the extraction technique was successful, considerably less than 2 ug of aflatoxin would be present in the 16 eggs given to each duckling, i.e., the eggs contained less than 0.125 ug or 21 ug/kg, according to calculations presented by Purchase (1972).

Brown and Abrams (1965) reported that the eggs from hens receiving rations containing 500 ug/kg or 700 ug/kg of aflatoxins were not toxic to ducklings when fed at a rate of 2

eggs/duckling/day. Similarly, Abrams (1965) reported that no aflatoxins were present in eggs from hens maintained on rations containing 750 ug of aflatoxins/kg.

Kratzer et al. (1969) found no aflatoxins in the meat, liver or blood of broilers fed a diet containing 1,600 ug/kg of aflatoxins for 60 days prior to slaughter. They also did not detect any aflatoxins in the eggs, meat, liver or blood of White Leghorn hens fed a ration containing 2,700 ug of aflatoxins/kg for a period of 48 days. They stated that the method used would detect as little as 3 to 5 ug/kg of aflatoxins.

Sims et al. (1970) failed to detect aflatoxins in the eggs and liver of hens receiving aflatoxins in the feed at dose levels of 2, 4, and 8 ug/kg of feed, and in the eggs from hens receiving comparable quantities of crude aflatoxins per os. Van Zytveld et al. (1970), however, detected aflatoxins and/or their metabolites in the liver and skeletal muscles of 15 out of 45 chickens which had been administered oral daily doses varying between 0.085 and 1.1 mg of mixed aflatoxins for 6 weeks. Aflatoxins were found mainly in chicks that died or grew poorly during the test and in only one bird that reached market weight.

In order to study the metabolism and tissue distribution of aflatoxins in the laying hen, Sawhney et al. (1973) administered 11.26 mg of ^{14}C-AFB_1 as a single oral dose to laying hens. The distribution of radioactivity was determined at one, four and seven days after administration of

the aflatoxin dose. Seven days after treatment, 70.61% of the dose had been recovered in the excreta. The major pathway of excretion seemed to be via the bile into the intestine, since at both one and four days the specific activity of the bile was greater than that of any tissue. All components of eggs laid at various times had higher ^{14}C activity in comparison to those at ten hours after ovulation. The activity in egg white decreased 14 hours after oviposition and increased in the yolk and shell membrane from 10 hours onward. They proposed differences could be due to the physiology of egg formation and the structural and compositional differences in the egg components. At day one, the liver, reproductive organs and kidneys had a higher concentration than the gizzard, heart, wing, breast and leg muscle, all of which had about half the activity of liver. Seven days after the oral dose all tissues still possessed activity, and there was an increase in activity of the small ova, spleen, kidney, breast muscle, pancreas and heart. The presence of radioactivity in all the tissues at one and seven days indicated rapid absorption but slow elimination of aflatoxins by the laying hen. The rate of elimination of aflatoxins from the hens' body followed first order reaction kinetics with a half-life of 66.8 hours.

Mabee and Chipley (1973a) intubated 6 week-old broiler chickens with a daily dose of 100 ug of ^{14}C-AFB_1/kg of body weight for 14 days. The chickens were kiled 5 hours after administration of the final dose. The total radioactivity

detected in the liver, heart, gizzard, breast and leg samples accounted for only 9.36% of the total ^{14}C administered, with the rest being excreted. Of the total amount retained, 11.04, 9.83, 4.30, 12.52, 31.66 and 30.63% were detected in the blood, liver, heart, gizzard, breast and leg muscles, respectively. The samples were lyophilyzed and extracted with sodium acetate buffer. The supernatant fluid was treated with NaCl and then further extracted with ethyl acetate. In the combined sample, 81.2% of the radioactivity was confined to the sodium acetate buffer extract. Further treatment of the sodium acetate extract with β-glucuronidase and subsequent chloroform extraction revealed that 31.5% of the total radioactivity present was transferred to the chloroform. The presence of AFM_1 in the chloroform extract was confirmed by TLC. The authors concluded that broiler chickens can metabolize the majority of AFB_1 when administered at relatively low doses. Furthermore, they concluded that aflatoxin conjugates are the predominant form of metabolites produced, and proposed this as possible explanation for the conflicting reports dealing with isolation of aflatoxins from tissues or the potential toxicity ot tissues from animals receiving aflatoxins.

Mabee and Chipley (1973b) used the same methods to study aflatoxin retention and excretion in White leghorn pullets. The total radioactivity detected in tissue samples accounted for only 7.85% of the total ^{14}C administered. The average level of radioactivity detected in the blood, liver, heart,

gizzard, breast and leg muscle was 19.5, 16.1, 3.9, 7.2, 26.4 and 26.9%, respectively, of the total amount retained by the layers. The radioactivity observed in the chloroform extract from each lyophilyzed sample was about 10% of the total. Thus, they concluded that aflatoxin conjugates are the predominating metabolites. AFM_1 glucuronides constituted 38.9% of the total conjugates extracted by ethyl acetate. The authors proposed that other forms of metabolites of AFB_1, probably as sulfate conjugates, were present. In order to further characterize the distribution and metabolism of ^{14}C-AFB_1 in broiler and layer chickens, Chipley et al. (1974) treated the ethyl acetate extracts of all samples with carboxypeptidase A, leucine aminopeptidase, pepsin or trypsin. They found an average of 50% of the ^{14}C detected in the acetate extracts was either a liberated peptide or amino acid conjugate of ^{14}C-AFB_{2a}, which was identified by comparison of R_f values on thin layer chromatograms (TLC) and by absorbance maxima with an AFB_{2a} standard.

Jacobson and Wiseman (1974) were the first to detect aflatoxin residues in the eggs of hens fed rations spiked with unlabeled AFB_1. They fed a group of laying hens consecutively with rations containing 100 ug of AFB_1/kg of feed for 10 days, 200 ug of AFB_1/kg of feed for 12 days, and finally 400 ug of AFB_1/kg of feed for 15 days. Egg samples were analyzed by a modification of the method of Jacobson et al. (1971), which is sensitive to 0.1 ug/kg of AFB_1. Measurable amounts of AFB_1 were detected in the eggs during

the three feeding periods, with average values of 0.23, 0.78 and 1.4 ug/kg for the 100, 200 and 400 ug levels, respectively. No aflatoxin was detected in eggs analyzed after storage for 9 months at 3°C, thus, they concluded that the toxin is not stable for long periods of time. Analysis of separated egg whites and yolks indicated average amounts of 2.2 and 3.6 ug/kg, respectively.

Lötzsch et al. (1976) fed Japanese quail for 30 days with rations containing 40 to 8,000 ug of AFB_1/kg of feed, and approximately five times as much AFG_1. Measurable amounts of AFB_1 (average 0.09 ug/kg) were found in the eggs from the quail maintained on the ration containing 100 ug/kg of AFB_1. As the amount of AFB_1 in the feed increased, the aflatoxin residues in the eggs increased to a maximum of about 2 ug/kg. Egg production of quail fed more than 8,000 ug/kg of AFB_1 stopped within 5 days. Later, Lötzsch and Leistner (1976) fed white and brown laying hens with aflatoxin spiked diets. White laying hens were fed rations containing 40 to 5,000 ug of AFB_1/kg and 52 to 1,500 uf of AFG_1/kg for 8 weeks. Another group was fed a ration containing 10,000 ug of AFB_1/kg and 13,000 ug of AFG_1/kg for 7 days. After feeding for 29 days without added aflatoxins, this group was fed a ration containing 5,000 ug of AFB_1/kg and 6,500 ug of AFG_1/kg for 7 days. The whites and yolks of 3 eggs were pooled together and analyzed separately. The aflatoxin content in the whole egg was calculated using a normal hen's egg white to yolk ratio as 67/33. Brown layers were fed rations containing

100 and 10,000 ug of AFB_1 and 31 to 3,100 ug of AFG_1 per kg of diet for 3 to 4 weeks.

In the same study aflatoxins were first detected in the eggs of white hens receiving 3,000 ug of AFB_1/kg of feed or 1,550 ug of AFG_1/kg of feed, with average values of 0.03 ug of AFB_1/kg and 0.02 ug of AFG_1/kg. The maximum amounts of AFB_1 and AFG_1 were detected in the eggs of hens fed the most highly contaminated ration and were about 0.4 ug/kg. In the eggs of brown layers, however, aflatoxin residues were first detected when the hens were fed rations containing 5,000 ug of AFB_1 and 3,100 ug of AFG_1 per kg. At the highest feeding levels, maximum residues recovered from the eggs were only about 0.2 ug/kg of AFB_1 and 0.05 ug of AFG_1/kg. They found that with both white and brown layers, aflatoxin residues were detected primarily during the first half of the feeding period. At the highest levels of aflatoxin feeding, egg production stopped after a few days. Egg production of quail and of white and brown hens decreased following aflatoxin ingestion but increased again towards the end of aflatoxin feeding.

Lötzsch and Leistner (1976) also administered single oral doses of aflatoxins ranging from 1.13 to 2.14 mg/kg of body weight to brown laying hens. Aflatoxin residues were first detected when the oral dose was 1.86 mg/kg (approximately equivalent to consumption of feed containing 17,000 ug of AFB_1/kg), and caused residues of AFB_1 up to 7 days after treatment. An interesting finding was that when white hens

were successively fed two different aflatoxin levels, the latter caused somewhat lower aflatoxin residues in eggs than the comparable one fed alone. The authors suggest that this may reflect development of resistance to aflatoxins as was also observed for egg production during aflatoxin feeding.

From this series of studies Lötzsch and Leistner (1977) concluded that quail are the most sensitive species for the transmission of aflatoxin residues from feed to eggs (critical concentration = 100 ug of AFB_1/kg of feed), followed by the white laying hen (critical concentration = 3,000 ug of AFB_1/kg of feed) and the brown hen (critical concentration = 5,000 ug of AFB_1/kg of feed). Furthermore, they concluded that the transmission of aflatoxins into eggs poses no potential hazard to public health.

Stoloff and Trucksess (1978) conducted a survey for AFB_1 in eggs and egg products obtained during January and July of 1977 from 35 establishments in the southern part of the United States. This survey was conducted as a result of concern for aflatoxin transmission to animal products because corn used as feed was found to be contaminated with aflatoxins. The method used had a detection limit of about 0.05 ug of AFB_1/kg. Of 112 samples analyzed, AFB_1 was found in only one sample of liquid egg at a level of 0.06 ug/kg.

Hughes et al. (1979) fed laying hens with diets containing 500 ug of AFB_1/kg of feed and with the same corn after ammoniation, which reduced the level of AFB_1 to about

3 ug of AFB_1/kg of feed. No aflatoxin residues were detected in eggs laid by hens fed either of these rations.

Exarchos and Gentry (1982) intubated laying pullets with doses ranging from 1.0 to 0.1 mg of AFB_1/kg of body weight for 14 days. Another group received 5.0 mg/kg for four days. Egg whites and yolks for each group were pooled, freeze-dried and sequentially extracted with hexane and chloroform. The concentrated extracts without any further cleanup were spotted on TLC plates for detection of AFB_1. In spite of the high levels of AFB_1 fed, they failed to detect AFB_1 during dosing or for up to 10 weeks following the treatment. The authors indicated, however, that this would not exclude the presence of AFB_1 metabolites that might still be toxic, but are not detected by the procedure used. No analysis of the tissues was performed. However, they proposed that the toxin was taken up by the organs and tissues, where it was held for several weeks and probably metabolized to cause a delayed effect on egg production.

In order to relate aflatoxin residues in eggs and tissues to aflatoxin intake via feed, Trucksess et al. (1983) fed 18 hens with a ration spiked with 8,000 ug of AFB_1/kg of feed for 7 days. At this time, half of the group was sacrificed while the remainder were sacrificed after an additional period of 7 days on an aflatoxin-free diet. One day after aflatoxin feeding started, AFB_1 and aflatoxicol, but no AFM_1 were found in the eggs. The levels of AFB_1 and aflatoxicol, when detected, were similar over the entire experimental

period. A maximum of about 0.2 ug/kg was reached by day 4 or 5 of aflatoxin feeding and remained at this level until aflatoxin withdrawal. After withdrawal, aflatoxin residues in the eggs decreased rapidly. AFB_1 was present up to 6 days after withdrawal (0.02 ug/kg) and low levels of aflatoxicol (0.01 ug/kg) were present up to the end of the experiment. The AFB_1 and aflatoxicol levels in the ova of hens sacrificed on day 7 were similar to the levels found in the eggs collected on days 6 and 7. No AFB_1 was detected in any of the eggs or in the ova of hens sacrificed after 7 days on the aflatoxin-free diet. At the end of aflatoxin feeding, AFB_1 and/or aflatoxicol were found in all tissues analyzed, while AFM_1 was only found in the kidneys. Liver and ova contained the highest levels of AFB_1 and aflatoxicol at the end of aflatoxin-feeding. AFB_1 was the only aflatoxin detected in the blood, and aflatoxicol the only aflatoxin detected in muscle. Seven days after aflatoxin withdrawal, AFB_1 was found in one of nine livers (0.08 ug/kg), and aflatoxicol (0.01-0.04 ug/kg) in eight of nine muscle samples, but no aflatoxins were found in any other tissues.

The retention of aflatoxin residues in tissues of turkey poults fed a diet containing 500ug of AFB_1/kg for 21 days was examined by Gregory et al. (1983). As revealed by high performance liquid chromatographic (HPLC) analysis, free and conjugated AFB_1 and AFM_1 were the principal tissue residues, while aflatoxicol was detected in some samples. Water soluble conjugates comprised 55-90% of the total

residues. In liver, an average of 0.10 ug/kg and 0.28 ug/kg of AFB_1 was detected in the nonpolar and aqueous phase after hydrolysis, respectively. Residue levels in the liver were higher than in muscle tissues, although all were very low. Clearance of the aflatoxin residues was followed over a three day period of feeding an aflatoxin-free diet. Clearance of AFB_1 occurred rapidly, with a half-life of 1.4 days in liver. Free and conjugated aflatoxins were still detected in liver samples on the third day after withdrawal, but not in the breast or thigh sample.

Furtado et al. (1982) studied the withdrawal time required for clearance of aflatoxins from pig tissues. Two trials using 20 pigs in each were conducted. The spiked diets contained 551 and 355 ug/kg of AFB_1 and AFB_2, respectively. After feeding for 42 days, aflatoxins were found widely distributed in all tissues of the pigs fed the spiked diet, and in the liver and kidneys of the control pigs in trial 1, which were fed a ration that was later found to be naturally contaminated with 20 and 31 ug/kg of AFB_1 and AFB_2, respectively. The blood showed the lowest level of residual aflatoxin, followed by the spleen, muscle and heart. The highest concentration of aflatoxins was found in the liver and kidneys. One day after placing the pigs on an aflatoxin-free diet, there was a significant decrease in the aflatoxin levels in all organs and tissues. After two days, only one pig contained trace amounts of aflatoxins in the tissues. After four days, there were no detectable levels

of aflatoxins in any of the tissues analyzed.

Miller et al. (1982) performed an acute and a chronic experiment in swine to study the clearance time of aflatoxin residues from the tissues. In the acute test, 8 pigs were given a single oral dose of 1.2 mg of aflatoxins/kg of body weight. Aflatoxins B_1, B_2, G_1, G_2 and M_1 were detected by HPLC, with residue levels correlating with times after administration of the dose. Residues were present in livers and kidneys at 12 hours after dosing, but in muscle, residues were detected only after 24 hours. No aflatoxin residues were detected in any pig 72 hours post-dosing. In the chronic experiment, young pigs were fed rations containing 0, 400 and 800 ug of aflatoxins/kg for 10 weeks. Aflatoxin residues were present in the livers and kidneys of pigs fed both aflatoxin spiked diets, but only in the muscle of the pigs fed the 800 ug/kg diet.

Chen et al. (1984) determined tissue levels of aflatoxins and the amount of time necessary for tissue clearance from broiler chickens fed an aflatoxin contaminated diet containing 2,057 and 1,323 ug/kg of AFB_1 and AFB_2, respectively. After 35 days of feeding the aflatoxin spiked ration, all tissues had aflatoxin residues, either as the original compounds, AFB_1 and AFB_2, or as their metabolites, AFM_1 or AFM_2. The highest levels were present in the livers and kidneys, although total aflatoxin residues in all tissues were less than 3 ug/kg. Four days after placing the chickens on an aflatoxin-free diet, there were no detectable levels

of aflatoxins in any of the tissues, thus indicating that broiler chickens rapidly metabolize and remove aflatoxins from their tissues.

Stability of Aflatoxins in Foods

Although aflatoxins are fairly stable molecules partial or total degradation may occur in contaminated foods and feeds under certain conditions (Doyle et al., 1982). Most of the initial research was done in an effort to design procedures by which aflatoxin contaminated feed grains could be treated to destroy the toxin residues and still retain their organoleptic and nutritive value without leaving harmful residues. Since AFM_1 has been found in commercial milk (Kiermeier et al., 1977b; Polzhofer, 1977) and cheeses (Kiermeier et al., 1977a; Polzhofer, 1977), recent studies have looked at the fate and stability of AFM_1 during milk and cheese processing (Applebaum and Marth, 1982; Wiseman and Marth, 1983a, 1983b).

Several studies have indicated that aflatoxins are very stable to heat (Peers and Linsell, 1975; Goldblatt, 1971). Mann et al. (1967) studied the effect of heat and moisture on aflatoxins in oilseed meals. There was little reduction at temperatures of 60° to 80°C, but marked reduction occurred at 100°C. Destruction of aflatoxins was enhanced by increasing heating times and by increasing moisture content. For example, heating a meal containing 30% moisture for 2.5 hours at 100°C

resulted in degradation of approximately 85% of the aflatoxins. When similar meal containing only 6.6% moisture was heated at the same temperature for the same period of time, only about 50% of the aflatoxin was degraded. Peers and Linsell (1975) observed that AFB_1 was not degraded in peanut or corn oil until the temperature approached 250°C.

The stability of aflatoxins under conditions simulating commercial dry and oil roasting of peanuts, which had been artificially contaminated with various levels of aflatoxins, was studied by Lee et al. (1969). Dry roasting was done at temperatures ranging from 121° to 204°C for 5 to 30 min. Oil roasting times varied from 3 to 7 min with temperatures between 163° and 174°C. Depending on the roasting conditions and initial aflatoxin levels, reductions ranging from 45 to 83% were observed. Waltking (1971) roasted commercially rejected peanuts in a pilot plant simulating conditions used for making peanut butter. They noted that aflatoxins B_1 and G_1 were more affected than aflatoxins B_2 and G_2, showing average losses of 40 to 50% and of 20 to 40%, respectively.

Luter et al. (1982) investigated the effectiveness of microwave roasting for reducing aflatoxin contamination in peanuts. They found that heating the nuts to a temperature of at least 150°C consistently reduced aflatoxin contamination by 95%. The rate of heating appeared to have very little or no effect on the degree of aflatoxin destruction. Microwave roasting also resulted in a satisfactory degree of browning, while protein and fatty acid content were not measurably

affected.

Conway et al . (1978) showed that a 40 to 80% reduction in aflatoxin content could be attained by a single passage of corn through a continous roaster in which temperatures ranged between 145° and 165°C. In a second experiment, the combined effects of heat and ammonia treatments were evaluated. A 57% reduction in aflatoxin content was reported when the corn was tempered with aqueous ammonia at a concentration of 0.5% NH_3 on a dry weight basis and then passed through the corn roaster. When the corn was retempered and again passed through the roaster, reductions of over 90% were achieved.

Ammoniation has generated a great interest as it is an effective and economically feasible means for reducing aflatoxin content of a variety of feedstuffs (Marth and Doyle, 1979). Biological data from growing rats (Southern and Clawson, 1980), swine (Jensen et al., 1977), lactating cows (McKinney et al., 1973), laying pullets (Hughes and Jones, 1979) and trout (Brekke et al., 1977) indicate that ammoniated corn can be safely used in animal feeding. Details on the use of ammoniation to destroy aflatoxins in corn have been recently reviewed by Norred (1982). This process however, has not been approved for use on human foods.

Ulloa-Sosa and Schroeder (1969) showed that boiling of corn with lime water as is traditionally done for the preparation of 'tortillas' in Mexico, Guatemala and other Latin American countries removed about two-thirds of the aflatoxins originally present in corn. Corn is the most important staple

in the diet of the majority of the population in these countries, thus it is very fortunate that the traditional method of preparation destroys aflatoxins and protects humans from aflatoxin consumption.

In studies with naturally contaminated raw milk, McKinney et al. (1973) reported that approximately 40% of the AFM_1 originally present disappeared after 4 days of storage at $0^{\circ}C$, and that after 6 days destruction was about 80%. On the other hand, Stoloff et al. (1975) concluded that AFM_1 in artificially contaminated milk was stable through a storage period of 17 days at $4^{\circ}C$. Kiermeier and Mashaley (1977) studied the degradation of AFM_1 in naturally and artificially contaminated milk. They also found that reduction occurred faster in naturally contaminated than in artificially contaminated milk. AFM_1 has been found to slowly degrade during frozen storage (McKinney et al., 1973; Stoloff et al., 1975; Kiermeier and Mashaley, 1977).

Allcroft and Carnaghan (1963) found that pasteurization at $80^{\circ}C$ for 45 sec or at $70^{\circ}C$ for 30 min, or roller drying of the 'toxic milk' from cows fed an aflatoxin-contaminated diet did not reduce its toxicity to 1-day old ducklings. Similar results were obtained by Stoloff et al. (1975) and van Egmond et al. (1977) who observed no loss of AFM_1 after different forms of heat treatment on milk. In contrast to these studies, Purchase et al. (1972) showed that processing of milk reduced its aflatoxin content and that the higher the temperature used, the smaller the amount of residual aflatoxins. Thus,

pasteurization at 62°C for 30 min reduced AFM_1 content by 32% and at 80° for 45 sec by 64%.

Wiseman and Marth (1983b) found no change in the AFM_1 content of naturally or artificially contaminated milk heated at 64°C or 84°C even after 2 hours of heat treatment, or in naturally contaminated milk heated at 100°C for 2 hours. Overnight storage of the heated milks did not reduce AFM_1 content. Differences between natural and artificial contamination, as well as variations in analytical techniques may explain the variability of results reported by different researchers. Pasteurization appears to have only a small effect on inactivating AFM_1 in milk. However, sterilization of milk causes some loss of AFM_1 (Purchase et al., 1972; Kiermeier and Mashaley, 1977).

Applebaum and Marth (1982) manufactured cottage cheese from milk naturally contaminated with AFM_1 and found that the concentration of AFM_1 in the curd was from 7.9 to 8.3 times higher than in the skim milk used in their manufacture. The whey and wash water from each trial contained less AFM_1 than did the original skim milk. The concentration of AFM_1 in the cheese did not decrease during a 2 week storage period at 7°C. They concluded that cottage cheese manufactured from milk naturally contaminated with AFM_1 could contain unacceptable levels of the toxin.

Van Egmond et al. (1977) found that AFM_1 could be recovered in slightly greater amounts from yogurt than from the original milk. They suggested that the increased AFM_1

content of yogurt probably resulted from more complete recovery. Wiseman and Marth (1983a) studied the behavior of AFM_1 in yogurt, buttermilk and kefir. In contrast to van Egmond et al. (1977), they reported that the AFM_1 content of yogurt was essentially the same as that in the initial milk, even after 6 weeks of storage at 7°C. In the preparation of buttermilk, they noted that in the first 3 trials the AFM_1 content appeared to increase after fermentation and remained high after 4 days of storage. In the second 3 trials, no increase in AFM_1 occurred after fermentation. The AFM_1 content of kefir decreased after fermentation, and although it appeared to increase slightly during storage, it did not return to the original levels after storage at 7°C for 2 weeks. The authors concluded that if AFM_1 is present in milk used to manufacture cultured dairy products, it still remains in these products. They indicated that variability in recovery of AFM_1 was a problem in these experiments as was the case for cheese as reported by Brackett and Marth (1982), and that a greater understanding of the casein-AFM_1 association is needed before its behavior in dairy products can be completely understood.

Strzelecki (1973) reported a slight decrease in the recovery of aflatoxins from raw ham, cured ham and salami. Using different time periods and storage conditions, they found recoveries of 19% from salami, 16% from raw ham and 7% for cured ham, with the amount retained being related to the aflatoxin level added initially to the products. Murthy

et al. (1975) also noted a decrease in the recovery of AFB_1 injected into beef with increasing storage periods. They suggested that incomplete extraction due to the interaction of aflatoxins with the meat constituents during storage probably accounted for the losses.

Furtado et al. (1982) studied the effects of cooking and/or processing upon levels of aflatoxins in meat from pigs fed a contaminated diet for 42 days. Although some destruction of aflatoxins B_1 and B_2 occurred during cooking and/or processing, the maximum amount of inactivation did not exceed 41% of the total, and was generally in the range of 15 to 30%. They concluded that aflatoxins are quite stable during cooking and/or processing.

Jacobson and Wiseman (1974) stored eggs laid by hens fed an aflatoxin contaminated diet at $3^{\circ}C$ for a period of 9 months. The average level of aflatoxins in the eggs before storage was 1.4 ug/kg. No aflatoxins could be detected in the eggs after storage, thus, they concluded that the toxin is not stable for long periods of time.

EXPERIMENTAL

FEEDING TRIAL

Preparation of the Diet

In order to prepare the spiked diet, 750 mg of pure AFB_1 (Polysciences, Inc.) and 380 mg of crystalline AFB_2 (Calbiochem-Behring Corp.) were dissolved in chloroform and diluted to 1 L in a volumetric flask. The aflatoxin solution was then stirred in the dark for one hour and divided into two-500 mL portions, which were added to 1 kg of ground commercial layer mash (Ralston Purina, St. Louis, MO), that had been dried over night in a 100°C oven. The specifications for the nutrient composition of the layers mash are shown in Tables 2 and 3. Initial mixing was done with a Kitchen Aid Mixer Model k5A (Hobart MFG Co.) for two hours. The chloroform was evaporated in the dark under the hood for four additional hours.

The two-1 kg batches of aflatoxin-spiked mash were thoroughly mixed and divided into five-400 g lots. Each lot was then mixed with additional layers mash in a stainless steel Wenger Mixer Model 61129 (Wenger MFG Co.) and divided into ten bags containing approximately 22 kg each. To ensure mixing and homogeneity of aflatoxins in all of the bags, their contents were remixed in groups of two. The feed was packed in black plastic bags containing approximately 11-12 kg each and stored at 5°C until used. The unspiked mash used for the control group and during the clearance period was

Table 2 - Nutrient Specifications of the Layer Hen Mash

Nutrient	Specification
ABS* Weight	1.0
Min* Protein(1	1.255
Min Lysine(1	0.053
Min Methionine(1	0.024
Min Methionine + Cystine(1	0.041
Min Tryptophan (1	0.012
ABS Metabolizable Energy(2	1225-1400
Min Total Fat(1	0.0
Max Fiber(3	10.0
Max Ash(3	15.0
Min Calcium(1	0.255
Max Calcium(1	-
Min Available Phosphorus(1	0.037
Min Total Phosphorus(3	0.0
Min Sodium(1	0.0155
Max Sodium(1	0.02
ABS Trace Minerals(3	-
Min Xanthophyl(4	7.0
ABS Vitamin Mix(3	-

*ABS = absolute or exact; Min = minimum; Max = maximum

1) Expressed as percent of metabolizable energy

2) Kilocalories per pound

3) Expresed as percent of total ration

4) Expressed in mg per pound of feed

Table 3 - Vitamin and Trace Mineral Mix for Layer Mash

Nutrient	Amount per pound of feed
Vitamin A, I. U.	4,000
Vitamin D_3, I. C. U.	1,112.5
Riboflavin, mg	3.5
Pantothenic acid, mg	6.0
Niacin, mg	12.5
Choline chloride, mg	200.0
Folic acid, mg	0.5
Vitamin B_{12}, mg	0.006
Vitamin E, I. U.	3.5
Menadione Sodium Bisulfate, mg	0.75
Manganese, mg	30
Iodine, mg	0.70
Copper, mg	1.80
Iron, mg	1.40
Zinc, mg	30.0

also stored under refrigeration until fed.

Experimental Birds

One hundred White Leghorn pullets of the Shaver strain were purchased from a commercial pullet grower at approximately 18 weeks of age. When received, they were housed in individual wire cages in the Poultry Research Center at Michigan State University. The pullets were subjected to a 15 hr/day light regime and allowed free access to feed and water.

Egg production records were kept daily during the prestudy period to select hens with a uniform rate of egg production for the aflatoxin feeding trial. At approximately 27-28 weeks of age, the hens were weighed using a Toledo hanging scale, Model 2110. Eighty-four hens, selected on the basis of body weight and egg production, were randomly distributed into one group of 16 hens and 8 groups of 8 hens each. Two extra hens in each treatment (aflatoxin-fed and control) were included. The group of 16 hens and 6 groups of 8 hens were fed the aflatoxin spiked mash for 4 weeks, while the other two groups were fed the unspiked mash. The spiked mash contained 3,310 ug of AFB_1 and 1,680 ug of AFB_2 per kg of feed.

Egg production was recorded daily. Eggs were identified by hen number, dated, weighed, and stored at $3^{\circ}C$ until analyzed for aflatoxin residues. Feed consumption was determined weekly.

All hens were weighed at the end of the aflatoxin

feeding period. At this time, 16 hens in the aflatoxin group and 8 controls were sacrificed. Breast, legs, liver, gizzard, kidneys, spleen, heart and ovaries of the sacrificed birds were removed, weighed, frozen and stored at -20°C until analyzed for aflatoxin residues. After slaughtering, the excised tissues were examined for gross lesions.

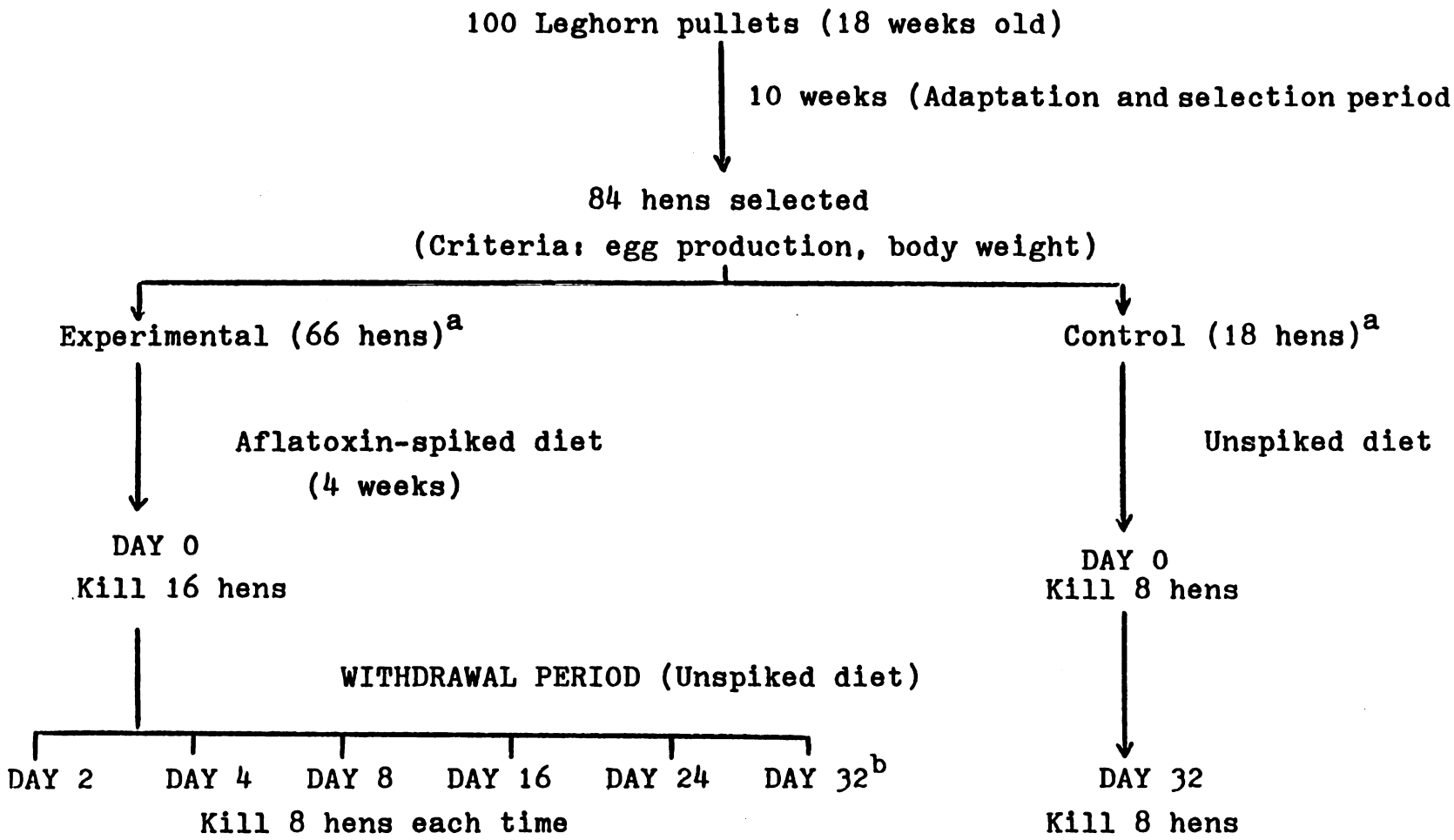
The remaining aflatoxin-fed hens (48) were fed the aflatoxin free mash to determine the time necessary to return to normal egg production and size, as well as to obtain egg and tissue clearance of aflatoxin residues. Eight hens were sacrificed at 2, 4, 8, 16, 24 and 32 days after replacing the aflatoxin-spiked diet with the uncontaminated feed. On day 32, the remaining 8 control hens were also sacrificed. All hens were weighed again at their corresponding killing time. Figure 5 schematically presents the experimental design.

ANALYSIS OF AFLATOXINS

Analysis of Aflatoxins in Eggs

Sample Preparation

Whole Eggs. Eggs were weighed, shelled and separated using a plastic egg separator (Ecko Housewares Co.) to determine the percentage of shell, albumen and yolk. Complete separation was difficult because the eggs were not very fresh at the time of analysis. The albumen and yolk of two to three eggs from the same day's production of each experimental



a) Two extra hens in each group (experimental and control)

b) One hen died during the third week of aflatoxin feeding. Probable cause, lymphoid leucosis

Figure 5 - Experimental Design of the Feeding Trial

group, were then remixed to give samples of approximately 90 to 100 g, which were used for aflatoxin analysis.

Separated Eggs. The combined yolks and albumen from five to six eggs were analyzed separately. The sample size in each case was also 90 to 100 g.

Extraction of Aflatoxins from Eggs

Extraction and analysis of aflatoxins from whole and separated eggs were carried out according to a modification of the procedures of Trucksess and Stoloff (1979) for the determination of aflatoxins B_1 and M_1 in liver, and of Gregory and Manley (1981) for aflatoxin analysis in animal tissues and products.

A total of 42 mL of saturated NaCl solution (40 g NaCl in 100 mL distilled H_2O) were added to about 100g of egg in a 250 mL beaker, which was placed in a 60°C water bath for 25 min. At the end of this heating period, the temperature of the water bath was increased to 72°C and the sample was held in the water bath for an additional 20 min. The egg sample was transferred to a blender jar, approximately 10 g of citric acid were added and the mixture was blended for 1 min in a Waring blender at moderate speed. Then 300 mL of acetone were added to the homogenate, with part of the acetone being used to wash both the sample beaker and the walls of the blender jar. The mixture was blended for 2 min at moderate speed followed by 1 min at high speed. The

material was then filtered through fast filtering prefolded filter paper (Whatman 114v) and the measured filtrate (230-250 mL) was transferred to a 500 mL Erlenmeyer flask. Then 20 mL of $Pb(OAc)_2$ solution (200 g $Pb(OAc)_2 \cdot 3\ H_2O$ in 500 mL H_2O containing 3 mL of acetic acid and made to volume of 1 L with distilled H_2O) and 150 mL distilled H_2O were added. The solution was stirred for 1 min, using a magnetic stirring device. Then 12 g of $(NH_4)_2SO_4$ and 10 g of Celite were added separately and in sequence to the solution with 0.5 min stirring after each addition. The solution was allowed to stand for about 5 min before filtering into a 500 mL graduated cylinder using fast filtering prefolded paper.

Purification of Aflatoxins

Liquid-Liquid Partition

The filtrate (325-350 mL) was transferred to a 500 mL separatory funnel to which 100 mL of petroleum ether (30-60°C b.p.) were added. The separatory funnel was shaken vigorously for 1 min and the layers allowed to separate. The lower aqueous-acetone fraction was drained into a clean 500 mL separatory funnel, while the upper petroleum ether containing lipid and liposoluble pigments was discarded. The aqueous-acetone solution was sequentially extracted with 50 mL of chloroform and 50 mL of chloroform-acetone (1:1) with 1 min of vigorous shaking after each solvent addition. The lower chloroform-acetone layer after extraction was

collected in a 500 mL round bottom flask. The remaining aqueous phase was then discarded. The combined chloroform-acetone extract was then evaporated to dryness in a rotary evaporator (Büchi, Switzerland) with a water bath temperature of 37°C.

Cleanup by Silica Gel Column Chromatography

A 25 x 250 mm Fischer and Porter modular chromatographic column was filled with chloroform. Approximately 10 g of anhydrous Na_2SO_4 were added to the column. A slurry of 10 g of silica gel 60 (70-230 mesh ASTM, E. Merck Reagents) in 50 mL of chloroform was added and the chloroform was drained to the top of the silica gel. Air bubbles were forced out using a mechanincal vibrator. The column was filled again with chloroform and 15 g of anhydrous Na_2SO_4 were carefully layered on top of the silica gel while the chloroform was drained. This prevented mixing of the silica gel and Na_2SO_4.

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 using 5 mL of chloroform-hexane (1:1) each time, and the washings were added to the column. The column was then drained to the top of the packing and the eluate was discarded. Low polarity interfering materials were eluted from the column with 100 mL of ether-hexane (3:1) and then discarded.

The aflatoxins were eluted from the silica gel column

with 150 mL of chloroform-methanol (95:5). The eluate was collected in a 500 mL round bottom 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 a 2 dram vial 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 40°C water bath. Overheating of the extract was avoided to prevent sample decomposition. The aflatoxin extract was dissolved in 100 uL of benzene-acetonitrile (98:2).

The vial was tightly closed with a Teflon lined screw cap. The samples were held in the vials at -20°C until removed for quantification of the aflatoxins by thin layer chromatography. At the time of analysis, the sample vial was vigorously shaken for 1 min in a test tube shaker (Vortex-Genie, Scientific Industries, Inc.).

Thin Layer Chromatography

Qualitative Thin Layer Chromatography

Samples were screened in order to speed up and reduce the expense of analysis. Thin layer chromatography (TLC) plates (Sil-G-HR-25 Brinkman Instruments Co.) were scored and spotted as shown in Figure 6. Two samples were applied to each plate as shown. A 20 uL sample of the aflatoxin extract was applied to the sample spot with a 25 ul syringe

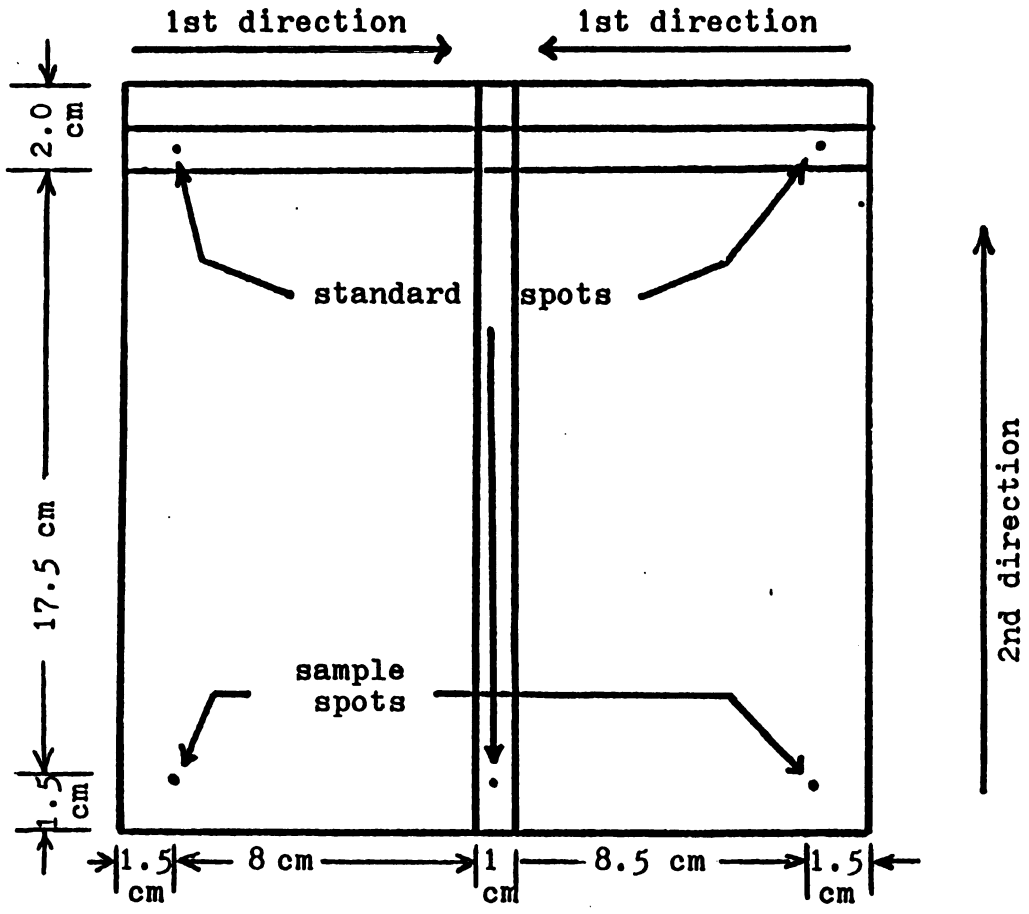


Figure 6 - Spotting and Scoring Pattern for Two-Dimension Double 10x20cm TLC (Screening)

(Hamilton Co.). Standards of 2.5, 0.75, 2.0 and 1.0 ng of aflatoxins B_1, B_2, M_1 and M_2, respectively, were spotted on the plate in both directions of development.

The plates were developed in the first direction in sequence for the two samples with diethyl ether-methanol-water (95:4:1) in an unlined, unequilibrated tank (Figure 6). After each run the plate was removed from the tank, dried under the hood for 2 min and transferred to a forced-draft oven at 45°C for 1 min. After evaporation of the solvent the plates were developed in the second direction with chloroform-acetone (83:17). In this case both samples were developed at the same time (Figure 6). After development, the plates were dried as described earlier and observed under long wavelength UV light in order to screen them for the presence of aflatoxin residues. Samples which were positive on screening were then quantitated as described below.

Quantitative Thin Layer Chromatography

Precoated 20 x 20 cm silica gel plates (Sil-G-HR-25, Brinkman Instruments Co.) were scored (Schoeffel Scoring Device SDA 303) to provide strips 10 mm wide, and were spotted as shown in Figure 7. Just before use, the plates were activated by drying in an oven at 100°C for 30 min which was followed by cooling for approximately 10 min. A 20 uL sample of the aflatoxin extract was applied to the sample spot with a 25 uL syringe (Hamilton Co.). Standards of 2.5, 0.75, 2.5, 0.75, 2.0 and 1.0 ng of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2,

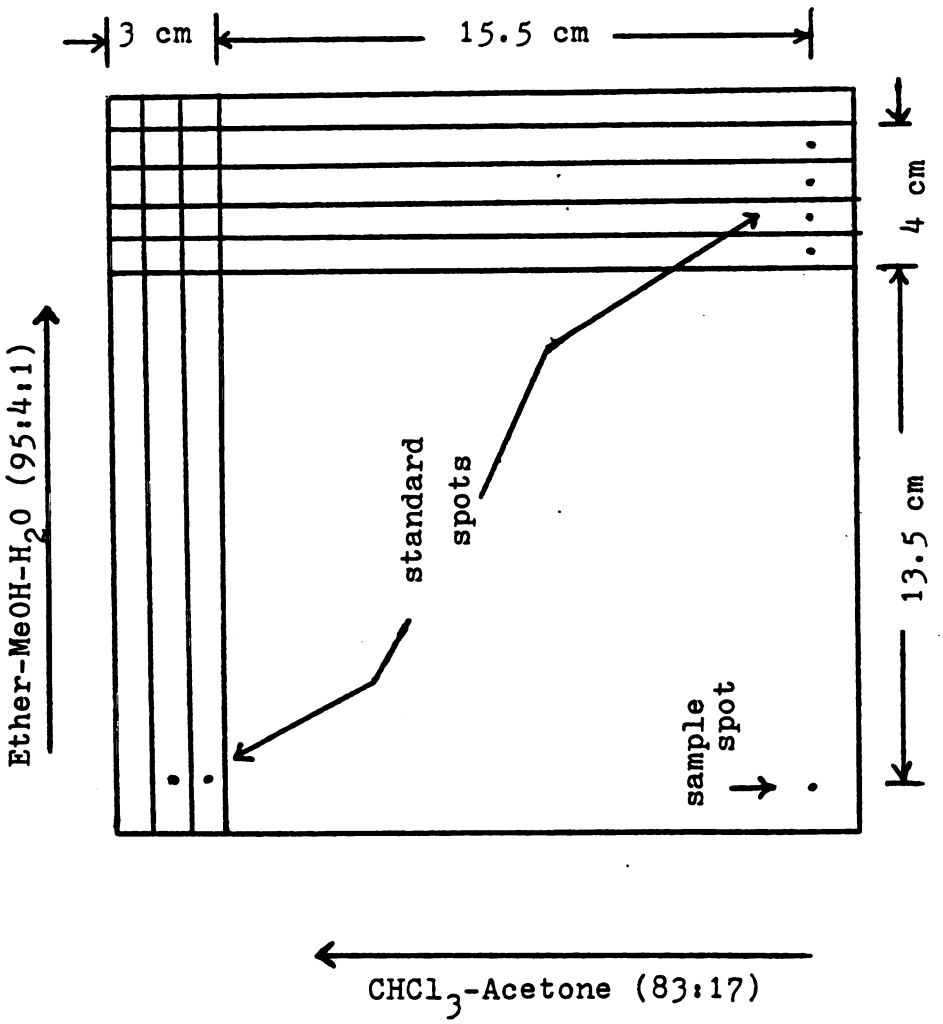


Figure 7 - Spotting and Scoring Pattern for Two-Dimensional 20 x 20 cm TLC plates

respectively, were spotted on the plates in the inner channel in the first direction and in the two inner channels for the second direction.

Aflatoxin B_{2a} standards were prepared in the plate on the outer channel in the first direction, and in the two outer channels in the second direction. In each of these, 1.25 ng of AFB_1 were spotted and then overspotted with 1 uL of trifluoroacetic acid (TFA)-chloroform (1:1). The plate was allowed to stand in the dark for about 5 min at room temperature. Then the plate was dried in a forced-draft oven at 45°C for 10 min. The AFB_{2a} standard was prepared before spotting the other standards and sample to prevent decomposition by the heating step.

The plates were developed in the first dimension with diethyl ether-methanol-water (95:4:1) in a sealed unequilibrated tank. After development in the first direction was completed, the plate was removed from the tank and dried under a hood for about 2 min. Drying was completed by placing the plate in a forced-draft oven at 45°C for 1 min. Development in the second dimension was done immediately with chloroform-acetone (83:17).

After development was completed, the plates were removed and dried as described before. After drying, the plates were examined visually under UV light and prepared for densitometric analysis. Each spot was localized within 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 direction of development.

Densitometric Analysis

Aflatoxin spots on the TLC plate were quantified using a double beam spectrophotometer SD3000-4 (Schoeffel Instruments) equipped with a 3380-A integrator (Hewlett Packard). The average of two readings of the aflatoxin reference standards spotted within the two strips parallel to the second direction of development was used for densitometric comparison when calculating the concentration of the sample spots. The reading of both standard and sample spots was done by placing the plate on the carrier and localizing the position of each spot with the beam of light set at 540 nm (green light). Then each spot was scanned by driving parallel to the second dimension. The spectrodensitometer was operated in the reflectance mode with the excitation wavelength set at 365 nm. Emitted fluorescence (425 nm) was collected with a secondary filter (430 nm band) which screens out all UV light and allows only emitted or visible fluorescent light to pass into the phototube.

The concentration of aflatoxins in the samples were calculated according to the formula:

$$\frac{ug}{kg} = \frac{B \times Y \times S \times V}{Z \times X \times W}$$

where: B = area of the aflatoxin peak in the sample spot;

Y = concentration of aflatoxin standard in ug/mL;

V = dilution of the sample extract in uL;

S = uL of sample extract;

Z = area of the aflatoxin standard peak;

X = uL of sample extract spotted on the plate;

and W = grams of sample in the final extract.

The weight of sample represented in the final aflatoxin extract (W) was calculated on the basis of the water content of the sample, the volume of the extracting solvents added to the sample homogenate and the volume of filtrate collected in the two filtration steps.

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

$$W = \frac{V_1}{V_{acetone} + V_{NaCl} + \underset{\text{in sample}}{\text{mL } H_2O}} \times \frac{V_2}{V_{\underset{\text{sln}}{Pb(OAc)_2}} + V_{H_2O} + V_1} \times W_s$$

Ws represents the weight of sample analyzed, and V_1 and V_2 are the volumes of the first and second filtrate, respectively. In the analysis of eggs, the volume of acetone was 300 mL, NaCl was 42 mL, $Pb(OAc)_2$ was 20 mL and water was 150 mL.

Analysis of Aflatoxins in Tissues

Sample Preparation

The tissues were stored at -20°C until they were defrosted and prepared for analysis. Leg and breast muscle samples were deboned and the collagenous tissues were removed. The muscle samples were then passed through a meat grinder (Hobart Manufacturing Co.) using a plate having 0.48 cm diameter holes. The internal organs (heart, kidney, gizzard,

liver and spleen) were cut into small pieces and placed into the blender jar for extraction. The ovary samples included ovarian tissue, ova and partially formed eggs contained in the oviduct. These were chopped and mixed manually, and analyzed for aflatoxin residues by the method used for eggs.

At slaughter, the blood of the sacrificed hens was combined and treated with EDTA to prevent clotting. However, some clotting occurred and thus the samples were centrifuged. The separated serum and blood clot were analyzed separately.

Extraction of Aflatoxins from Tissues

The extraction and analysis of aflatoxins from the tissues were carried out according to a modification of the procedure of Trucksess and Stoloff (1979) as reported by Chen (1983). Blood samples were extracted by the method of Furtado (1980), which is similar to the one used for the analysis of the tissues herein, except for the larger sample size (100 g), and proportionately larger amounts of reagents.

About 25 g of tissue were blended for 2 min in a Waring blender at moderate speed with 20 mL of NaCl-citric acid solution (35g NaCl + 4.8g citric acid in 100 mL H_2O). Then, 150 mL of acetone were added to the homogenate, with part of the acetone being used to wash both the sample beaker and the sides of the blender jar. The tissue was blended for 3 min at moderate speed, followed by 2 min at high speed. The material was then filtered through fast filtering prefolded filter paper (Whatman 114v), and the filtrate was collected in a

300 mL Erlenmeyer flask. The blender jar was then washed with an additional 50 mL of acetone. After filtration the tissue residue was discarded and the flask containing the filtrate was stoppered and placed in the freezer at -20°C for a minimum of 3 hours to precipitate the excess fat.

The precipitated fat was removed by filtration, using the same type of filter paper. The filtrate was measured and transferred to another 500 mL Erlenmeyer flask. Then, 15 mL of $Pb(OAc)_2$ solution (200 g $Pb(OAc)_2 \cdot 3H_2O$ in 500 mL of H_2O containing 3 mL acetic acid and made to a volume of 1 L with H_2O), and 70 mL of H_2O were added. The solution was stirred using a magnetic stirring device for 1 min. Then, 5 g of $(NH_4)_2SO_4$ and 6.5 g of Celite were added separately and in sequence to the solution with 0.5 min stirring after each addition. The solution was allowed to stand for about 5 min before filtering into a 250 mL graduated cylinder using fast filtering prefolded filter paper.

Purification of the Aflatoxin Extract

Liquid-Liquid Partition

Purification of the filtrate from the tissues was performed as described earlier for the analysis of eggs (p. 75), except that 50 mL of petroleum ether were used instead of 100 mL as was the case for the eggs.

Cleanup by Silica Gel Column Chromatography

The procedure followed for the tissues was essentially the same as that described earlier for egg analysis (p. 76) with three exceptions. First, only 50 mL of ether-hexane (3:1) were used to elute low polarity interferring materials, second, 150 mL of chloroform-methanol (97:3) were used to elute the aflatoxins from the column, and third, 50 uL of benzene-acetonitrile (90:10) were used to dissolve the aflatoxin extract.

Quantitative 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 7. A 20 uL sample of the aflatoxin extract was applied to the sample spot with a 25 uL syringe (Hamilton Co.). Standards of aflatoxins B_1, B_2, M_1 and M_2, were spotted on the plates in both directions of development about 1.5 cm from the edges.

The plates were developed in the first direction with chloroform-acetone (87:13) in a sealed and unequilibrated tank. After the development in the first direction was completed, the plates were removed from the tank and dried under a hood for about 2 minutes. The drying was completed by placing the plate in a forced-draft oven for 1 min at 45°C. The plates were immediately developed in the second dimension with ether-methanol-water (95:4:1). 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. Each spot was localized within 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 direction of development.

Densitometric Analysis

Aflatoxin spots on the TLC plate were quantified using a double beam spectrophotometer SD3000-4 (Schoeffel Instruments) equipped with a 3380-A integrator (Hewlett Packard) as described earlier for eggs (p. 82) with appropriate changes in the equations to calculate aflatoxin content in tissue samples.

Analysis of Aflatoxins in the Feed

Analysis of aflatoxins in the feed was carried out according to a modification of the AOAC method (1965). A 50 g sample of feed was weighed into a 500 mL Erlenmeyer flask. Then 25 mL of water, 25 g of Celite and 250 mL of chloroform were added. The flask was shaken for 30 min 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 silica gel TLC plates and quantified as described before for the analysis of aflatoxins in the eggs.

Confirmatory Tests for Aflatoxins

General Test for Aflatoxins

The presence of aflatoxins at low levels was indicated by spraying the TLC plate after development and identification of the spots under UV light with 25% sulfuric acid (v/v) (Przybylski, 1975). The plate was then dried in a forced-draft oven for 1 min. Changes in the characteristic fluorescence of aflatoxins B_1, B_2, M_1 and M_2 from blue and blue violet to yellow provided additional indication of aflatoxins. Spots with similar UV fluorescence to aflatoxins, which do not turn to yellow after H_2SO_4 treatment, are not due to aflatoxins. However, materials other than aflatoxin can turn yellow. Thus, this tests confirms absence of aflatoxin but does not consitute a definite proof of their presence (AOAC, 1975a).

Aflatoxin B_1

After two-dimensional development of the TLC plate as described earlier, the spot corresponding to AFB_1 was identified by comparison with AFB_1 standards in both directions of development, marked and quantified. When doubt existed as to the nature of the spot, the AFB_{2a} derivative was formed in the plate. The suspect spot corresponding to AFB_1 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 served as a guide to apply about 2.5 ng of AFB_1 standard close to the AFB_1 spot. The 1 uL of trifluoroacetic acid (TFA)-chloroform (1:1) was applied to both of the aflatoxin spots. The plate was allowed to stand in the dark at room temperature for about 5 min and was dried in a forced-draft oven at 45°C for 5 min. After cooling the plate in the dark, another 2.5 ng of AFB_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). When development was completed, the plate was dried and examined for the formation of AFB_{2a}. The chromatographic equivalence of the sample and the aflatoxin standard spots after treatment with TFA was used as a confirmatory test for identifying AFB_1.

Aflatoxin M_1

When development in the second direction was completed, the AFM_1 spot in the sample was marked in the silica gel on the left with a pencil along the direction of development. Another pencil mark was made about 3 cm apart to the right and close to the AFM_2 spot. The second mark was used as a guide for applying 2.0 ng of AFM_1 standard. One uL of TFA-chloroform (1:1) was then applied to both the sample spot and the AFM_1 standard. The plate was allowed to stand in

the dark at room temperature for about 5 min and then dried in a forced-draft oven at 75°C for 5 min. After cooling the plate to room temperature, another 1 ng of AFM_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 of development using chloroform-acetone (85:15). After development, the plate was dried and examined under UV light for the formation of AFM_1 derivative in order to ascertain if the R_f was lower than that of the unreacted AFM_1 standard. The chromatographic equivalence of the sample and the AFM_1 standard spot after treatment with TFA confirmed the identity of AFM_1.

Three Dimensional Chromatography

This procedure was used to compare the chromatographic mobilities of aflatoxin spots which are extracted from the TLC plate after two-dimensional development, with the chromatographic mobility of authentic standards in one or more additional solvent systems.

The aflatoxin spots in the sample were extracted from the plate as follows: A ball of glass wool was introduced into a 5 3/4 inches long glass disposable pipet, and a layer of about 0.5 cm of anhydrous Na_2SO_4 was added. The tip of the glass pipet was then attached to rubber vacuum tubing connected to a water aspirator. The spot on the TLC plate was observed under UV lights and marked on the silica gel with four pencil marks to define its boundary. Then about

2 uL of water were applied to the aflatoxin spot. The silica gel layer containing the aflatoxin was then removed by scraping it from the plate. The vacuum generated inside the pipet was adequate to aspirate and collect the flaked silica gel spot. The glass pipet was then disconnected from the aspirator and the aflatoxin was eluted from the silica gel using 3 mL of acetone. The eluate was collected in a 1 dram vial and the acetone evaporated under a gentle stream of nitrogen The extract was dissolved in 30 uL of chloroform, and was then spotted on a 20 x 20 cm TLC plate along with known standards. The plate was then developed with chloroform-acetone-isopropanol (85:10:5) to establish chromatographic equivalence with a third solvent system. This technique was particularly useful when trying to confirm the presence of a definite aflatoxin or aflatoxin metabolite, or in trying to establish the identity of an unknown spot.

Preparation of Reference Standards

Aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 were obtained from Sigma Chemical Company, St. Louis, MO., and used as reference standards. Aflatoxin standards were prepared according to the AOAC method (1975b). The solvent used for aflatoxins B_1, B_2, G_1 and G_2 standards was benzene-acetonitrile (98:2). The aflatoxin concentrations were 0.5, 0.15, 0.5 and 0.15 ug/mL, respectively. Aflatoxins M_1 and M_2 standards were prepared

in benzene-acetonitrile (90:10) at concentrations of 0.4 and 0.2 ug/mL, respectively. The standard solutions were stored in a desiccator at -20°C.

Moisture Analysis

The A.O.A.C. (1980) procedure for determining total solids in eggs was used. Five grams of liquid egg were accurately weighed into a previously dried (overnight at 98-100°C) and tared covered aluminum dish. The cover was removed and most of the water evaporated by heating on a steam bath. Drying was completed in a vacuum oven ($P < 25$ mm) for 6 hours. The dried sample was cooled in a desiccator and weighed. Loss in weight was reported as moisture per hundred grams of egg.

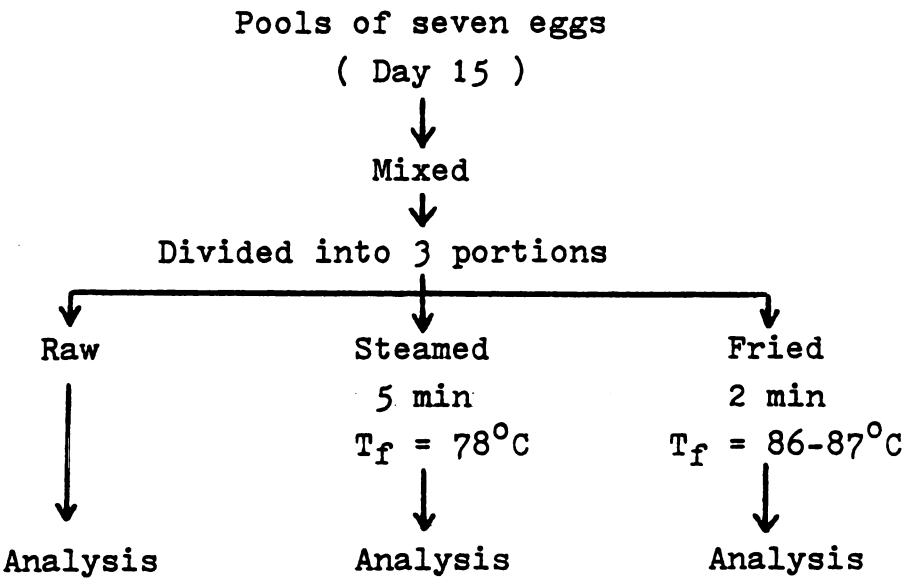
To determine the moisture content of steamed and fried eggs, two to three grams of finely comminuted sample were used. The initial evaporation step in the steam bath was omitted. Two replicates were run for each sample analyzed.

Cooking of Egg Samples

The effect of cooking on the levels of aflatoxins in the contaminated eggs was evaluated. Three sample pools of seven eggs from day 15 of aflatoxin feeding were made. Each pool was thoroughly mixed and divided into three samples. One of these was the control or raw sample. Another sample was steamed in an aluminum egg poacher (Mirro Corporation) for 5 minutes. The final temperature at the center of the

steamed egg was 78°C. The third sample was fried for 2 min with 5 g lard in an electric frying pan heated to 160°C. The final temperature at the center of the fried egg was 86-87°C. Moisture was determined in each sample as described earlier and the value was used in the calculation of weight of sample in the final extract in order to account for moisture changes during cooking. All samples were analyzed for aflatoxins by the modified egg method as described earlier herein.

The experimental design of the cooking experiments is summarized in the following figure:



The effect of cooking was also evaluated on spiked samples because of the very low levels in the naturally contaminated eggs. Samples were spiked with aflatoxins B_1, B_2, M_1 and M_2, and were cooked and analyzed as described earlier.

Safety Procedures

All glassware and vials in contact with aflatoxins were rinsed with 5-6% NaOCl (household bleach) to destroy any residual aflatoxins. During all work with aflatoxins, plastic disposable gloves were worn. A respiration mask was worn when mixing and handling the spiked ration. The surface of work areas was routinely scanned with an UV lamp and any contaminated areas treated by washing thoroughly with bleach. All TLC plates used in aflatoxin analysis were soaked in NaOCl solution before discarding. Filter papers and sample residues resulting from aflatoxin analysis were soaked overnight with concentrated ammonium hydroxide solution before discarding. These treated waste materials were collected in plastic bags and placed inside tightly closed containers which were labeled and removed by the M. S. U. Animal Waste Disposal Unit. The discarded solvents derived from the analysis were collected in labeled 5 gal containers and removed by the M. S. U. Chemical and Biologcial Safety Unit. All work involving aflatoxins, i.e., extraction, spotting, development and drying of the TLC plates was done under a hood. Similarly, any work involving the use of toxic solvents, such as ammonia, chloroform, benzene, methanol and acetonitrile was also carried out under a hood.

Statistical Analysis

Performance data for the aflatoxin-fed and control hens was analyzed by analysis of variance (Snedecor and Cochran, 1967). Dunnett's test (Dunnett, 1955; 1964) was used to compare various means against a control value.

Organ weights as percentage of body weights for the aflatoxin fed hens during clearance were analyzed by one way analysis of variance (Snedecor and Cochran, 1967). If an F test proved significant ($P < 0.05$), the Dunnett test for comparisons with a control was applied to determine significant differences between means at different times and the mean at 0 days after aflatoxin withdrawal (Dunnett, 1955, 1964).

RESULTS AND DISCUSSION

FEEDING TRIAL

The response of the hens to ingested aflatoxins was evaluated in terms of egg production and egg weights and of body weights and organ weights at the time of death. Feed consumption was measured weekly and represents average intake. Average aflatoxin intake was calculated from average feed consumption data and the level of aflatoxins in the diet.

Egg production and average egg weights for the aflatoxin-fed and control groups were calculated on a weekly basis during the aflatoxin feeding period (first four weeks). During the withdrawal period, these parameters were calculated for every time period as follows: Period 1 corresponds to the first two days after removal from the aflatoxin contaminated feed, Period 2 - days 2 to 4, Period 3 - days 4 through 8, Period 4 - days 8 to 16, Period 5 - days 16 through 24, and Period 6 - days 24 through 32, when all remaining hens were sacrificed.

Feed and Aflatoxin Consumption

Feed consumption is presented in Figure 8 and shows that it declined in the aflatoxin-fed group, particularly during the third week. Similarly egg production of

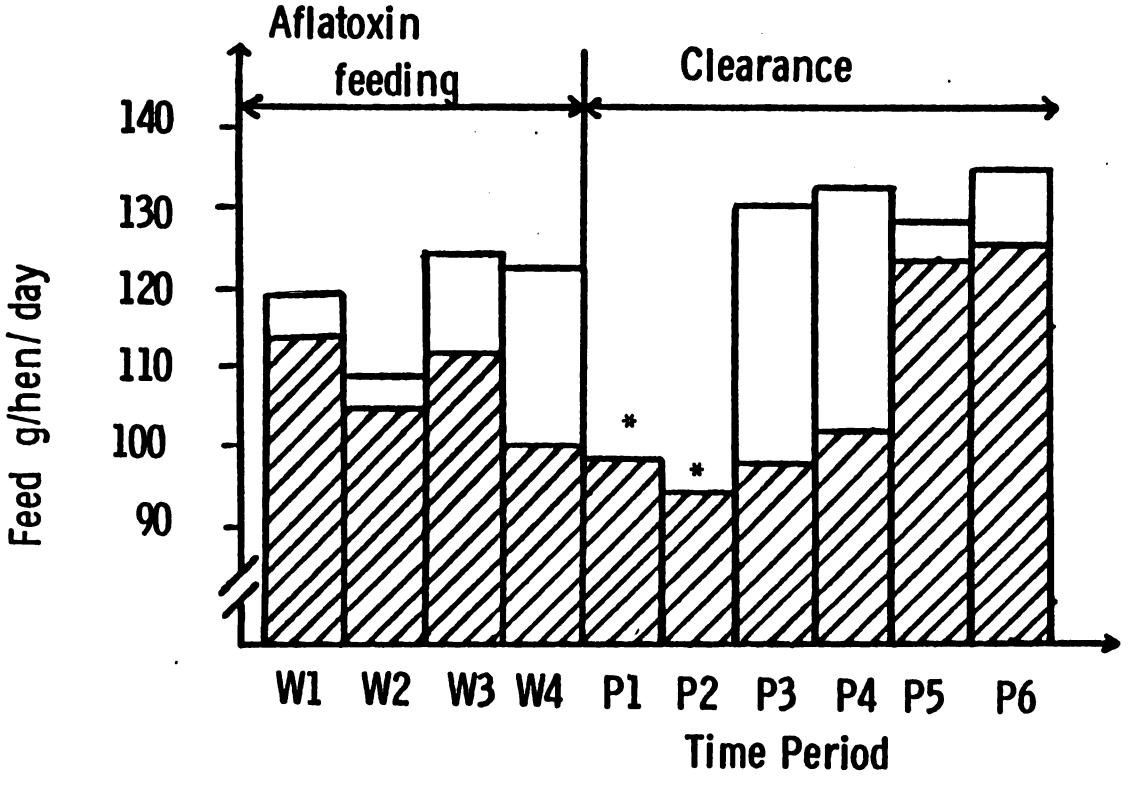


Figure 8 - Feed consumption by Laying Hens

* = No values for the control group available for these time periods

□ = Control; ▨ = Aflatoxin-fed

the group also decreased significantly during the third week. The decrease in feed consumption was reversed during the first week after withdrawal and eventually reached a value similar to that of the control group.

Although feed consumption data could not be analyzed statistically, during Period 3 the hens fed the aflatoxin-spiked ration showed a 26% reduction in feed intake in comparison to the controls.

Feed consumption values were used to estimate the average amounts of aflatoxins consumed by the hens during aflatoxin feeding. These results are presented in Table 4. The aflatoxin-spiked ration was found to contain 3,310 ug of AFB_1 and 1,680 ug of AFB_2 per kg. Total consumption of aflatoxins B_1 and B_2 was approximately 9,940 and 5,045 ug/hen, respectively. In expressing the amount of aflatoxins consumed, Armbrecht et al. (1971) have recommended use of the daily dosage rate (DR), which is the amount of toxin ingested daily per unit of body weight. This permits a better comparison of the effects of various detary levels since it corrects consumption for the body weight of the exposed animals. As shown in Table 4, the laying hens were exposed to a DR of 213 and 108 ug of aflatoxins B_1 and B_2 per kg of body weight, respectively.

Effect of Aflatoxins on Egg Production

Average egg production during the entire trial is summarized in Table 5. Egg production and egg weights of the aflatoxin-fed hens as a percentage of control values are shown

Table 4 - Aflatoxins Consumed by Laying Hens

Week	Aflatoxin B_1		Aflatoxin B_2	
	ug/hen/day	ug/hen	ug/hen/day	ug/hen
1	374	2,618	190	1,329
2	344	2,410	175	1,223
3	370	2,590	188	1,315
4	332	2,322	168	1,178
Total		9,940		5,045
Daily Dosage Rate, DR[1]		213		108

1) Calculated from the formula DR = Wa/0.5(Ws + We)t, where (Wa) = ug of aflatoxin ingested during the interval of time (t) in days, starting weight (Ws) and ending weight (We) in kg (Armbrecht et al., 1971).

in Table 6. The data indicate that egg production decreased significantly during the third week of aflatoxin feeding, reaching approximately 82% of the control value, and continued to decrease until it reached a minimum of 37% by days 2 to 4 (Period 2) after removal from the aflatoxin-spiked ration. No significant difference in egg production between the aflatoxin-fed group and the control was found after one week on the aflatoxin-free diet. Although egg production was only 76, 86 and 78% of control during Periods 4, 5 and 6 of clearance, respectively, the difference in comparison to the control was not significant ($P < 0.05$).

Figure 9 shows egg production curves of the aflatoxin-fed and control hens during the entire feeding trial.

Table 5 - Effect of Aflatoxins on Egg Production

Time Period	Percent Egg Production[1]					
	Aflatoxin-fed			Control		
	n	Mean[2]	S.E.[3]	n	Mean[2]	S.E.[3]
Aflatoxin Feeding						
Week 1	63	92.8	1.19	16	93.7	2.36
Week 2	63	89.9	1.14	16	93.8	2.26
Week 3	63	78.8[a]	1.56	16	96.5[b]	3.09
Week 4	63	57.7[c]	2.45	16	95.6[d]	4.86
Withdrawal Trial						
Period 1 (0-2 days)	47	46.8[e]	5.08	8	93.7[f]	12.30
Period 2 (2-4 days)	39	34.6[g]	4.95	8	93.7[h]	10.92
Period 3 (4-8 days)	31	41.2[i]	5.67	8	87.5[j]	11.16
Period 4 (8-16 days)	23	64.3	8.29	8	84.4	14.06
Period 5 (16-24 days)	15	68.3	9.85	8	79.5	13.49
Period 6 (24-32 days)	7	57.3	15.69	8	73.5	14.68

1) Assuming 100% production = one egg/day during each time period.

2) Values in the same line with different superscripts differ significantly ($P < 0.01$)

3) Standard error of the mean, S.E. $= \sqrt{\frac{\text{Error mean square}}{n}}$

Table 6 - Effect of Aflatoxins on Egg Production and Egg Weights During the Feeding Trial

Time Period	Percent of Control Value[1]	
	Egg Production	Egg Weights
Aflatoxin feeding		
Week 1	99.0	100.0
Week 2	95.8	98.3
Week 3	81.7	96.8
Week 4	60.4	92.5
Withdrawal Trial		
Period 1 (0-2 days)	49.9	87.8
Period 2 (2-4 days)	36.9	90.7
Period 3 (4-8 days)	47.1	92.2
Period 4 (8-16 days)	76.2	96.1
Period 5 (16-24 days)	85.9	101.1
Period 6 (24-32 days)	78.0	101.0

1) Calculated from: $\frac{\text{Average value in aflatoxin group}}{\text{Average value in control group}} \times 100$ for both egg production and egg weights.

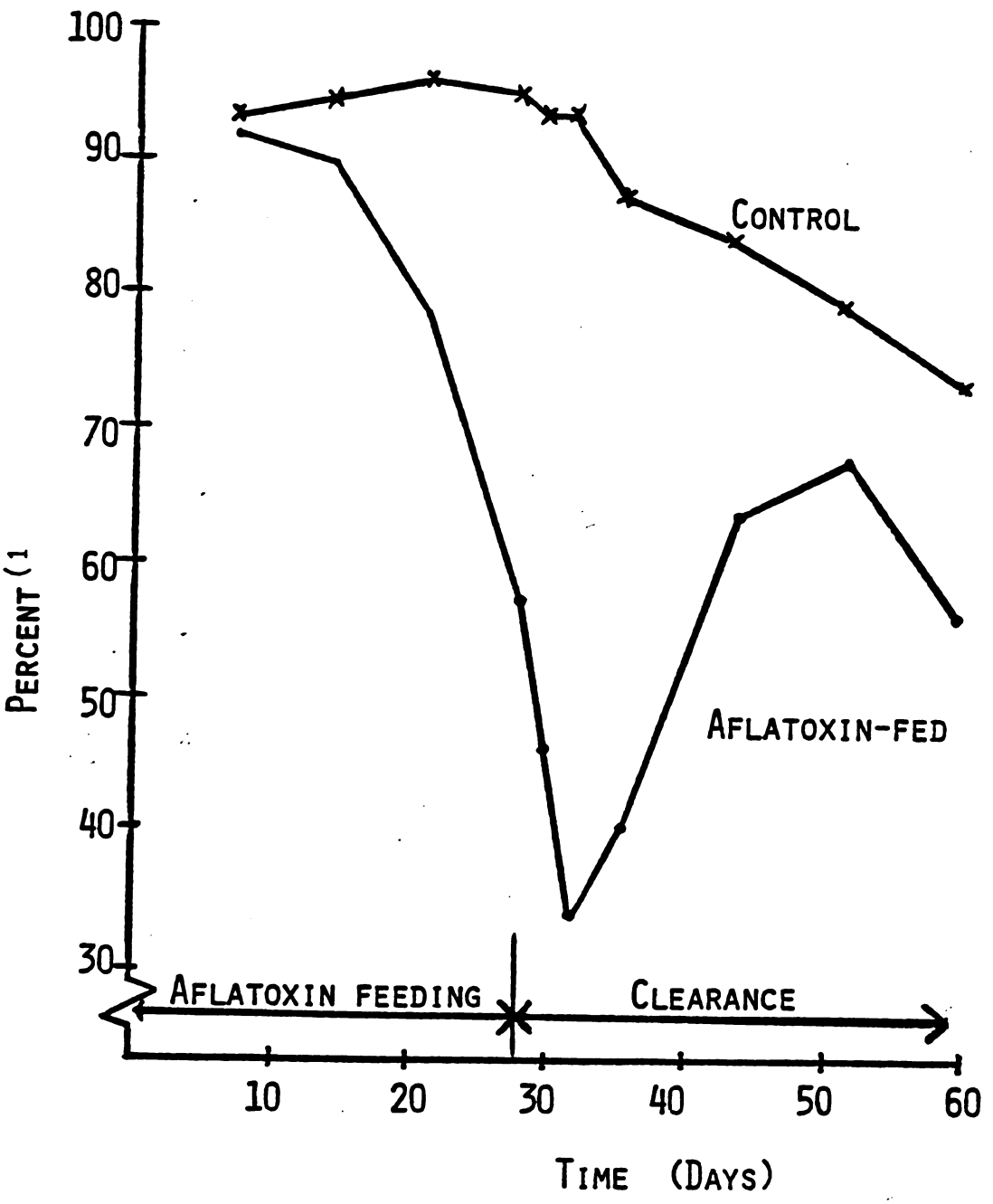


Figure 9 - Effect of Aflatoxins on Egg Production

1) 100% = one egg/hen/day

As illustrated, egg production of both the control and aflatoxin-fed groups decreased during the experiment. The aflatoxin-fed group, however, showed a dramatic decline during aflatoxin feeding, which was reversed after removal from the aflatoxin-spiked diet.

The onset and magnitude of the decrease in egg production, as well as the time required to return to normal egg production after removal of aflatoxins from the diet, are influenced by the level and duration of aflatoxin feeding (Hamilton and Garlich, 1972; Lötzsch and Leistner, 1976; Howarth and Wyatt, 1976). The delayed effect of aflatoxins on egg production observed here was also reported by Lötzsch and Leistner (1976), Howarth and Wyatt (1976) and Exarchos and Gentry (1982). Huff et al. (1975) proposed that the delayed effect may be the result of a compensation mechanism by which the reduced amounts of plasma lipids and proteins, which are precursors of egg lipids and proteins, may be preferentially channeled to ova already committed to maturation. Moreover, Garlich et al. (1973) noted that the decline in egg production associated with feeding high levels of aflatoxins can occur even after the hens are consuming an aflatoxin-free ration. After feeding lower amounts for longer periods of time, however, Lötzsch and Leistner (1976) reported that recovery in egg production may occur even before removal of the contaminated feed. They proposed that the laying hen may become adapted to the toxin and suggested that egg production is not a sensitive indicator of exposure to aflatoxin

contamination since a decline can occur after aflatoxins have disappeared from the diet.

Effect of Aflatoxins on Egg Weight

Egg weights were recorded daily and averaged for each hen over the same time intervals, i.e., weekly during the aflatoxin feeding period (first four weeks), and for every time interval after removal from the aflatoxin-spiked diet. As shown in Table 7, the average egg weight was significantly reduced during the fourth week of aflatoxin feeding and reached a minimum during the first two days after removal from the aflatoxin-spiked diet. The decline in egg weight was reversed after two days on the aflatoxin-free diet as shown in Figure 10 and reached control values by the second week.

Huff et al. (1975) proposed that the production of smaller eggs by aflatoxin-fed hens may also be a mechanism by which the hen compensates for the decrease in plasma proteins and lipids. They concluded that the decrease in plasma lipids and proteins observed in their study and by Garlich et al. (1973) for laying hens and by Tung et al. (1972) in young chicks resulted from an impairment of lipid transport and from decreased protein and fatty acid synthesis in the liver.

The average weight of eggs laid by the aflatoxin-fed hens reached a minimum of 53 g, which corresponded to approximately 88% of the controls (Table 6). Even though a 12%

Table 7 - Effect of Aflatoxins on Egg Weight

Time Period	Egg Weight, g					
	Aflatoxin-fed			Control		
	n	Mean[1]	S.E.[2]	n	Mean[1]	S.E.[2]
Aflatoxin Feeding						
Week 1	63	58.7	0.51	16	58.7	1.01
Week 2	63	58.1	0.52	16	59.1	1.04
Week 3	63	56.9	0.54	16	58.8	1.08
Week 4	61	54.6[a]	0.60	16	59.0[b]	1.17**
Withdrawal Trial						
Period 1 (0-2 days)	33	53.0[c]	0.92	8	60.4[d]	1.87**
Period 2 (2-4 days)	23	53.4[e]	0.98	8	58.9[f]	1.66**
Period 3 (4-8 days)	22	55.5[g]	1.03	8	60.2[h]	1.70*
Period 4 (8-16 days)	19	58.6	0.95	7	61.0	1.57
Period 5 (16-24 days)	13	61.8	1.36	7	61.1	1.86
Period 6 (24-32 days)	5	61.9	2.74	7	61.3	2.32

1) Values in the same line with different superscript differ significantly; $P < 0.01$ (**) or $P < 0.05$ (*)

2) Standard error of the mean, S.E. $= \sqrt{\frac{\text{Error mean square}}{n}}$

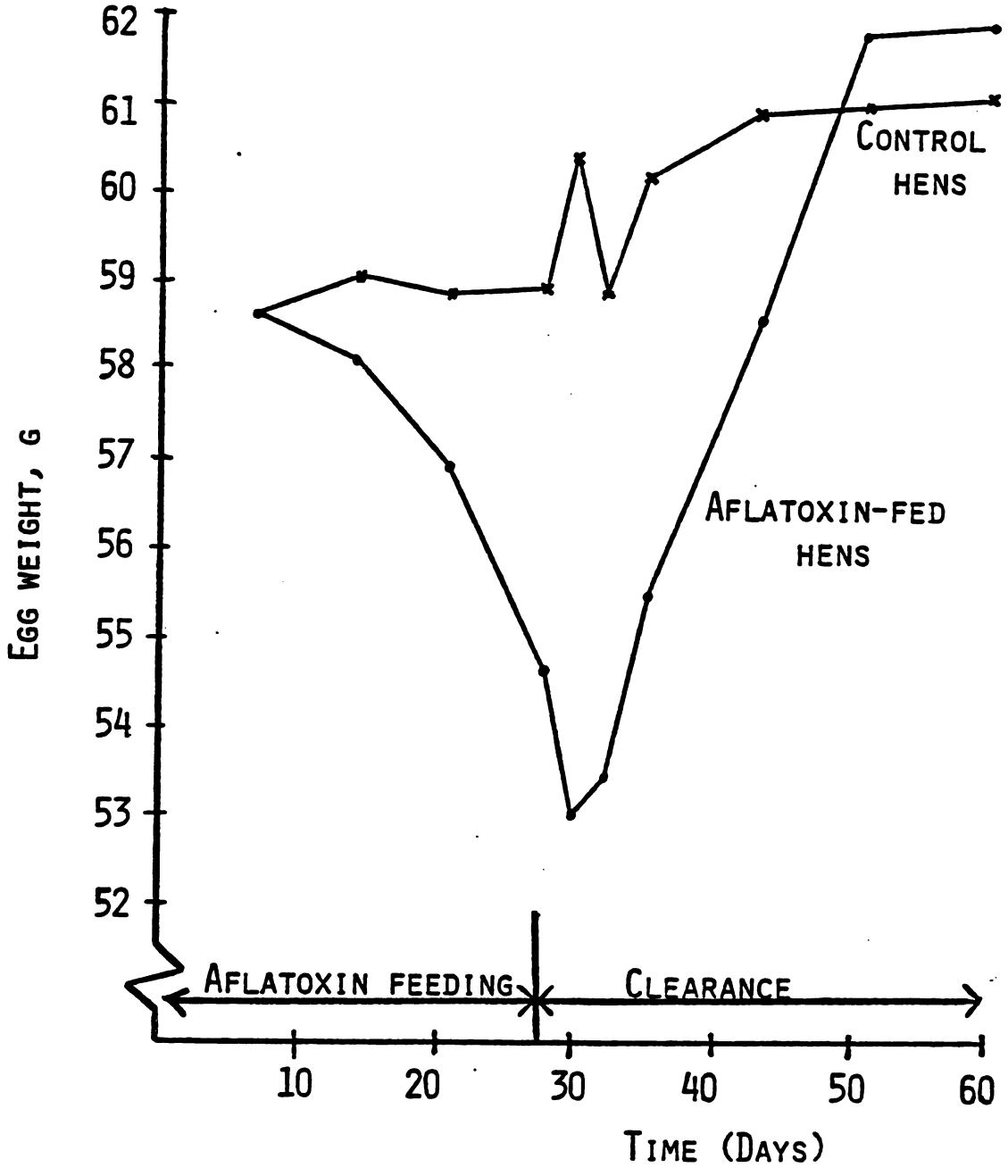


Figure 10 - Effect of Aflatoxins on Egg Weight

decrease in egg weight does not appear to be as dramatic as the decrese in egg production (37% of control values), the size reduction was enough to downgrade the eggs from large (56.7 to 63.8 g/egg) to medium (49.6 to 56.6 g/egg) for the last week of aflatoxin feeding through the first week after removal from the spiked diet.

Figure 11 presents the effects of aflatoxin feeding on egg production and egg weights in comparison to control values. The decrease in egg production and egg size showed delayed, but almost simultaneous expression, which was significant after 3 and 4 weeks of aflatoxin feeding, respectively. As pointed out earlier, the magnitude of the effects is different, being more dramatic for egg production. Both egg production and egg weights were not significantly different from controls during the second week of withdrawal from the contaminated feed. However, egg production was only 76% of the controls, while egg size was 96% of the control values. It appears from these results that the effect of aflatoxins on egg size and is less dramatic than that for egg production. These results are in general agreement with those of previous studies showing a more pronounced effect on egg production than on egg weights by aflatoxins (Huff et al., 1975; Washburn and Wyatt, 1978; Howarth and Wyatt, 1976).

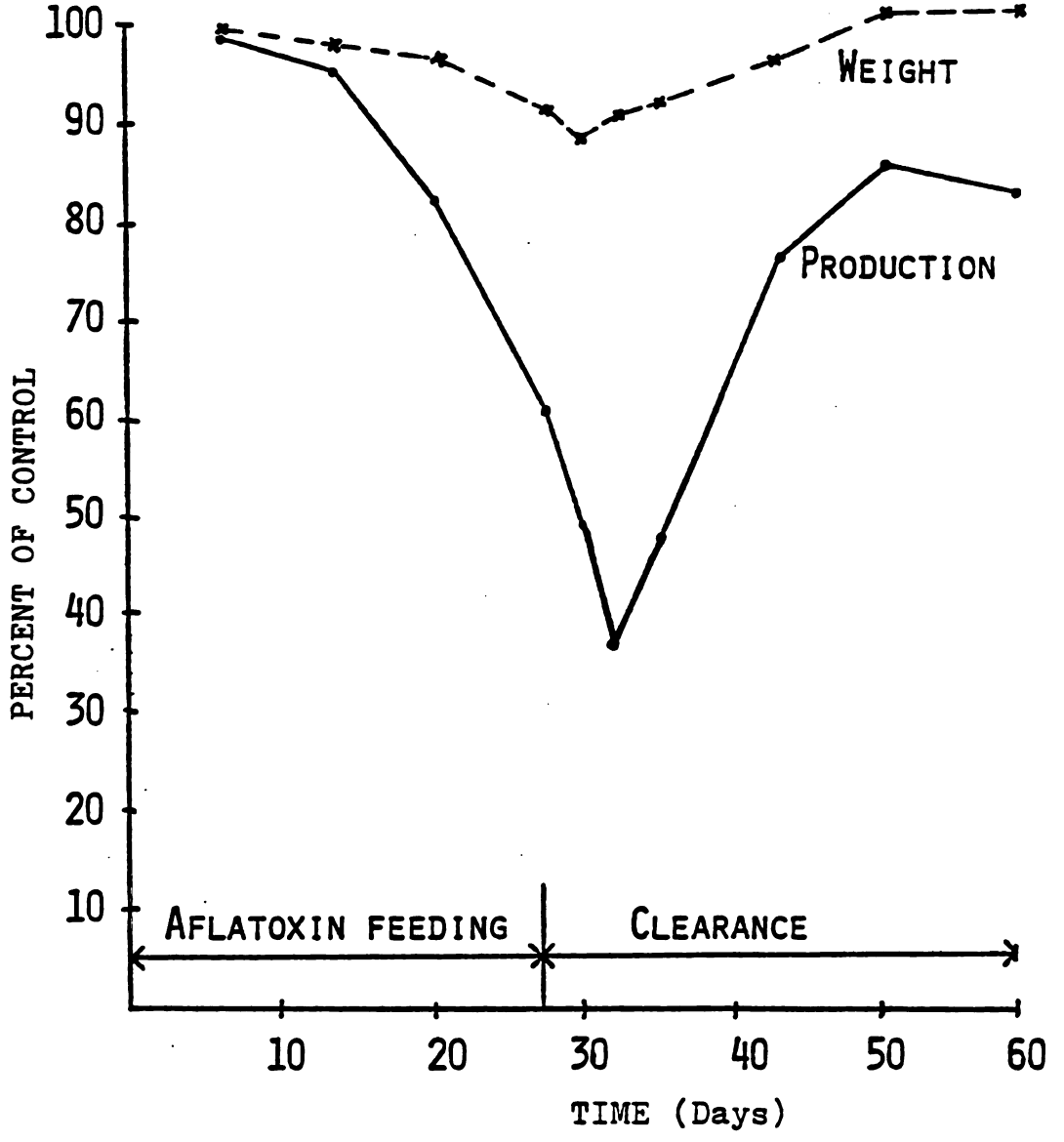


Figure 11 - Effect of Aflatoxins on Egg Production and Egg Weight Expressed as Percent of Control

Effects of Aflatoxins on Body Weights

The mean weights for the control and the aflatoxin-fed groups at the beginning of the feeding trial were 1,637 and 1,636 g, respectively, with a maximum average difference for all groups ammounting to 11 g. At the end of the aflatoxin feeding trial, the average weights for the controls and the aflatoxin-fed hens were similar (1,687 and 1,690 g, respectively). When final weights were compared regardless of killing time, no significant difference was found between the controls and the aflatoxin-fed hens, which averaged 1,727 and 1,665 g, respectively.

Figure 12 shows the average weights of the hens at different times. No control hens were killed at 2, 4, 8, 16 or 24 days after withdrawal from the aflatoxin-spiked diet. Thus, control hens were weighed only at the beginning and end of the aflatoxin feeding period and at the end of the withdrawal period. Weight changes of the aflatoxin-fed hens during the withdrawal trial shown in Figure 13 indicated that they lost weight during the first two weeks after withdrawal, while their weight remained practically constant during the last two weeks. Control hens showed a slight weight gain during the same time period.

The possibility that the weight losses of the treated hens after withdrawal may be due to a decrease in the size of their livers was examined. Table 8 summarizes the

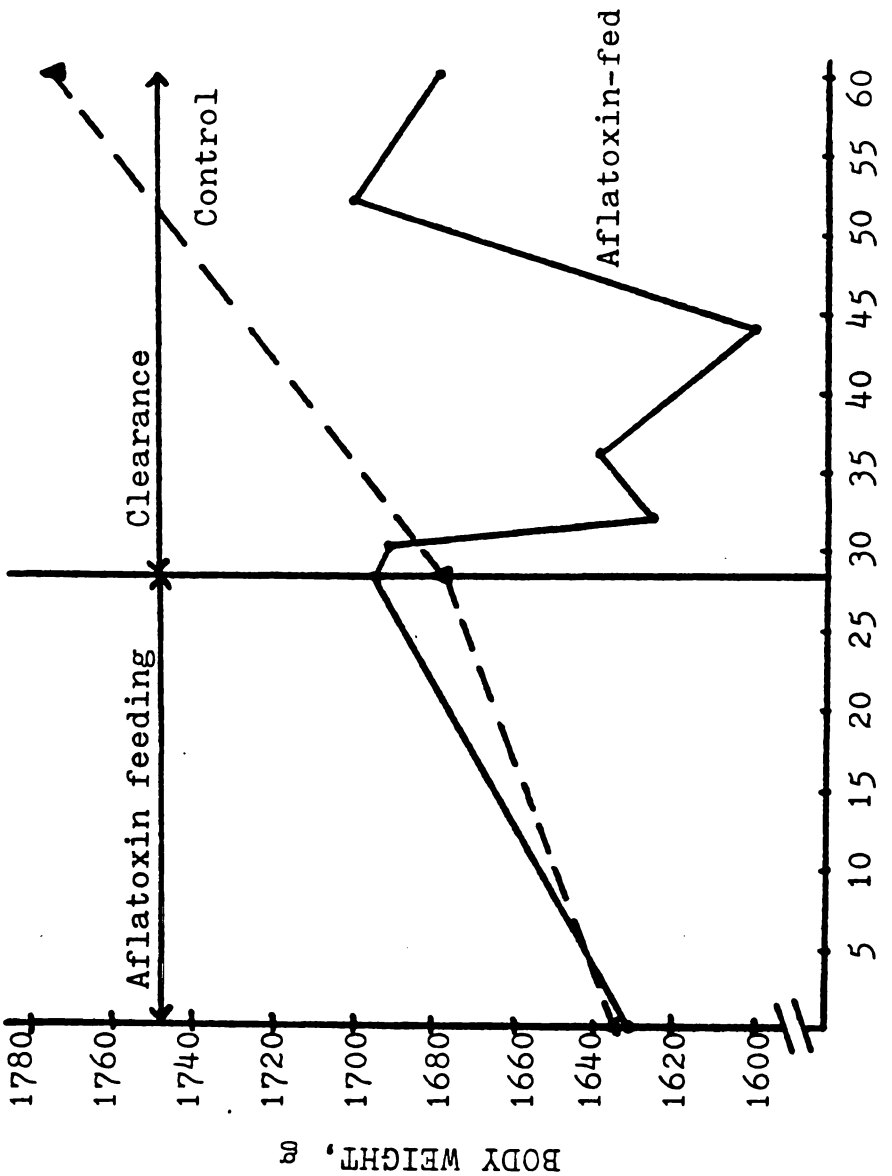


Figure 12 - Body Weight of Hens During the Feeding Trial

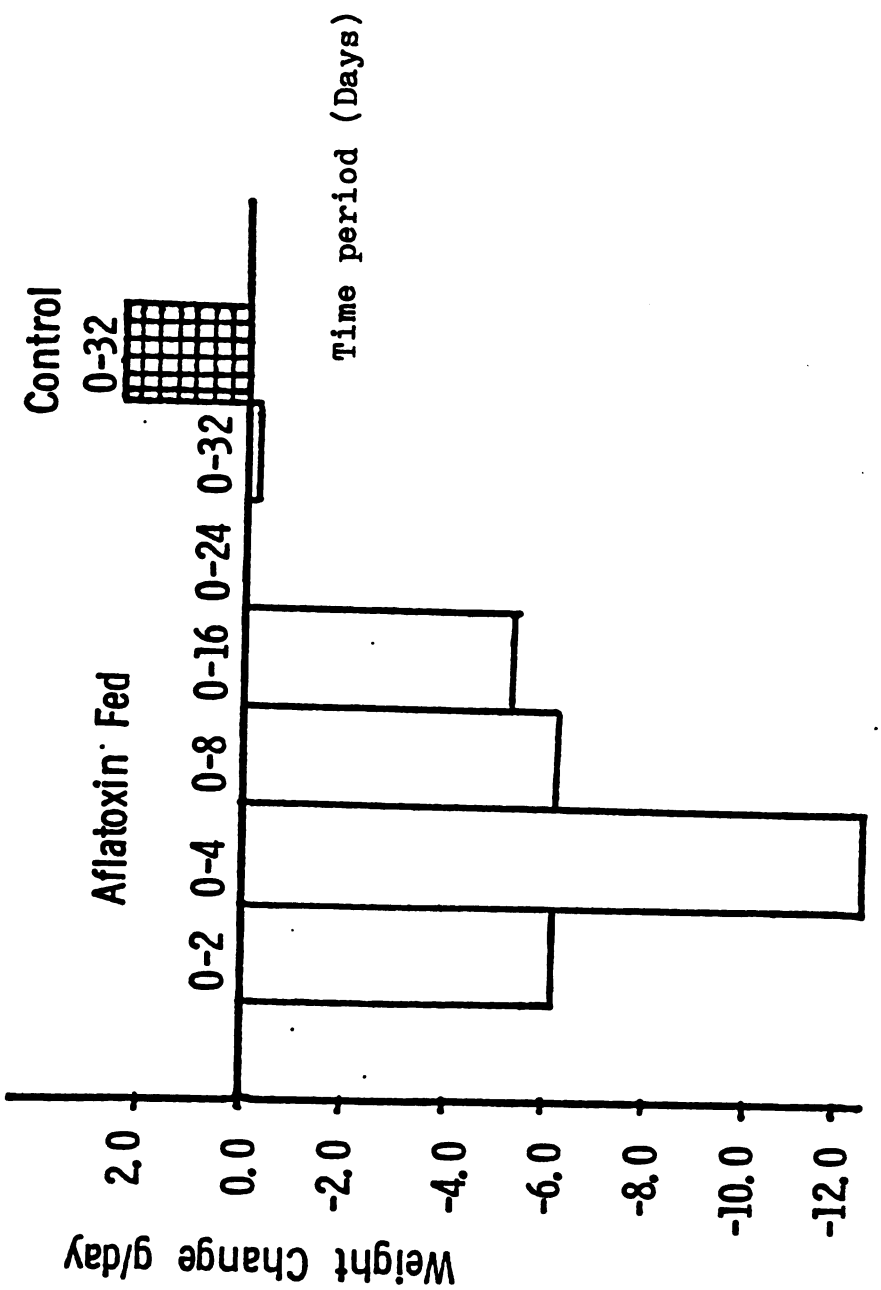


Figure 13 - Changes in Body Weight of Hens During the Withdrawal Trial

average liver weights of the hens killed at different times during the aflatoxin withdrawal period. The data indicate

Table 8 - Average Liver Weights of Aflatoxin-Fed Hens During Clearance

Days of Clearance	Liver Weight (g)	
	Mean	S.D.[1]
0	57.9	10.55
2	68.5	14.98
4	64.2	29.69
8	50.0	11.85
16	35.6	7.59
24	37.8	5.87
32	32.2	3.42

1) S. D. = standard deviation

that the liver weights continued to increase for two days after the hens were removed from the aflatoxin-spiked diet. Thus, changes in liver weight did not explain the weight loss suffered by the hens during the first four days after aflatoxin withdrawal. It seems more reasonable to suggest that this effect was related to lower feed consumption (Figure 8) and the general unthriftiness caused by the aflatoxins (Allcroft and Carnaghan, 1963).

Most studies on the effects of aflatoxins in laying hens have failed to demonstrate a significant influence on body weights. The only study in which a significant weight

reduction is reported occurred when the aflatoxins were administered per os, and did not occur when a comparable amount was fed (Sims et al., 1970). Weight loss and reduced growth, however, have been reported in growing chickens fed aflatoxins (Brown and Abrams, 1965; Gumbmann et al., 1970).

Gross Observations on Tissues

At slaughter, the hens were inspected for gross pathological lesions by Dr. Theo Coleman, Professor of Poultry Science at Michigan State University. The weights of the excised internal organs (liver, gizzard, spleen, heart, ovaries and kidneys) were recorded. The most obvious lesions found in the aflatoxin-fed hens killed at the end of the aflatoxin feeding period occurred in the liver and ovaries. Observations are summarized in Tables 9 and 10. Livers were enlarged and appeared slightly to very pale with some areas of petechial hemorrhages or with larger hemorrhages close to the edges.

The liver is the target organ for aflatoxins in many animal species (Hayes, 1980). Enlargement and fatty infiltration, which causes tha pale color of the liver, are both related to a higher lipid content resulting from impaired lipid transport during aflatoxicosis. Similar results have been reported in chickens (Tung et al., 1972; Van Zytveld et al., 1970; Chen et al., 1984), pigs (Keyl and Booth, 1971; Furtado et al., 1979), beef cattle (Keyl and Booth, 1971)

Table 9 - Effect of aflatoxins on the Liver of Hens[1]

Days of Withdrawal	n[2]	OBSERVATION		
		Pale liver	Hemorrhages	
			Petechial	Large Areas
0	16	16	5	4
0 Control	8	4	0	0
2	8	8	8	5
4	8	4	0	3
8	8	2	2	4
16	8	3	1	0
24	8	1	0	0
32	7	1	0	0
32 Control	8	1	0	0

1) Numbers in the table indicate the number of hens showing lesions at slaughter. Control values at 0 and 32 days are also included.

2) Total number of hens slaughtered

and quail (Gumbmann et al., 1970). In laying hens, Sims et al. (1970) observed enlarged, pale yellow livers with petechial and larger hemorrhages after aflatoxin feeding. Similarly, Van Zytveld et al. (1970) observed that the affected livers of aflatoxin-fed chicks were pale yellow and 'mottled' with small reddish spots.

Table 10 - Effect of Aflatoxins on Ova Development

Days of Withdrawal	Proportion of Hens Having Large Ova (Ø > 25 mm) at Death
0 Treated	0/16
0 Control	8/8
2	5/8
4	4/8
8	8/8
16	7/8
24	8/8
32 Treated	5/8
32 Control	6/8

Three of the 16 hens killed at 0 days had reabsorbed ova. As shown in Table 10 none of the aflatoxin-fed hens had ova larger than 25 mm in diameter at the end of the aflatoxin feeding period. Control hens showed an average of 6 large ova (Ø > 25 mm) at this time. Eight days after aflatoxin withdrawal, all hens sacrificed had large ova, thus in-

dicating that their laying potential had returned to normal. These results are in agreement with those of Trucksess et al. (1983), who reported that the livers and ovaries of laying hens were affected most during aflatoxin feeding.

Effects of Aflatoxins on Organ Weights

The average organ weights as a percentage of body weight at the end of the aflatoxin feeding period (0 days) and at the end of withdrawal (32 days) for the aflatoxin-fed and control hens are shown in Table 11. These results demonstrate that at the end of the aflatoxin feeding period the size of the livers, kidneys, ovaries, spleens and hearts from the aflatoxin-fed hens differed significantly from those of the control. Livers, kidneys and spleens were enlarged by approximately 61, 14 and 20% over control values, respectively. Ovaries and hearts of treated hens were smaller than those of controls by 27 and 7.5%, respectively. At the end of the withdrawal period, the weights of all organs, except for the hearts, were not significantly different from the controls, indicating that recovery had occurred.

Although the liver is the main target organ, AFB_1 is known to cause damage to other tissues and organs (Hayes, 1980; Wogan, 1973). Van Zytveld et al. (1970) reported pale, swollen and enlarged kidney in poultry fed aflatoxin contaminated rations. Chen (1983) reported that the kidneys of aflatoxin-fed chicks were 46% heavier than those of the

Table 11 - Effect of Aflatoxins on Organ Weights Expressed as Percent of Body Weight

Organ	Days after Withdrawal	Aflatoxin-fed			Control			
		n	Mean[1]	S.E.[2]	n	Mean[1]	S.E.[2]	
Liver	0	16	3.41[a]	0.12	8	2.12[b]	0.17	**
	32	7	1.93	0.14	8	2.18	0.13	
Gizzard	0	16	1.56	0.04	8	1.53	0.05	
	32	7	1.55	0.08	8	1.42	0.08	
Kidneys	0	16	0.48[c]	0.01	8	0.42[d]	0.02	*
	32	7	0.40	0.03	8	0.37	0.03	
Ovaries	0	16	2.35[e]	0.23	8	3.24[f]	0.33	*
	32	7	3.17	0.62	8	3.21	0.58	
Spleen	0	16	0.12[g]	0.00	8	0.10[h]	0.01	*
	32	7	0.13	0.02	8	0.10	0.02	
Heart	0	16	0.37[i]	0.01	8	0.40[j]	0.01	*
	32	7	0.39[k]	0.02	8	0.44[l]	0.01	*

1) Values in the same line with different superscripts differ significantly; $P < 0.01$ (**) or $P < 0.05$ (*)

2) Standard error of the mean, $S.E. = \sqrt{\frac{\text{Error mean square}}{n}}$

control group. Smith and Hamilton (1970) observed enlargement of the spleen and pancreas in young broiler chickens fed aflatoxins. Enlargement of the spleen and kidneys in laying hens after aflatoxin feeding was reported by Howarth and Wyatt (1976), while Hamilton and Garlich (1971) did not detect any changes in the same organs in laying hens. These differences are probably due to variation in the strains of birds and in the levels and/or purity of the aflatoxins fed as well as to duration of feeding.

The significantly smaller weight of the hearts from the aflatoxin-fed hens at the end of aflatoxin feeding and also at the end of the withdrawal trial was unexpected. In contrast, Chen (1983) reported that the hearts of aflatoxin-fed chickens were slightly enlarged over those of controls ($P < 0.10$). Butler (1966) reported that the only change seen in the hearts of guinea pigs dosed with AFB_1 was an occasional area of fatty degeneration of the myocardium. Although aflatoxin residues were detected in the hearts of the hens in the present study, it is not clear if this effect was caused by aflatoxins. Mabee and Chipley (1973b) and Sawhney et al. (1973) also detected aflatoxin residues in the heart of aflatoxin-fed hens, but reported that there were no evident lesions in any of the tissues of the test hens.

Average organ weights as percent of body weight for the aflatoxin-fed hens during the withdrawal period are presented in Figure 14. Control values at 0 and 32 days of withdrawal are also indicated. The graph indicates that

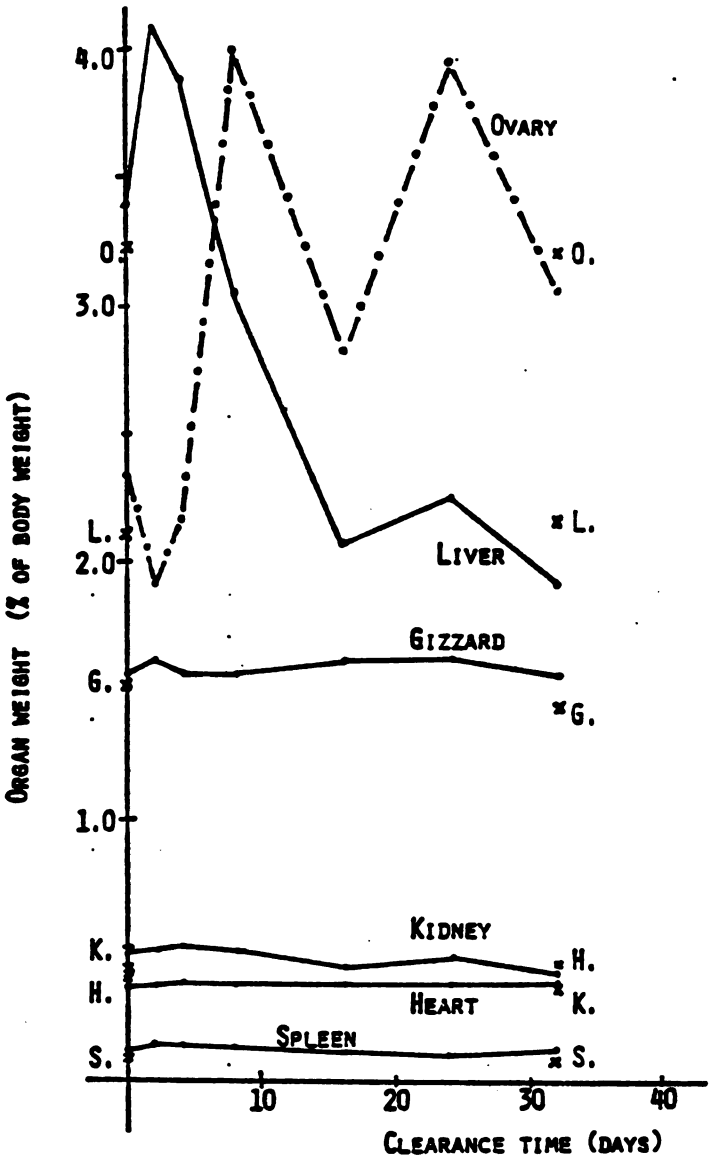


Figure 14 - Organ Weights as Percent of Body Weight of Hens During the Withdrawal Trial

Control values are indicated by an x at 0 and 32 days, O, L, G, H, K and S, representing ovaries, liver, gizzard, heart, kidneys and spleen, respectively.

the size of the spleen, heart, kidneys and gizzard showed little variation, whereas, the livers and ovaries changed markedly during withdrawal, but were similar to control values at 32 days.

Present results are in general agreement with previous reports indicating a relatively fast recovery in organ weights upon removal of aflatoxins from the feed (Blount, 1961; Smith and Hamilton, 1970; Hamilton and Garlich, 1972, Chen, 1983).

Organ weights during withdrawal were analyzed by one-way analysis of variance over time (Snedecor and Cochran, 1967). Significant F ratios ($P < 0.05$) were obtained for liver, ovaries and kidney, while heart, spleen and gizzard showed no significant change during withdrawal. Values having a significant F ratio were compared against the 0 days value (Dunnett, 1955, 1964) to establish when the difference was significant.

Average organ weights as percent of body weight for liver, kidneys and ovaries during withdrawal are shown in Table 12. The data indicate that liver and kidney size were significantly smaller ($P < 0.01$) at 16 and 32 days of withdrawal, respectively, than at 0 days. Ovary size, however, was significantly larger at 8 and 24 days of withdrawal than the 0 day value, but not at 16 and 32 days. When compared against control values, no statistical difference in ovary size was found at 32 days. This apparent discrepancy may be associated with a decrease in the laying potential of the hens as they became older (North, 1978), which could affect both the

Table 12 - Average Organ Weights as Percent of Body Weight of Laying Hens During Withdrawal

Days after Withdrawal	n	Average Organ Weights as Percent of Body Weight[1,2]		
		Liver	Kidney	Ovaries
0 days	16	3.40	0.48	2.35
2 days	8	4.09	0.49	1.95
4 days	8	3.89	0.50	2.20
8 days	8	3.05	0.49	4.02 **
16 days	8	2.09 **	0.41	2.85
24 days	8	2.26 **	0.46	3.97 **
32 days	7	1.93 **	0.40 **	3.17

1) Values at different times were compared against the 0 days value. Values differing from the 0 days value are indicated by (**): $P < 0.01$

2) Error mean squares are: liver = 0.579; kidney = 0.005; ovaries = 1.494

Standard error of the mean, S.E. $= \sqrt{\frac{\text{Error mean square}}{n}}$

aflatoxin-fed and control hens regardless of aflatoxin feeding. In addition, the statistical tests used in these comparisons were different and could affect sensitivity.

AFLATOXIN RESIDUES

Tentative Identification of Unknown Aflatoxin Metabolite in Eggs and Tissues of Laying Hens

In addition to aflatoxins B_1, B_2, M_1 and M_2, a blue violet fluorescent spot, which showed a lower mobility (R_f) than AFM_2 in both directions of development, was detected in eggs and tissue samples of hens fed the aflatoxin contaminated diet. The spot was not present in control eggs and showed a similar pattern of appearance and clearance to other aflatoxin residues detected in eggs and tissue samples. The unknown spot turned yellow under UV light on spraying the plate with 25% H_2SO_4, indicating that this compound may be an aflatoxin metabolite (A.O.A.C., 1975a).

Since the unknown spot had a lower R_f than aflatoxins B_1 and B_2, and aflatoxins M_1 and M_2 (Figures 1 and 2), a metabolite of higher polarity was suspected. The unknown spot was then extracted from the TLC plate and chromatographed in three solvent systems with other hydroxylated aflatoxin metabolites including aflatoxins M_1, M_2, Q_1 , B_{2a}, M_{2a} and aflatoxin B_1-dihydrodiol. The hemiacetal derivatives of

aflatoxins B_1 and M_1, namely aflatoxins B_{2a} and M_{2a}, were prepared in situ by addition of TFA/$CHCl_3$ (1:1) as described earlier herein. Aflatoxin B_1-dihydrodiol was prepared from AFB_1 as described by Swenson et al. (1974).

Results of the comparative chromatograms are presented in Table 13. The data indicate that the mobility of the unknown compound was very similar to that of AFB_{2a}.

Table 13 - Comparative Thin Layer Chromatography of the Unknown Aflatoxin Metabolite in Different Solvent Systems

Compound	R_f[1] in:		
	$CHCl_3$-Acetone (83:17)	$CHCl_3$-Acetone-Isopropanol (85:10:5)	Ether-MeOH-Water (90:8:2)
UNKNOWN	0.14	0.40	0.32
AFM_1	0.22	0.48	0.41
AFM_2	0.19	0.45	0.35
AFB_{2a}	0.19	0.41	0.32
AFM_{2a}	0.03	0.20	0.23
AFB_1-dihydrodiol	0.02	0.18	0.27

1) R_f (Relative mobility) = Distance of center of spot from origin/ Distance of solvent front from origin (Gänshirt, 1965)

Attempts were made to identify the unknown metabolite by mass spectrometry techniques (MS), including negative ion chemical ionization and electron impact. Relatively small amounts recovered from the TLC plates along with contamination by plasticizers precluded positive identification. However, since the mobility and fluorescence of the unknown spot were similar to those of AFB_{2a}, it was tentatively identified as AFB_{2a}. Thus standards for AFB_{2a} were prepared from AFB_1 treated with TFA/$CHCl_3$ (1:1) on each plate and the unknown spot was quantified as AFB_{2a}. These values are included in the tables for aflatoxin residues in eggs and tissues as an indication of relative amounts of this compound.

Aflatoxin Residues in the Eggs of Hens Fed a Contaminated Diet

Aflatoxin levels for the eggs of hens during the first week of feeding the aflatoxin contaminated diet are presented in Table 14. Each value presents the level of aflatoxins in a composite sample of two eggs that were randomly selected and pooled together for analysis. The levels of aflatoxins in the eggs on days 10, 14, 18, 22, 25 and 28 of the aflatoxin feeding trial are shown in Table 15. Mean values of aflatoxin residues are also shown in both tables.

The data presented indicate that the transfer of aflatoxins from the feed into eggs occurred rapidly after initial feeding of the contaminated ration. Aflatoxins B_1, B_2, M_2

Table 14 - Aflatoxin Residues Detected in Eggs During the First Week of Aflatoxin Feeding

Day	Sample	Aflatoxin (ug/kg)[1]				
		B_1	B_2	M_1	M_2	B_{2a}
1	1	0	0	0	tr	tr
	2	tr	0.01	0	0.01	0.02
	3	tr	tr	0	0.01	0.03
Mean		tr	tr	0	tr	0.02
2	1	tr	0.03	0	0.01	0.05
	2	tr	0.02	0	0.03	0.07
	3	0.02	0.01	0	0.01	0
Mean		tr	0.02	0	0.02	0.04
3	1	0.03	0.03	0	0.03	0.07
	2	0.07	0.03	0	0.02	0.05
	3	0.02	0.03	0	0.03	0.06
Mean		0.04	0.03	0	0.03	0.06
4	1	0.05	0.04	0.01	0.05	0.07
	2	0.06	0.04	0.01	0.03	0.08
	3	0.01	0.02	0	0.03	0.05
Mean		0.04	0.03	tr	0.04	0.07
5	1	0.06	0.03	tr	0.05	0.09
	2	0.05	0.03	tr	0.03	0.05
	3	0.05	0.02	0	0.03	0.05
Mean		0.05	0.03	tr	0.04	0.06
6	1	0.01	0.03	0	0.02	0.07
	2	0.02	0.03	0	0.05	0.11
	3	0.02	0.03	0	0.02	0.05
Mean		0.02	0.03	0	0.03	0.06
7	1	0.04	0.03	tr	0.05	0.05
	2	0.05	0.03	0.01	0.02	0.06
	3	0.03	0.04	0	0.02	0.07
Mean		0.04	0.03	tr	0.03	0.06

1) tr = trace amounts, visible but too small to quantitate (< 0.01 ug/kg)

Table 15 - Aflatoxin Residues Detected in Eggs During the Aflatoxin Feeding Period

Day	Sample	Aflatoxin (ug/kg)[1]				
		B_1	B_2	M_1	M_2	B_{2a}
10	1	0.07	0.03	0.02	0.03	0.10
	2	0.05	0.04	tr	0.04	0.11
	3	0.05	0.02	0.01	0.01	0.07
Mean		0.06	0.03	0.01	0.03	0.09
14	1	0.06	0.05	0.01	0.02	0.07
	2	0.03	0.03	0.03	0.04	0.07
	3	0.05	0.03	0.01	0.01	0.07
Mean		0.04	0.04	0.02	0.02	0.07
18	1	0.10	0.06	tr	0.02	0.07
	2	0.03	0.03	tr	0.02	0.05
	3	0.03	0.03	0.01	0.02	0.07
Mean		0.05	0.03	tr	0.02	0.05
22	1	0.03	0.04	0.01	0.04	0.08
	2	0.01	0.04	tr	0.03	0.08
	3	tr	0.01	0	0.02	0.05
Mean		0.01	0.03	tr	0.03	0.07
25	1	0.09	0.07	0.02	0.07	0.11
	2	0.04	0.05	0.03	0.04	0.07
	3	0.03	0.02	0	0.02	0.06
Mean		0.05	0.05	0.02	0.04	0.08
28	1	0.05	0.04	0.01	0.02	0.08
	2	0.06	0.06	tr	0.03	0.06
	3	0.03	0.01	0	0.01	0.02
Mean		0.05	0.04	tr	0.02	0.05

1) tr = trace amounts, visible but too small to quantitate ($<$ 0.01 ug/kg)

and B_{2a} appeared in trace or low but measurable amounts in the eggs laid only one day after consumption of the aflatoxin-spiked ration. The mean values in Tables 14 and 15 show that the levels of aflatoxin residues rapidly increased and reached a maximum value, which remained relatively constant as long as the hens continued to consume the aflatoxin-spiked diet. Results agree with the findings of Trucksess et al. (1983).

In a longer feeding trial, Lötzsch and Leistner (1976) found that the maximum levels of aflatoxin residues were found after 8-10 days of aflatoxin feeding and decreased afterwards, even before removal from the contaminated diet. After feeding hens at a level of approximately 3,000 ug of AFB_1/kg of diet, they reported that the average level of AFB_1 in the eggs was 0.03 ug/kg, which is similar to the levels found in the present study. Jacobson and Wiseman (1974), however, reported average amounts of 2.2 and 3.6 ug of AFB_1/kg in the albumen and yolks of eggs laid by Arbor Arce hens fed with only 100 ug of AFB_1/kg diet.

Trucksess et al. (1983) fed hens a ration containing 8,000 ug of AFB_1/kg, a level about 2.4 times higher than that used in the present study. Unlike Trucksess et al. (1983), who did not find AFM_1 in any of the eggs analyzed, AFM_1 was found in some samples in the present study although the levels were very low. The failure of Trucksess et al. (1983) to detect AFM_1 could be due in part to less sensitive analytical methodology and/or to the naturally low levels of free AFM_1.

Extraction of AFM_1 from eggs is difficult and requires preheating according to Gregory and Manley (1981). They concluded that no AFM_1 could be recovered using the official A.O.A.C. method for AFB_1 in eggs (Trucksess et al., 1977) which uses no heating, no acidulant or protein precipitants other than a saturated solution of sodium chloride. The method used by Trucksess et al. (1983) has no heating step and uses only a saturated sodium chloride solution to precipitate the protein (Trucksess and Stoloff, 1984).

The method used in the present study for the analysis of eggs included a two-stage sequential heating step prior to extraction, and is a modification of the method for the analysis of aflatoxins in animal tissues (Trucksess and Stoloff, 1979). The need for the preheating step was seen by the fact that either poor or no recovery of added AFM_1 and AFM_2 occurred in spiked samples. Phase separation was also noted in samples heated in a boiling water bath for 20 min as recommended by Gregory and Manley (1981). This problem was solved by heating the samples in a 60°C water bath for 30 min, and then gradually increasing the temperature to 72°C over a 15 min period. After heating, recovery of AFM_1 and AFM_2 improved, but a slight decrease in recovery of AFB_1 and AFB_2 was noted.

As shown in Tables 14 and 15, the levels of AFB_2 in eggs were in general slighlty lower than those of AFB_1. Since the ratio of AFB_1 to AFB_2 in the feed was approximately 2.3, it appears that the metabolism of AFB_2 is less efficient. A similar observation was made by Furtado et al. (1982) in

relation to the levels of AFB_1 and AFB_2 in the liver and kidney of pigs fed rations contaminated with AFB_1 and AFB_2, and by Chen et al. (1984) in the tissues of broiler chickens.

The levels of AFM_2, the hydroxylated metabolite of AFB_2, were also higher than those of AFM_1, the hydroxylated metabolite of AFB_1. This again is in agreement with observations of Furtado et al. (1982) and of Chen et al. (1984), who concluded that the removal of aflatoxins B_2 and M_2 from the kidneys and livers of pigs and chickens, respectively is somewhat impaired in relation to that of aflatoxins B_1 and M_1. The relatively high levels of AFB_2 and AFM_2 may also be associated with the conversion of AFB_1 to AFB_2, and of AFM_1 to AFM_2. Patterson and Allcroft (1970) reported that AFB_1 and AFM_1 were reduced in vitro to the corresponding reduction products, AFB_2 and AFM_2, by liver preparations from chickens, ducklings, guinea pigs and mice.

Trucksess et al. (1983) reported the presence of aflatoxicol in eggs laid by hens fed an AFB_1-spiked diet. Aflatoxicol was determined after separation on a C_{18} reverse phase high performance liquid chromatographic system, because it could not be resolved from interferences by TLC. In the present study, the presence of aflatoxicol in the eggs could not be established, since extracts were resolved only by TLC. A strongly fluorescent compound normally present in eggs showed the same mobility than pure aflatoxicol in the chloroform/acetone (83:17) developing system, but differenct R_f in the ether/methanol/water (95:4:1) system. In addition to

being present in control eggs, this fluorescent compound showed no change in the color of its fluorescence when the plate was sprayed with 25% H_2SO_4, thus eliminating the possibility of it being an aflatoxin residue (A.O.A.C., 1975a).

Aflatoxin Residues in Egg Albumen and Egg Yolks

Egg albumen and egg yolk were analyzed separately during aflatoxin feeding to establish the distribution of aflatoxin residues in the liquid egg. Five to six eggs were separated and the combined yolks and albumen (about 90-100g) were analyzed separately by the same procedure followed in the analysis of whole eggs.

Levels of aflatoxins in albumen and yolks are presented in Table 16. It can be seen that the residue levels in both the albumen and yolks showed similar patterns to those observed in whole eggs. After two days of feeding the aflatoxin-spiked diet, trace to measurable amounts of all aflatoxins, except AFM_1, could be detected in both the albumen and yolks. The levels increased rapidly showing a maximum value around the 8th day of aflatoxin feeding.

Rapid growth of an ovum begins about 10 days before the yolk is released from the ovary (Nesheim *et al.*, 1979). Thus, it may be expected that maximum levels in the yolk would be reached after this period of time and they would remain relatively constant as long as aflatoxin feeding continues. However, a slight decrease in aflatoxin levels in both the

Table 16 - Aflatoxin Residues Detected in the Albumen and Yolks of Eggs Laid During Aflatoxin Feeding

Day	Sample[1]	Aflatoxin (ug/kg)[2]				
		B_1	B_2	M_1	M_2	B_{2a}
		ALBUMEN				
2	1	0.02	0.02	0	0.03	0.05
	2	tr	0.01	0	0.02	0.03
5	1	0.02	0.02	0	0.02	0.06
	2	0.01	0.02	0	0.01	0.04
8	1	0.06	0.03	tr	0.02	0.07
	2	0.06	0.03	0.02	0.02	0.05
15	1	0.04	0.02	tr	0.03	0.04
	2	0.02	0.02	tr	0.03	0.05
22	1	0.02	0.03	tr	0.02	0.08
	2	0.06	0.04	0.01	0.04	0.13
28	1	0.04	0.03	0.01	0.02	0.06
	2	0.03	0.02	0.01	0.02	0.04
		YOLK				
2	1	0.01	0.03	0	0.01	0.03
	2	0.02	0.02	0	0.02	0.03
5	1	0.02	0.02	0	0.02	0.08
	2	0.03	0.02	0	0.02	0.09
8	1	0.05	0.04	0.02	0.02	0.08
	2	0.05	0.02	0.01	0.03	0.09
15	1	0.04	0.02	0.01	0.01	0.07
	2	0.05	0.02	0.02	0.04	0.09
22	1	0.04	0.03	tr	0.03	0.08
	2	0.06	0.04	0.02	0.04	0.11
28	1	0.04	0.02	0.01	0.03	0.08
	2	0.04	0.02	0.02	0.01	0.06

1) Each sample was a composite of 5-6 eggs for every day

2) tr = trace amounts, visible but too small to quantitate (< 0.01 ug/kg)

albumen and yolks occurred toward the end of the feeding period. Similar results were reported by Lötzsch and Leistner (1976) who suggested that hens fed aflatoxins for relatively long periods of time may become adapted, which may result in lower residue levels in the eggs.

Comparison of the levels of the various aflatoxins in separated eggs indicates that similar amounts of aflatoxins B_2, M_1 and M_2 were recovered from albumen and yolks, while slightly higher amounts of AFB_1 were recovered from the yolks. Similar observations for the deposition of AFB_1 in eggs were reported by Lötzsch and Leistner (1976). Jacobson and Wiseman (1974) reported that the level of AFB_1 recovered from the yolk was 1.6 times higher than that in the albumen. Using ^{14}C-AFB_1, Sawhney et al. (1973) detected increasing levels of aflatoxins in egg components after administration of a single oral dose. After oviposition, the highest level of radioactivity was detected in shell membranes, followed by the yolks and albumen, respectively. No attempt was made to further characterize which metabolites were deposited. Trucksess et al. (1983) found the same levels of aflatoxins in the ova and eggs at the end of aflatoxin feeding. They proposed that transmission appeared to be relatively constant throughout the process of egg formation, although actual analysis of egg albumen and yolks was not carried out separately.

In the present study, the levels of AFM_2 were higher than those of AFM_1 in both the albumen and yolks. The presence of the 2,3-double bond in the AFM_1 molecule makes it

more reactive than AFM_2 toward biotransformation by microsomal enzymes in the liver (Swenson, 1981) and could account for the higher levels of free AFM_2 recovered. In addition, Mabee and Chipley (1973b) showed that most of the AFM_1 formed upon feeding AFB_1 to laying hens was found in the aqueous buffer extract and was bound to glucuronate and perhaps to other water soluble molecules.

AFB_{2a} was recovered in considerably higher amounts from the yolk than from the albumen. AFB_{2a} is known to bind very readily with proteins, amino acids and peptides at physiological and alkaline pH values. AFB_{2a} rearranges itself into dialdehydic phenolate resonance hybrid ions, which can then react with amino acids, peptides and proteins to form Schiff bases (Gurtoo and Campbell, 1974; Ashoor and Chu, 1975). The lower amounts of AFB_{2a} recovered from the albumen could be the result of real differences in deposition or from the higher amount bound to the protein in the albumen, since the protein in the albumen accounts for about 92% of the dry matter and only for about 50% in the yolk (Nesheim et al., 1979). As indicated by Brackett and Marth (1982) for cheese and by Murthy et al. (1975) for beef, the interactions between aflatoxins and proteins in animal products are not well understood. Thus, conclusive statements on the causes for the differences can not be made at this time.

As indicated earlier, the hens were exposed to a DR of 213 and 108 ug for aflatoxins B_1 and B_2, respectively.

Assuming an average weight of 1.6 kg/hen, daily consumption of AFB_1 and AFB_2 would be about 340 ug and 170 ug per hen, respectively. Comparison of these values to the levels recovered from eggs (Tables 14, 15 and 16) suggests that only small amounts of aflatoxins are transferred to the eggs either as the original aflatoxins or their unbound or free hydroxylated metabolites.

The level of total aflatoxins added to the diet in the present study was about 5,000 ug/kg, which is 50 times higher than the action level of 100 ug/kg of total aflatoxins officially set by the FDA for the rations of beef cattle, swine and poultry (Anonymous, 1982). Although contamination at the levels used in the present study is not encountered normally, it may occur under some circumstances. For example, a naturally occurring case of feed contamination at levels of about 83 and 110 mg of aflatoxins/kg of ration was reported by Hamilton (1971) in a commercial laying flock. On the basis of present results, it can be concluded that if aflatoxin residues in the feed are in compliance with action levels, the transmission of aflatoxins into the eggs of laying hens poses little or no potential public health hazard. This conclusion is supported by the results in previous reports (Sims et al., 1970; Lötzsch and Leistner, 1976, 1977).

Aflatoxin Residues in Eggs During Withdrawal

Whole Eggs

Aflatoxin levels in whole eggs during withdrawal from the aflatoxin-spiked diet are presented in Table 17. The data indicate that upon withdrawal aflatoxin residues disappear from the eggs as rapidly as they appear during aflatoxin feeding. Low levels of aflatoxins were detected at one and two days of clearance. After three days, one egg sample had traces of AFB_1 and AFB_2, and two samples had trace to low but measurable amounts of AFM_2 and AFB_{2a}. By the fourth day of withdrawal no detectable residues were found in any of the samples.

Trucksess *et al*. (1983) reported that aflatoxin residues in eggs decreased rapidly after feeding of the contaminated ration was discontinued. They detected no AFB_1 in eggs laid six days after withdrawal, although low levels of aflatoxicol (0.01 ug/kg) were present after seven days. Differences in the time required to achieve clearance between their study and the present experiment are probably due to variation in the levels and duration of feeding. In the present study, a lower level of aflatoxins combined with a longer feeding period may have resulted in adaptation of the hens to the toxin, and could be responsible for lower residue levels and faster clearance as reported by Lötzsch and Leistner (1977).

Table 17 - Aflatoxin Residues in Eggs After Withdrawal from the Aflatoxin-spiked Diet

Days after Withdrawal	Sample	Aflatoxin (ug/kg)[1]				
		B_1	B_2	M_1	M_2	B_{2a}
1	1	0.03	0.02	tr	tr	0.03
	2	0.02	0.01	0	0.01	0.04
	3	0.03	0.02	0	tr	0.03
Mean		0.03	0.02	0	tr	0.03
2	1	tr	tr	tr	0	0.02
	2	0.02	tr	0	tr	0.04
	3	0.01	tr	0	tr	0.03
Mean		0.01	tr	0	0	0.03
3	1	tr	tr	0	0.01	0.02
	2	0	0	0	tr	tr
	3	0	0	0	0	0
Mean		0	0	0	tr	0.01
4	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
Mean		0	0	0	0	0

1) tr = trace amounts, visible but too small to quantitate (< 0.01 ug/kg)

Egg Albumen and Egg Yolks

Egg albumen and egg yolks were analyzed separately during withdrawal to establish if there were differences in clearance patterns between the egg components. Since egg production by the aflatoxin-fed hens was very low at the end of aflatoxin feeding and some hens had been sacrificed, only a few eggs were available for analysis. Therefore the albumen and yolks at three and four days of withdrawal could not be analyzed.

Levels of aflatoxins in albumen and yolks after withdrawal are shown in Table 18. The data indicate that clearance of aflatoxin residues from the albumen occurred faster than from the yolks. Only trace amounts were detected in the albumen at two days, and no residues were present at five days. In contrast, measurable levels were present in the yolks at two days and traces were still detected in one sample up to six days after removal from the contaminated ration.

The presence of residues in the yolks of eggs laid five to six days after withdrawal may be due to deposition during the initial days of ova maturation (Nesheim et al., 1979). Maturation of the yolks of eggs laid five to six days after withdrawal would have begun four to five days before withdrawal and thus could have accumulated some aflatoxin residues. Sawhney et al. (1973) reported that large ova (> 10 mm) retained ^{14}C activity four days after administration of a

Table 18 - Aflatoxin Residues Detected in the Albumen and Yolk of Eggs After Withdrawal from the Aflatoxin-Spiked Ration

Days after Withdrawal	Sample	Aflatoxin (ug/kg)[1] B_1	B_2	M_1	M_2	B_{2a}
		ALBUMEN				
1	1	0.02	0.01	0	0.01	0.02
	2	tr	tr	0	tr	0
2	1	tr	tr	0	tr	tr
	2	tr	tr	0	tr	
3,4[2]						
5	1	0	0	0	0	0
	2	0	0	0	0	0
6	1	0	0	0	0	0
	2	0	0	0	0	0
		YOLK				
1	1	0.03	0.01	tr	0.01	0.05
	2	0.02	0.01	0.01	tr	0.05
2	1	0.01	tr	0	0.01	0.05
	2	0.02	0.01	0	0.01	0.04
3,4[2]						
5	1	0	0	0	0	0
	2	tr	tr	0	tr	tr
6	1	tr	tr	0	0	tr
	2	0	0	0	0	tr
7	1	0	0	0	0	0
	2	0	0	0	0	0

1) tr = trace amounts, visible but too small to quantitate ($<$0.01 ug/kg)

2) Not enough eggs available for separate analysis

single oral dose of ^{14}C-AFB_1, and that the activity was present in small ova (< 10 mm) up to seven days after dosing. Trucksess et al. (1983) reported that aflatoxin residues were present in the ova of hens sacrificed at the end of aflatoxin feeding, but had disappeared after 7 days of clearance. Similar results were obtained in the present study (Table 27).

Growth of the ovum and addition of the albumen free of aflatoxins to the whole egg have a dilution effect, which may account for faster clearance of aflatoxin residues. In a normal egg, the yolk constitutes only about 36% of the liquid portion (Powrie, 1976). Furthermore, eggs laid by hens fed aflatoxins are smaller as a result of a decrease in yolk size (Huff et al., 1975). Thus, the contribution of the yolk to the aflatoxin content of the whole egg during clearance was probably below detection limits.

Results support the conclusion that aflatoxin residues in eggs decrease rapidly after withdrawal of the contaminated diet. Clearance of aflatoxin residues can be achieved within three to four days from either the whole egg or the egg white and within six to seven days from the egg yolk.

Effects of Cooking on the Levels of Aflatoxins in Eggs

The effects of cooking on the levels of aflatoxins in the eggs of hens fed an aflatoxin contaminated diet are presented in Table 19. Eggs from day 15 of aflatoxin feeding were selected for analysis since this was an intermediate

Table 19 - Effect of Cooking on Aflatoxin Levels in Eggs From Hens Fed an Aflatoxin-Spiked Diet

SAMPLE	Aflatoxin Level (ug/kg)				
	B_1	B_2	M_1	M_2	B_{2a}
Replication 1					
Raw	0.03	0.01	tr	0.01	0.04
Steamed	0.03	0.01	tr	0.04	0.06
Fried	0.03	0.02	tr	0.03	0.04
Replication 2					
Raw	0.02	0.03	tr	0.02	0.07
Steamed	0.02	0.02	tr	0.02	0.05
Fried	0.02	0.02	tr	0.03	0.03
Replication 3					
Raw	0.01	0.01	tr	0.01	0.05
Steamed	0.02	0.02	tr	0.01	0.03
Fried	0.02	0.02	0.01	0.02	0.03

time in the feeding trial and adequate numbers of eggs were available.

As indicated by the data, no major changes in the aflatoxin levels of the eggs were observed upon cooking. Most levels remained the same, others showed a slight decrease and some increased with cooking, even after correcting for moisture losses. Problems were encountered in establishing the effects of processing on eggs naturally contaminated by aflatoxins since the amounts present were very low and the method was not sufficiently sensitive and/or precise to detect small differences at the low levels.

Variability in the recovery of AFM_1 during processing of dairy products has been reported to be a problem in experiments designed to study the effects of processing on AFM_1 levels (Van Egmond et al., 1977; Wiseman and Marth, 1983a; Brackett and Marth, 1982). Wiseman and Marth (1983a) reported inconsistent recoveries between trials in processing of cultured dairy products when working at levels as high as 3 to 6 ug og AFM_1/kg. They concluded that a better understanding of the interactions between AFM_1 and casein will be necessary in order to explain the results of processing. Similarly, interactions between aflatoxins and the egg proteins are not understood and may explain the variability in results from the present study.

In a second experiment, control eggs were spiked with 0.5, 0.15, 0.4 and 0.2 ug/kg of aflatoxins B_1, B_2, M_1 and M_2, respectively, and cooked as described for the naturally

contaminated eggs. Although the levels of aflatoxins were higher than for the naturally contaminated eggs, they were considerably lower than those used by Wiseman and Marth (1983a) in the study of cultured dairy products. Results for the spiked eggs are presented in Table 20 as percent recoveries. Values ranged from 40 to 108% indicating considerable variability. Similar to the naturally contaminated eggs, there was no consistent trend in the effects of cooking. With two different methods of cooking (steaming and frying), AFB_1 remained constant for both in the first replication and increased in the second. In the third replication, AFB_1 recovery decreased with steaming but increased with frying. The differences on comparing these results were not statistically significant due to small number of samples and the large degree of variability.

Higher recoveries of aflatoxins from processed or aged dairy products than from the corresponding raw milk or unaged products have been reported in several studies (Van Egmond et al., 1977; Wiseman and Marth, 1983a; Brackett and Marth, 1982). Furtado et al. (1981) also noted a slight increase in the average residual levels of aflatoxins upon frying of sliced pork bellies.

In the present study, temperatures reached at the center of the steamed and fried eggs were 78 and 86-87°C, respectively. Total cooking times were 5 min for steamed eggs and 2 min for the fried eggs. Results of the present study indicate that aflatoxins were quite heat stable during

Table 20 - Percent Recoveries of Spiked Aflatoxins During Cooking of Eggs

SAMPLE	% Recovery of Aflatoxins[1,2]			
	B_1	B_2	M_1	M_2
Replication 1				
Raw	46	62	101	69
Steamed	41	66	92	64
Fried	43	48	47	72
Replication 2				
Raw	62	54	94	73
Steamed	81	60	94	88
Fried	81	62	94	70
Replication 3				
Raw	52	46	86	108
Steamed	40	63	81	71
Fried	62	52	91	76
Mean of Replications				
Raw	53	54	94	83
Steamed	54	63	89	74
Fried	62	54	77	73

1) Spiking levels of raw egg were 0.5, 0.15, 0.4 and 0.2 ug/kg of aflatoxins B_1, B_2, M_1 and M_2, respectively. Corrections were made to account for moisture losses during cooking.

2) $$\% \text{ Recovery} = \frac{\text{Amount recovered from sample}}{\text{Amount added to sample}} \times 100$$

cooking. Little or no reduction would be expected during cooking of eggs under these conditions due to the heat stability of aflatoxins (Goldblatt, 1971). In support of these reults,Peers and Linsell (1975) have shown that AFB_1 is not degraded in peanut and corn oils until the temperature reaches 250°C, which is close to its decomposition temperature. Likewise, Allcroft and Cranaghan (1963) found that pasteurization of 'toxic' milk at 80°C for 45 sec or at 70°C for 30 min, or roller drying did not reduce its toxicity to 1-day old ducklings. Wiseman and Marth (1983b) also found no change in the AFM_1 content of naturally contaminated milk after heating for 2 hours at 100°C.

Furtado et al (1981) reported that cooking and/or processing of pork had some effect in lowering the levels of aflatoxins B_1 and B_2. Although inactivation was in the range of 15 to 30%, it was not statistically significant. The authors concluded that aflatoxins are quite stable during cooking and/or processing and that the procedures used were not effective in reducing the levels in contaminated meat, which is in essential agreement with results of the current study.

Present results suggest that steaming or frying had no major effect on the levels of aflatoxins present in the raw eggs. This was probably a result of the stability of the aflatoxins to heat in combination with low cooking temperatures and short cooking times. At the low levels of aflatoxins present in the raw eggs, the sensitivity and precision

of the method were not high enough to detect consistent differences. The action level officially set by the FDA in raw and processed foods for human consumption is 20 ug/kg. At the levels of aflatoxins found in the eggs of hens consuming a spiked ration (< 0.5 ug/kg), the significance of the small changes on cooking are of doubtful importance. This provides further support for concluding that the potential hazard for human exposure to aflatoxins by consumption of raw or cooked eggs is negligible.

Aflatoxin Residues in the Tissues and Organs of Hens Fed an Aflatoxin-Contaminated Diet

Aflatoxin levels for the tissues and organs of hens sacrificed at the end of the aflatoxin feeding period are presented in Table 21. Although 16 treated hens were slaughtered at this time, analysis were carried out on the tissues of only eight randomly selected hens. Extra hens had been included in the aflatoxin feeding experiment to have enough muscle samples for processing experiments. As shown in Table 21, aflatoxin levels in breast and leg muscles were very low. Thus, processing was not carried out, since it was evident that at these low levels the method used is not sensitive and/or precise enough to detect the small changes occurring during processing.

As shown in Table 21, measurable amounts of both AFB_1 and AFB_2 were carried over to all tissues and organs of the

Table 21 - Aflatoxin Residues Detected in the Tissues of Aflatoxin-Fed Hens at the End of the Aflatoxin Feeding Period

Hen # or Sample	Aflatoxin Level[1] (ug/kg)									
	B_1	B_2	M_1	M_2	B_{2a}	B_1	B_2	M_1	M_2	B_{2a}
	BREAST					LEG				
202	0	0	0	0	tr	0.03	0.03	0	tr	0.02
205	0	0	0	0	tr	0.04	0.03	0	0.01	0.04
207	0	tr	0	0.02	0.01	0.01	0.22	0	0.01	0.04
208	0	tr	0	0	tr	0.02	0.02	0	tr	0.04
210	0.05	0.05	tr	0.02	0.03	0.11	0.07	0.01	0.02	0.03
211	tr	0.01	0	tr	0	0.02	0.04	tr	0.01	0.02
216	0	0	0	0	0.01	0.02	0.02	0	tr	0.02
233	0	0	0	tr	0.01	tr	0.02	0	tr	0.03
Mean[2]	0.01	0.01	0	0.01	0.02	0.03	0.06	0	0.01	0.03
	LIVER					GIZZARD				
202	0.08	0.03	tr	0.01	2.21	0.35	0.25	0	0	tr
205	0.22	0.09	0	0.01	0.54	1.09	0.73	0.01	0.02	0.07
207	0.44	0.25	0.05	0.10	3.87	0.93	0.52	0	0.02	0.10
208	0.07	0.04	0	tr	0.35	0.36	0.22	0	0	0.04
210	0.26	0.26	0	0.01	0.35	1.29	0.68	0	0	0
211	0.12	0.07	NR	NR	1.16	0.48	0.18	tr	tr	0.07
216	0.20	0.23	0.04	0.04	1.35	0.54	0.34	tr	tr	0.06
233	0.20	0.09	0.05	0.05	2.32	0.34	0.16	tr	0.01	0.07
Mean[2]	0.20	0.13	0.02	0.03	1.52	0.67	0.38	0	0.01	0.05
	OVARIES[3]					SPLEEN[4]				
202	0.07	0.03	0.01	0.02	0.04					
205+210	0.15	0.04	0.02	0.01	0.02					
208	0.04	0.02	0.01	0.02	0.03					
211	0.04	0.03	tr	0.01	0.02					
216	0.08	0.02	tr	tr	0.03					
233	0.03	0.02	0	tr	0.04					
Mean[2]	0.07	0.03	0.01	0.01	0.06	0.25	0.10	tr	0.01	0.10
	HEART					KIDNEYS				
I[5] (207,208, 205,216)	0.08	0.05	0	0	0.06	0.27	0.16	0.07	0.03	1.94
II[5] (233, 211, 205,210)	0.18	0.05	tr	0.02	0.05	0.87	0.30	0.11	0.08	2.29
III[5] (203,212, 206,209)			NA			0.53	0.20	0.10	0.07	1.93
IV[5] (213,201, 204,214)			NA			0.27	0.12	0.11	0.08	2.30
Mean[2]	0.13	0.05	0	0.01	0.05	0.49	0.20	0.10	0.07	2.12
	BLOOD CLOT					BLOOD SERUM				
Pool A[6]	0.06	0.07	tr	0.01	0.02	0.02	0.05	0	tr	0.02
Pool B[6]	0.12	0.10	tr	0.01	0.04	0.03	0.04	0	tr	0.01
Mean[2]	0.09	0.08	tr	0.01	0.03	0.02	0.04	0	tr	0.01

1) tr = trace amounts - visible but too small to quantitate (< 0.01 ug/kg)
NR = chromatogram not well resolved, NA = not analyzed

2) Means calculated assuming tr = 0.0058, which is the largest amount which would not be measurable

3) Ovaries of hens 205 and 210 were combined for analysis. The ovary of hen 207 was not analyzed because it was too small

4) The spleens from all 16 hens were combined for analysis (30.6 g)

5) Sample represents a composite of heart and kidney samples for the hens indicated in parenthesis

6) Pool A = Combined blood of hens 201, 203, 205, 207, 209, 210, 211 and 216
Pool B = Combined blood of hens 202, 204, 206, 208, 213, 214, 212, and 233

hens fed the contaminated diet. The lowest levels were detected in breast and leg muscles and in blood serum, followed by the ovaries and the blood clot. Higher levels were detected in all other excised organs.

The highest levels of both AFB_1 and AFB_2 were detected in the gizzard, followed by the kidneys, liver, spleen and heart, respectively. The high levels of AFB_1 and AFB_2 in the gizzard may have been caused in part by direct contamination from the feed during slaughtering as reported by Chen et al. (1984). Additional evidence for this in the present study is the high ratio between the amounts of aflatoxins B_1 and B_2 in comparison to the total aflatoxins levels detected in the gizzard and, conversely, the low metabolite to parent or ingested residues ratio (Table 22). Sawhney et al. (1973) also reported a high concentration of ^{14}C activity in the crop and gizzard of hens dosed orally with ^{14}C-AFB_1. However, they concluded that this suggested either slow absorption or passage, or both, from this segment of the alimentary canal.

The levels of aflatoxins B_1 and B_2 in the blood clot were considerably higher than those in the serum. This is in agreement with results of Kumagai et al. (1983), who showed that upon incubation of blood from different animal species with ^{14}C-AFB_1, high levels of radioactivity were associated with blood cells. They suggested that AFB_1 may bind some component of the blood cells. Since the radioactivity detected in the plasma was present in the plasma protein fraction, they proposed that in vivo AFB_1 may be carried by

both the plasma proteins and the blood cells.

Aflatoxins M_1 and M_2, the metabolites of AFB_1 and AFB_2, respectively, were detected in low amounts in all organs and tissues. The highest levels of these metabolites were found in the kidneys and liver. The compound tentatively identified as AFB_{2a} was the most abundant metabolite detected in all tissues of aflatoxin-fed hens. The highest levels were found in kidneys and liver, while the lowest amounts occurred in breast and leg muscles and in the blood serum and clot.

Results of the present study are in general agreement with reports by Sawhney et al. (1973) and Mabee and Chipley (1973b), who detected ^{14}C activity in all tissues and fluids analyzed from laying hens dosed or fed with ^{14}C-AFB_1. Similarly, Trucksess et al. (1983) reported the presence of AFB_1, AFM_1 and aflatoxicol in various tissues of hens fed a diet contaminated with AFB_1. Chen et al. (1984) also reported that aflatoxins were deposited, either as the original compound fed (AFB_1 and AFB_2) or as their metabolites (AFM_1 and AFM_2), in all tissues of chickens fed an aflatoxin-spiked diet.

Aflatoxin B_{2a}, the hemiacetal derivative of AFB_1, is considered to be a metabolite of AFB_1 (Patterson and Roberts, 1970; Gurtoo and Campbell, 1974), although it was also a major urinaty metabolite in rats injected with AFB_2 (Dann et al., 1972). Patterson and Roberts (1970) reported that the liver of chicks, guinea pigs and mice metabolized AFB_1 into small amounts of AFM_1 (5-10%), whereas, the major metabolite

(90%) was AFB_{2a}. Later, Patterson and Roberts (1972) stated that the yields of the hemiacetal were difficult to assess because of binding with free amino groups in proteins and amino acids to form Schiff base compounds. In the present study, the use of strong protein and nucleic acid precipitants, such as ammonium sulfate and lead acetate, in combination with with acidulating agents and competitive reactants, such as citric acid and saturated sodium chloride solution, may account for the detection of AFB_{2a}. These reagents may cause dissociation of the bonds formed with cellular components and thus release AFB_{2a}. This possibility is supported by findings of Ashoor and Chu (1975), who have shown that the interactions of AFB_{2a} with amino acids, proteins and liver microsomes in vitro are reversible and pH dependent.

Neal et al. (1981) have pointed out that earlier reports on the production of AFB_{2a} (Patterson and Roberts, 1970; Gurtoo and Dahms, 1974) were erroneous being based on identification of AFB_1-dihydrodiol as AFB_{2a} due to the similarity of their ultraviolet spectra (Swenson et al., 1973). In the present study, however, the unknown metabolite was tentatively identified as AFB_{2a}, since upon comparison of its chromatographic mobility in different solvent systems, it had an R_f similar to AFB_{2a} and not to AFB_1-dihydrodiol. Further evidence indicating the formation of AFB_{2a} by chickens and laying hens dosed with ^{14}C-AFB_1 was presented by Chipley et al. (1974). They reported that upon enzymatic treatment ethyl acetate extracts of tissues and organs of dosed birds,

an average of 50% of the ^{14}C detected was a liberated peptide or amino acid conjugate of ^{14}C-AFB_{2a}. Identification was based on comparison of the R_f values and absorbance maxima of the isolated metabolite, which were identical to those of a prepared standard of AFB_{2a}. Gregory et al. (1983) detected compounds having the chromatographic behavior and fluorescence properties of AFB_{2a} and AFM_{2a} in tissues of turkey poults fed a diet containing 500 ug of AFB_1/kg for 21 days. Dashek et al. (1983) also reported an unidentified fluorescent compound with an R_f similar to AFB_{2a} which was present in the blood, liver and excreta of quail dosed with mixed aflatoxins. They reported, however, that the UV absorption spectra of the presumed AFB_{2a} failed to yield the expected absorption maxima.

Furtado et al. (1979) tentatively identified AFB_{2a} in the tissues of pigs fed aflatoxins. No positive identification was made due to the lack of standards. However, in a later study, Furtado et al. (1982) concluded that the spot previously identified as AFB_{2a} was really AFM_2 on comparison of its chromatographic mobility with authentic AFB_{2a} and AFM_2 standards. Aflatoxins M_2 and B_{2a} are very similar chemically and structurally (Figure 2), differing only by the position of the hydroxy group in the molecule. Thus, they show similar chromatographic characteristics and exhibit similar blue-violet fluorescence under UV light. Positive identification of this metabolite by mass spectra will be required to definitely establish whether the compound isolated in the present study is AFB_{2a}.

Total amounts of ingested aflatoxins and their metabolites deposited in the tissues and organs, as well as some relationships between them are presented in Table 22. As shown, the highest concentration of aflatoxin residues was found in the liver and kidneys. These results agree with those reported by Furtado et al. (1982) and by Chen et al. (1984), who found that upon feeding aflatoxin-spiked diets to pigs and chickens, respectively, the highest amounts of aflatoxins were detected in the liver and kidneys.

The high capacity of the liver and kidneys to concentrate toxic compounds in comparison to other organs is probably related to the important role they play in elimination of xenobiotics (Klaassen, 1980). Both the kidney and the liver have the capacity to excrete and metabolize many chemicals, although the main metabolizing organ is the liver (Klaassen, 1980). In the present study, the higher proportion of free metabolites ($M_1 + M_2 + B_{2a}$) over the total or the parent ($B_1 + B_2$) aflatoxins found in the liver and kidneys in comparison to other organs (Table 22) indicates their importance in aflatoxin metabolism by the laying hen. Thus, the liver and kidney had on average 4.8 and 3.3 times more metabolites than parent aflatoxins, respectively. This probably reflects the transformation of AFB_1 and AFB_2 to metabolites by these organs.

As indicated in Table 22, the breast and ovaries contained approximately the same amounts of both the parent aflatoxins and their metabolites, while the other tissues had

Table 22 - Average Total Amounts and Ratios Between Aflatoxins B_1 and B_2 and Their Metabolites M_1, M_2 and B_{2a} Deposited in Tissues of Hens Fed an Aflatoxin-Spiked Diet[1]

Tissue	Aflatoxins (ug/kg)[2]			Ratio		
	Parent	Metabolites	Total	Parent / Total	Metabolites / Total	Metabolites / Parent
Liver	0.33	1.57	1.90	0.17	0.83	4.76
Kidneys	0.69	2.29	2.98	0.23	0.77	3.32
Breast	0.02	0.02	0.04	0.50	0.50	1.00
Ovaries	0.10	0.08	0.18	0.56	0.44	0.80
Leg	0.09	0.04	0.13	0.69	0.31	0.44
Heart	0.18	0.06	0.24	0.75	0.25	0.33
Spleen	0.35	0.11	0.46	0.76	0.24	0.31
Blood Clot	0.17	0.04	0.21	0.81	0.19	0.24
Blood Serum	0.06	0.01	0.07	0.86	0.14	0.17
Gizzard	1.05	0.06	1.11	0.95	0.05	0.06

1) Data were calculated from mean values in Table 21

2) Parent aflatoxins correspond to the amount of AFB_1 and AFB_2; Metabolites to AFM_1, AFM_2 and AFB_{2a} in the tissues at the end of aflatoxin feeding. Total = Parent + Metabolites

higher parent molecule residues. This is probably due to the higher polarity and increased water solubility of the hydroxylated metabolites, which are more easily removed from the tissues than unmetabolized aflatoxins B_1 and B_2. Excretion of the hydroxylated metabolites may be either as the original compound, or more likely as aflatoxin conjugates after binding to various endogenous compounds (Mabee and Chipley, 1973b; Neal et al., 1981).

The low proportionof hydroxylated metabolites in the blood components in the present study may indicate that if they are present, they are more likely bound to protein or other components and remain in the water soluble portion of the extract (Mabee and Chipley, 1973b). The gizzard had the lowest metabolite concentration, which as discussed earlier may indicate contamination from the feed during slaughter (Chen et al., 1984).

Results of the present study demonstrate that only a small fraction of the aflatoxins consumed was deposited in the tissues of hens, either as the original aflatoxins or as their metabolites. These results are in agreement with those of Mabee and Chipley (1973b) and of Sawhney et al. (1973), who indicated that laying hens can metabolize the majority of AFB_1 when administered at relatively low doses. Sawhney et al. (1973) reported that 7 days after administration of a single oral dose of ^{14}C-AFB_1, 70.61% had been recovered in the excrement. Similarly, Mabee and Chipley (1973b) reported recovery of only 7.85% of the total

radioactivity administered in the tissues of the laying hens while the rest was excreted. They also noted that only 10% of the radioactivity present in the sample extracts was chloroform soluble and concluded that aflatoxin conjugates are the predominant metabolites. They suggested that formation and deposition of conjugates may explain conflicting reports dealing with the levels of aflatoxins from animal tissues.

In the present study efforts were concentrated on the determination of free aflatoxins because the mutagenicity, genotoxicity and acute toxicity of the water soluble conjugates is very low in comparison to that of free residues (Wei et al., 1978; Jaggi et al., 1980). The importance of water soluble conjugates, however, can not be disregarded, since conjugated aflatoxins can be liberated from the tissues in the presence of the appropriate enzyme(s) (Mabee and Chipley, 1973a; Chipley et al., 1974, Wei et al., 1978) and may contribute to the risk of chronic toxicity during long term ingestion of contaminated tissues.

The total amounts of aflatoxin residues in each tissue were calculated from multiplying the total concentration in each tissue (Table 22) by the average weight of the corresponding tissue. These values and the contribution of each tissue as a percentage of the total aflatoxins recovered are presented in Table 23. As shown, the highest absolute amount of aflatoxins was found in the liver followed by the gizzard, leg and kidneys. The small size of the kidneys accounts for

Table 23 - Average Calculated Values for Aflatoxins Found in the Tissues of Hens Sacrificed at the End of Aflatoxin Feeding

Tissue	Average weight, g[1]	Total Aflatoxin Content, ug[2]	Percent of Total Recovered[3]
Liver	53.8	0.101	43.3
Kidneys	7.9	0.023	9.9
Breast	185.2	0.007	3.0
Ovaries	49.0	0.009	3.9
Leg	195.5	0.025	10.7
Heart	6.5	0.016	6.9
Spleen	1.8	0.009	3.9
Blood[4]	101.8	0.014	6.0
Gizzard	25.9	0.029	12.4
Total		0.233	100.0

1) Average weight of each tissue from 8 aflatoxin-fed hens sacrificed at 0 days.

2) Total Aflatoxin Content (ug)= Total aflatoxin concentration (ug/kg from Table 22) x Organ weight (g)/1,000

3) $$\% \text{ of total recovered} = \frac{\text{Total aflatoxins in a tissue}}{\text{Total aflatoxin content of all tissues}} \times 100$$

4) Blood volume was calculated under the assumption that it represents about 6% of the weight of a mature hen (Nesheim et al., 1979)

their low net contribution in spite of having the highest total concentration (Table 22). In contrast, leg muscle, which contained only about 4% of the concentration present in the kidneys, contributed a larger percentage of the total because leg weight was about 25 times that of the kidneys. Results demonstrate that in absolute terms very small amounts of aflatoxins were actually present in tissues of hens fed an aflatoxin-contaminated diet.

Rodricks and Stoloff (1977) summarized a large number of studies on carry over of aflatoxin residues from feed to edible tissues of food producing animals. They tabulated the data in terms of the ratio between the level of aflatoxins in the feed and the level in the tissue or animal product. This was intended to provide regulatory and public health officials with the information necessary to control human exposure to aflatoxins by placing restrictions on the aflatoxin levels in feeds. Table 24 presents the calculated ratios derived from the present study for kidneys, liver and leg muscle. The lowest ratios between the parent aflatoxin in feed and parent aflatoxin in tissue occurred in the kidneys and liver, respectively. This reflects the ability of these organs to concentrate the ingested aflatoxins as discussed earlier. When the metabolites present in the tissues were included, the ratio decreased markedly, particularly for AFB_1. This suggests that deposition of the parent aflatoxins is low while higher amounts of metabolites are present, probably as a result of the ability of laying hens to

Table 24 - Ratios of Aflatoxins B_1 and B_2 in the Feed in Relation to Aflatoxins B_1, B_2, M_1, M_2 and B_{2a} in the Tissues of Hens[1]

Tissue	Aflatoxin in Feed	Aflatoxin in Tissue[2]	Ratio of Level in Feed to Level in Tissue
Liver	B_1	B_1	16,550
	B_1	$B_1+M_1+B_{2a}$	1,902
Liver	B_2	B_2	12,923
	B_2	B_2+M_2	10,500
Kidney	B_1	B_1	6,755
	B_1	$B_1+M_1+B_{2a}$	1,221
Kidney	B_2	B_2	8,400
	B_2	B_2+M_2	6,222
Leg	B_1	B_1	110,333
	B_1	$B_1+M_1+B_{2a}$	55,167
Leg	B_2	B_2	28,000
	B_2	B_2+M_2	24,000

1) Higher values indicate less residues from a given amount of aflatoxins in the feed

2) It is assumed that AFM_1 and AFB_{2a} are only metabolites of AFB_1, and that AFM_2 is the only metabolite of AFB_2 in the tissues

metabolize and excrete these compounds on administration at relatively low doses (Mabee and Chipley, 1973b; Sawhney et al., 1973).

Three studies summarized by Rodricks and Stoloff (1977) give values of 59, 537 and > 1,860 for the ratio between AFB_1 in the feed and AFB_1 in the liver of hens. These values are all smaller than the 16,550 derived in the present study, but point out the great amount of variability that can be encountered on estimating this parameter. The variability that can occur is emphasized by Patterson et al. (1980), who reexamined the ratios between AFB_1 in the feed and AFM_1 in cow's milk estimated by Rodricks and Stoloff (1977), and found an overall range of 34 to 1,600. They also reported a similar degree of variation between 6 cows that they studied in a single experiment (Patterson et al., 1980).

As indicated by Rodricks and Stoloff (1977), the differences in the feed/tissue ratios may be due to a number of factors which are known to quantitatively and in some cases even qualitatively affect tissue deposition of xenobiotics. These include: 1) the species and breed of animals; 2) dose, mode and duration of exposure; 3) nutritional and health status of the animal; and 4) length of time between cessation of exposure and collection of samples. Lötzsch and Leistner (1977) suggested that long term feeding of aflatoxins to laying hens resulted in adaptation to aflatoxins with lower levels being deposited in their eggs. This type of response would probably also have an effect on feed/tissue ratios.

Total aflatoxin residues in the tissues analyzed in the present study were below 3 ug/kg (Table 22), and thus were all less than the action level of 20 ug/kg officially set by the FDA for aflatoxin levels allowable in foods and food ingredients intended for human consumption. Furthermore, the total aflatoxin levels found in the breast and leg muscles, which would be more commonly consumed than organ meat, had even lower values and averaged less than 0.2 ug/kg. On the basis of these results it can be concluded that there is little risk for humans of acute toxicity from exposure to aflatoxins in the tissues of aflatoxin-fed hens. Nevertheless, the possibility of long term or chronic exposure to free aflatoxins and those which could be released from the water soluble conjugates can not be disregarded. This is in agreement with the conclusion that aflatoxin residues deposited in the tissues of pigs (Jaggi et al., 1980; Furtado et al., 1982) and of broiler chickens (Chen et al., 1984) constitute little risk to humans consuming them.

Aflatoxin Residues in Tissues and Organs of Hens During Withdrawal

Upon removal of the contaminated feed, aflatoxin residues in the tissues of hens decreased considerably in a short period of time. Two days after withdrawal from the contaminated feed, no aflatoxins were detected in the spleens and

hearts of the laying hens (Table 25). All other tissues analyzed had trace to low but measurable amounts of one or more of the aflatoxin residues. The levels, however, were far lower than those found in the corresponding tissues at the end of the aflatoxin-feeding period, particularly in the gizzard, kidneys and liver, which were the organs with the highest levels at 0 days. Thus, the average level of AFB_1 in kidneys decreased from 0.49 ug/kg at 0 days to 0.01 ug/kg after two days on the aflatoxin-free diet.

The marked decrease in the content of aflatoxins B_1 and B_2 in the liver and kidneys is probably due to the important role these organs play in xenobiotic metabolism and excretion (Klaassen, 1980). Williams et al. (1965) and Millburn et al. (1967) tentatively concluded that compounds with high molecular weight (> 300) and containing two or more aromatic rings tended to be excreted into the bile. Several studies using ring labeled AFB_1 have indicated that aflatoxins are preferentially excreted through the bile (Sawhney et al., 1973; Harland and Cardeilhac, 1975; Falk et al., 1965).

The metabolic activity of the liver and kidneys may also be responsible for the higher levels of AFB_{2a} present 2 days after withdrawal, since AFB_{2a} is a known metabolite of both AFB_1 (Patterson and Roberts, 1970; Gurtoo and Campbell, 1974) and AFB_2 (Dann et al., 1972). AFB_{2a} has been tentatively identified in the tissues and excreta of laying hens dosed with ^{14}C-AFB_1, either in its free form (Harland and Cardeilhac, 1975) or conjugated to peptides or amino acids

Table 25 - Aflatoxin Residues Detected in the Tissues of Hens After 2 Days of Withdrawal From the Aflatoxin-Spiked Diet

Hen # or Sample	Aflatoxin Level[1] (ug/kg)									
	B_1	B_2	M_1	M_2	B_{2a}	B_1	B_2	M_1	M_2	B_{2a}
	BREAST					LEG				
217	0	tr	0	0	tr	tr	0.03	0	0	tr
218	0	0	0	0	tr	0	0	0	0	0
219	0	tr	0	0	tr	tr	0.01	0	0	0.02
220	tr	0.02	0	0	tr	0	tr	0	0	tr
221	0	0	0	0	tr	tr	0.01	0	0	tr
222	0.01	0.02	0	NR	NR	0.01	0.02	0	0	tr
223	tr	0.02	0	0	tr	tr	0.02	0	tr	tr
224	tr	tr	0	0	0	0	0	0	tr	0
Mean[2]	0	0.01	0	0	0	0	0.01	0	0	0.01
	LIVER					GIZZARD				
217	0	0	0	0	NR	TR	0.01	0	0	0
218	0	0	0	0	0.09	0	tr	0	0	0
219	0	0	0	0	0.01	tr	0.01	0	0	0
220	0	0	0	0	0.04	0	0	0	0	0
221	tr	0.02	0	tr	0.05	0.03	0.03	0	0	0
222	0	0	0	0	tr	0.04	0.05	0	0	tr
223	0	0.01	NR	NR	0.06	0	tr	0	0	tr
224	0	0	0	0	0.02	0	0	NR	NR	NR
Mean[2]	0	0	0	0	0.03	0.01	0.01	0	0	0
	OVARIES					SPLEEN[3]				
217	tr	tr	0	tr	0.01					
218	0.03	0.02	0.01	0.02	0.05					
219	0.03	0.01	0	0	tr					
220	0.01	0.01	0	0	tr					
221	0.03	0.02	0	0.01	0.02					
222	0.02	0.03	0	0	0					
223	0.02	0.02	0	tr	0.02					
224	0.08	0.02	0	0	0.02					
Mean[2]	0.03	0.02	0	tr	0.02	0	0	0	0	0
	HEART					KIDNEYS				
I[4] (218,220, 222,223)	0	0	0	0	0	0	0.01	0	0	0.03
II[4] (217,219, 221,224)	0	0	0	0	0	0.02	0.01	0	0	0.03
Mean[2]	0	0	0	0	0	0.01	0.01	0	0	0.03
	BLOOD CLOT[5]					BLOOD SERUM[5]				
All hens	0.05	0.03	0	0	tr	0.01	0.01	0	0	tr

1) tr = trace amounts - visible but too small to quantitate (< 0.01 ug/kg)
NR = chromatogram not well resolved
2) Means calculated assuming tr = 0.0058, which is the largest amount which would not be measurable
3) The spleens from all 8 hens were combined for analysis
4) Sample represents a composite of heart and kidney samples for the hens indicated in parenthesis
5) From combined blood of all 8 hens

where it accounted for as much as 50% of the ^{14}C label upon enzymatic tratment of the sodium acetate extracts of tissues and excreta (Chipley et al., 1974). Although the metabolite detected in the present study was tentatively identified as AFB_{2a}, controversy over its identity has arisen recently (Neal et al., 1981). It appears, however, that the metabolite in the present study is the same compound reported and identified as AFB_{2a} by other researchers working with laying hens (Harland and Cardeilhac, 1975; Mabee and Chipley, 1973b; Chipley et al., 1974).

As discussed previously, contamination of the gizzard during slaughter with aflatoxins B_1 and B_2 from the feed may have been responsible for the high levels of AFB_1 and AFB_2 present on day 0. Chen et al. (1984) also reported contamination of chicken gizzards from the feed during slaughter. As shown in Table 25, the levels of aflatoxins B_1 and B_2 in the gizzard decreased markedly after two days on the aflatoxin-free diet. This may be due to the fact that contamination from the feed was no longer possible.

After two days on the aflatoxin-free diet, the highest levels of both AFB_1 and AFB_2 were detected in the blood clot. The presence of aflatoxins in the blood may be expected, since the blood functions in the transport of nutrients, metabolites, hormones and waste products in the hen (Nesheim et al., 1979). The higher levels in the clot are in agreement with the results of Kumagai et al (1983) who reported that blood cells appear to carry more AFB_1 than plasma proteins.

Aflatoxins B_1 and B_2 were still detected in the breast, leg, gizzard and ovaries of almost all hens sacrificed 2 days after withdrawal. The ovaries had relatively high levels of aflatoxins B_1, B_2 and B_{2a}. Similarly, Sawhney et al. (1973) noted an increase with time after dosing on the specific activity of the small ova from hens administered ^{14}C-AFB_1. In the present study, aflatoxins M_1 and M_2 were detected in only a few samples. The low levels of these metabolites in the tissues are probably due to their high polarity and water solubility, which contribute to their excretion. Mabee and Chipley (1973b) reported that AFM_1 in the tissues of laying hens fed aflatoxins is present mainly in the form of a conjugated glucuronide and perhaps also bound to sulfate, both of which are soluble in aqueous extracts and are not recovered by traditional chloroform extraction.

Four days after withdrawal from the aflatoxin contaminated diet, trace to measurable amounts of aflatoxins were detected in only a few tissues of some hens (Table 26). One hen, 227, had trace to measurable amounts of aflatoxins B_1 and B_2 in the breast, leg and liver. Upon reviewing some other performance parameters of this hen, it was noted that she had stopped laying with no evident signs of recovery. In fact, the ovaries weighed only 7.2 g, and thus were to small to be analyzed. Her liver was still significantly enlarged at slaughter, comprising about 6.75% of her body weight compared to a control value of approximately 2.1%. The large size of the liver may have had a dilution effect

Table 26 - Aflatoxin Residues Detected in the Tissues of Hens After 4 Days of Withdrawal From the Aflatoxin-Spiked Diet

Hen # or Sample	Aflatoxin Level[1] (ug/kg)									
	B_1	B_2	M_1	M_2	B_{2a}	B_1	B_2	M_1	M_2	B_{2a}
	BREAST					LEG				
225	0	0	0	0	0	0	0	0	0	0
226	0	0	0	0	0	0	0	0	0	0
227	tr	tr	0	NR	NR	0.01	0.02	0	0	0
228	0	0	0	0	0	0	0	0	0	0
229	0	0	0	0	0	0	0	0	0	0
230	0	0	0	0	0	0	0	0	0	0
231	0	0	0	0	0	0	0	0	0	0
232	0	0	0	0	0	0	0	0	0	0
Mean[2]	0	0	0	0	0	0	0	0	0	0
	LIVER					GIZZARD				
225	0	0	0	0	0	0	0	0	0	0
226	0	0	0	0	0	0	0	0	0	0
227	tr	tr	0	0	tr	0	0	0	0	0
228	0	0	0	0	0	0	0	0	0	0
229	0	tr	0	0	0.04	0	tr	0	0	NR
230	0	0	0	0	0.07	0	0	0	0	0
231	0	0	0	0	tr	0	0	0	0	0
232	0	0	0	0	0.08	0	0	0	0	0
Mean[2]	0	0	0	0	0.02	0	0	0	0	0
	OVARIES					SPLEEN[3]				
225	0	0	0	0	0					
226	0	0	0	0	0					
227[4]			NA							
228	0	tr	0	0	tr					
229	0	0	0	0	0					
230	0	0	0	0	tr					
231	0	0	0	0	0					
232	0	0	0	0	0.01					
Mean[2]	0	0	0	0	0	0	0	0	0	0
	HEART					KIDNEYS				
I[5] (226,228, 229,230)	0	0	0	0	0	0	0	0	0	0
II[5] (225,227 231,232)	0	0	0	0	0	0	0.01	0	0	tr
Mean[2]	0	0	0	0	0	0	0	0	0	0
	BLOOD CLOT[6]					BLOOD SERUM[6]				
All hens	0	0	0	0	0	tr	0.01	0	0	0

1) tr = trace amounts - visible but too small to quantitate (< 0.01 ug/kg)
NR = chromatogram not well resolved, NA = not analyzed

2) Means calculated assuming tr = 0.0058, which is the largest amount which would not be measurable

3) The spleens from all hens were combined for analysis

4) The ovary of hen 227 was not analyzed because it was too small

5) Sample represents a composite of heart and kidney samples for the four hens shown in parenthesis

6) From combined blood of all hens

on the residue levels and could account for the fact that only trace amounts of aflatoxins were detected in this organ. Hen 227 also lost 225 g of body weight during the 4 days of clearance. From these observations it is evident that this hen appeared to be more sensitive to aflatoxins and had not recovered before slaughter.

No aflatoxin residues were detected in the heart or spleen of hens sacrificed four days after withdrawal from the spiked ration. However, blood serum contained some AFB_2 and one kidney sample contained traces of AFB_{2a} and AFB_2. AFB_{2a} was detected in 5 liver and 3 ovary samples on day 4.

Both AFB_{2a} and AFB_1-dihydrodiol may react with proteins, peptides and amino acids at physiological and alkaline pH values to form Schiff bases (Gurtoo and Dahms, 1974; Neal et al., 1981). Aflatoxin residues bound to tissue components probably remain in the tissues longer than the free or conjugated aflatoxins. This may explain the persistence of AFB_{2a} residues in the present study and may account for the fact that radioactivity was still detected in the excreta and tissues of hens dosed with ^{14}C-AFB_1 at 7 days after dosing (Sawhney et al., 1973). Under the extraction conditions used in the present study, dissociation of the bonds of AFB_{2a} to cellular components, which have been reported to be reversible and pH dependent (Ashoor and Chu, 1975), could result in the release and detection of AFB_{2a} over a longer period of time.

No aflatoxin residues were detected in the breast, leg,

gizzard and ovaries of hens sacrificed after 8 days on the aflatoxin-free diet (Table 27). Four liver samples had trace to measurable, but low levels of either aflatoxins B_1, B_2 or B_{2a}. One of the composite kidney samples contained some AFB_2 and AFB_{2a}, and the blood clot had AFB_1, AFB_2 and traces of AFB_{2a}. However, by day 16, no aflatoxins were recovered from the kidneys, blood clot or serum.

Performance data for hens showing aflatoxin residues after 8 days on the aflatoxin-free diet were examined. Hen 240, which had residues of aflatoxins B_1, B_2 and B_{2a} in the liver, had stopped laying during the third week of aflatoxin feeding. The ovary size of this hen at death was normal, however, and there were large and medium ova present, which would indicate that recovery had begun. Liver size appeared to be normal , although it was still slightly pale and showed some petechial hemorrhages, which may account for decreased ability of this hen to metabolize aflatoxins that were found in the liver.

Results of the present study indicate that differences exist between individual hens in the amount of time required to achieve tissue clearance upon removal of aflatoxins from the feed. Parameters like egg production and the size and condition of the liver and ovaries serve as indicators of recovery from aflatoxin exposure and appear to be related to the likelihood of detecting aflatoxin residues in the tissues of hens. Similarly, Van Zytveld *et al.* (1970) detected aflatoxins in the tissues of chickens that grew poorly or died

Table 27 - Aflatoxin Residues Detected in the Tissues of Hens After 8 Days of Withdrawal from the Aflatoxin-Spiked Diet

Hen # or Sample	Aflatoxin Level[1] (ug/kg)									
	B_1	B_2	M_1	M_2	B_{2a}	B_1	B_2	M_1	M_2	B_{2a}
	BREAST					LEG				
235	0	0	0	0	0	0	0	0	0	0
236	0	0	0	0	0	0	0	0	0	0
237	0	0	0	0	0	0	0	0	0	0
238	0	0	0	0	0	0	0	0	0	0
239	0	0	0	0	0	0	0	0	0	0
240	0	0	0	0	0	0	0	0	0	0
241	0	0	0	0	0	0	0	0	0	0
242	0	0	0	0	0	0	0	0	0	0
Mean[2]	0	0	0	0	0	0	0	0	0	0
	LIVER					GIZZARD				
235	0	0	0	0	0	0	0	0	0	0
236	0	0	0	0	tr	0	0	0	0	0
237	tr	tr	0	0	0	0	0	0	NR	NR
238	0	0	0	0	0.02	0	0	0	0	0
239	0	0	0	0	0	0	0	0	0	0
240	tr	0.01	0	0	0.01	0	0	0	0	0
241	0	0	0	0	0	0	0	0	0	0
242	0	0	0	0	0	0	0	0	0	0
Mean[2]	0	0	0	0	0	0	0	0	0	0
	OVARIES									
235	0	0	0	0	0					
236	0	0	0	0	0					
237	0	0	0	0	0					
238	0	0	0	0	0					
239	0	0	0	0	0					
240	0	0	0	0	0					
241	0	0	0	0	0					
242	0	0	0	0	0					
Mean[2]	0	0	0	0	0					
	KIDNEYS									
I[3] (235,239, 237,242)	0	0	0	0	0					
II[3] (236,238, 240,241)	0	0.01	0	0	tr					
Mean[2]	0	tr	0	0	0					
	BLOOD CLOT[4]					BLOOD SERUM[4]				
All hens	0.02	0.03	0	0	tr	0	0	0	0	0

1) tr = trace amounts - visible but too small to quantititate (< 0.01 ug/kg)
NR = chromatogram not well resolved

2) Means calculated assuming tr = 0.0058, the largest amount that would not be measurable

3) Sample represents a composite of kidney samples for the hens shown in parenthesis

4) From combined blood of all 8 hens

during aflatoxin feeding, but residues were found in only one chicken reaching market weight.

Since it was noted that egg production and liver size appeared to be related to the likelihood of finding aflatoxin residues in the tissues of laying hens, the livers of hens 244 (slaughtered on day 16), hen 257 (slaughtered on day 24) and hen 261 (slaughtered on day 32) were analyzed. Liver size at death had returned to normal for these hens, although only hen 244 had resumed normal egg production. No residues were detected in the livers of hens 244 or 257, but 0.04 ug/kg of AFB_2 were detected in the liver of hen 261.

After finding AFB_2 residues in the liver of hen 261, the livers of all hens slaughtered on the last day of the experiment (32 days) were analyzed. No aflatoxin residues were detected in any of the livers of the other hens sacrificed at this time. The fact that hen 261 still had AFB_2 residues in the liver further indicates the existence of individual variation in response to aflatoxins, and was also manifested by gross observations at slaughter including an enlarged spleen, reabsorbed ova and weight loss over the entire clearance period.

Present results demonstrate that residue levels decrease rapidly, but complete tissue clearance may require longer periods or time in some hens which may be more sensitive to the effects of aflatoxins. Pier (1981) concluded that when animals of the same age and breeding are fed aflatoxins experimentally, there is often a marked variation in the effects

on different individuals in the same experimental group. Recently, Marks and Wyatt (1979) indicated that at least part of the within-breed resistance to the effects of aflatoxins may be hereditary. They reported that the offspring of quail selected for resistance to the effects of aflatoxin had significantly lower mortality on diets containing aflatoxins than those produced by unselected parents. In a study of genetic resistance of chickens to aflatoxins, Williams et al. (1980) also reported that individual responses of the birds were quite variable, suggesting that selection for resistant progeny could reduce aflatoxin sensitivity in poultry. It would be interesting to know, however, if resistant birds also deposit less tissue residues and achieve faster clearance.

Species differences in response to aflatoxins have been reported by several workers (Ciegler, 1975; Pier, 1981). Comparison of the present study with the results of Furtado et al. (1982) and of Chen et al. (1984) on the time required for aflatoxin clearance from tissues of pigs and chickens, respectively, further demonstrates the existence of species differences in the response to aflatoxins. Both studies reported clearance of aflatoxin residues from all tissues after 4 days on an aflatoxin-free diet. In the present study, however, clearance of aflatoxin residues in the breast, leg, gizzard and ovaries required 8 days of withdrawal, and for the kidneys and blood 16 days. One liver sample still had measurable levels of AFB_2 32 days after withdrawal.

Slow clearance of aflatoxin residues from the tissues of hens dosed with ^{14}C-AFB_1 has also been reported by Sawhney et al. (1973). These authors found that approximately 29% of the administered dose still remained in the tissues of laying hens 7 days after treatment. Trucksess et al. (1983) detected aflatoxicol in the muscle of most hens and AFB_1 in the liver of one hen 7 days after withdrawal of aflatoxin from the diet, but residues were not detectable in the kidneys, blood and ovaries at this time.

Studies using labeled aflatoxins have shown that most of the administered dose appears in the tissues and excreta of the dosed animals as water soluble conjugated aflatoxin metabolites (Bassir and Osiyemi, 1967; Mabee and Chipley, 1973a, 1973b). Harland and Cardeilhac (1975) reported that only 25% of the ^{14}C excreted in the bile and urine of hens dosed with ^{14}C-AFB_1 could be extracted with chloroform, while most (75%) of the ^{14}C excreted remained in the aqueous phase. Similarly, Mabee and Chipley (1973b) reported that on average 80% of the radioactivity in the excreta and tissues of layer hens was confined to the sodium acetate portion of the extracts. They concluded that metabolism of aflatoxins by animals, which results in the formation of conjugates, may account for the conflicting reports and failure to isolate aflatoxins in animal products.

Due to their high polarity and water solubility, aflatoxin conjugates would not be extracted by the solvents used in the present study. However, the possibility that aflatoxin

conjugates may remain in the tissues of hens following withdrawal from the contaminated feed is more unlikely than is the case for the free aflatoxins. Gregory et al. (1983) reported that both free and conjugated aflatoxins were cleared at rapid and similar rates from the tissues of turkey poults that had been fed a diet spiked with AFB_1.

Previous studies have not followed the clearance of aflatoxins from laying hens for such a long period of time. This allowed us to observe different response patterns to aflatoxins in terms of egg production, gross lesions and aflatoxin residues in different tissues. There were differences in the length of time required to achieve clearance of aflatoxin residues from different tissues of the laying hens. In fact, one liver still had measurable amounts of AFB_2 32 days after withdrawal from the contaminated feed. In general, however, no residues were detected in most tissues after 8 days on the aflatoxin-free diet.

The importance of water soluble conjugates and covalently bound aflatoxins can not be disregarded, since Wei et al. (1978) have shown that the intestinal microflora of the rat has the ability to hydrolyze aflatoxin conjugates and to release free toxins. This may contribute to the risk of chronic toxicity during long term exposure to contaminated tissues. More information on the presence, toxicity and metabolism of aflatoxin conjugates and adducts is needed to fully determine the importance of these residues in tissues of animals destined for human consumption.

SUMMARY

A feeding trial was conducted to determine the levels of aflatoxins deposited in the eggs and tissues of hens fed an aflatoxin-spiked diet for 4 weeks. The aflatoxin-spiked diet contained 3,310 ug of AFB_1 and 1,680 ug of AFB_2 per kg. The amount of time necessary to achieve egg and tissue clearance upon removal of the spiked diet was also ascertained over a period of 32 days.

Aflatoxin feeding resulted in a significant decrease in both egg production and egg weights by the third and fourth weeks of feeding, respectively. At the end of aflatoxin feeding, however, there was no significant difference in body weights for the treated and control hens.

After feeding aflatoxins for 4 weeks, 16 treated and 8 control hens were sacrificed, examined for gross lesions and selected tissues were analyzed for residues. The livers appeared slightly to very pale with some petechial hemorrhages or with large hemorrhages close to the edges. None of the ovaries of the treated hens had ova larger than 25 mm in diameter. The livers, kidneys and spleens of the aflatoxin fed hens were 61, 14 and 20% larger than those of controls, respectively. In contrast, the ovaries and hearts were 27 and 7.5% smaller than those of the controls.

Egg production and egg weights of the treated hens returned to control values by the end of the second week of clearance. After 32 days on the aflatoxin-free ration, only the hearts of the treated hens were different from the controls, remaining significantly smaller. All other organs had returned to control values.

Transfer of aflatoxins from the feed into eggs occurred rapidly. Residue levels in whole eggs increased to a maximum by 4-5 days and remained relatively constant throughout aflatoxin feeding. Aflatoxins B_2, M_1 and M_2 levels were similar in the yolk and albumen, while higher levels of AFB_1 and AFB_{2a} were recovered from the yolk. The mean value for the combined aflatoxins in the whole egg was less than 0.5 ug/kg. Clearance of aflatoxin residues from the albumen occurred faster than from the yolk. Due to an insufficient number of eggs, analysis of separated eggs could not be carried out on days 3 and 4 of withdrawal. However, no residues were detected in the albumen and the yolk after 5 and 7 days of withdrawal, respectively. No aflatoxin residues could be recovered from whole eggs after feeding the aflatoxin- free diet for four days, probably as a result of dilution.

The effects of cooking on the levels of aflatoxins in naturally and artificially contaminated eggs were studied. At the low levels of aflatoxins present, the sensitivity and precision of the method were not high enough to detect consistent differences. In general, however, no major effects on aflatoxins were observed with cooking.

Only a small fraction of the aflatoxins consumed was deposited in the tissues, either as the original compounds or as their metabolites. The aflatoxins were found widely distributed in all tissues. The compound tentatively identified as AFB_{2a} was the most abundant residue. The highest levels of aflatoxins were detected in the gizzard, kidneys and liver, with average combined concentrations of less than 3 ug/kg each. The lowest residue levels were detected in the breast, blood serum and leg. The breast muscle had a total concentration of less than 0.1 ug/kg.

Upon withdrawal from the contaminated feed, residue levels in the tissues decreased rapidly. However, complete tissue clearance required longer for some hens than for others, which could result from greater absorption or slower clearance. By two days after removal of the contaminated feed, aflatoxin residues in all tissues had decreased markedly, with no aflatoxins being detected in the heart or spleen. No aflatoxin residues were detected in the breast, leg, gizzard and ovaries of hens sacrificed 8 days after withdrawal, or in the kidneys and blood at 16 days. However, one hen out of seven still had measurable amounts of AFB_2 in the liver at 32 days after withdrawal.

Results of the present study indicated that differences exist between tissues and between individual hens in the amount of time required to achieve tissue clearance. However, few residues were detected in the eggs and in most tissues

after 4 and 8 days on the aflatoxin-free diet, respectively. Thus, there appears to be little or no risk to humans on consuming eggs or meat from hens 8 days after removal of aflatoxins from the feed.

LIST OF REFERENCES

Abbott, W.W. and Couch, J.R. 1971. Fatty liver syndrome. Proc. Distillers Feed Conf. 26:27.

Abrams, L. 1965. Mycotoxicosis. J. South Africa Vet. Med. Ass. 36:5.

Allcroft, R. and Carnaghan, R.B.A. 1962. Groundnut toxicity: Aspergillus flavus toxin (aflatoxin) in animal products: preliminary communication. Vet. Rec. 74:863.

Allcroft, R. and Carnaghan, R.B.A. 1963. Groundnut toxicity: An examination for toxin in human food products from animals fed toxic groundnut meal. Vet. Rec. 75:259.

Allcroft, R., Carnaghan, R.B.A., Sargeant, K. and O'Kelly, J. 1961. A toxic factor in Brazilian groundnut meal. Vet. Rec. 73:428.

Allcroft, R. and Raymond, W.D. 1966. Toxic groundnut meal: Biological and chemical assays of a large batch of a 'reference' meal used for experimental work. Vet. Rec. 79:122.

Allcroft, R. and Roberts, B.A. 1968. Toxic groundnut meal: The relationship between aflatoxin B_1 intake by cows and excretion of aflatoxin M_1 in milk. Vet. Rec. 82:116.

Allcroft, R., Rogers, H., Lewis, G., Nabney, J. and Best, E. 1966. Metabolism of aflatoxin in sheep: Excretion of the milk toxin. Nature (London) 209:154.

Allen-Hoffmann, B.L. and Campbell, T.C. 1977. The relationship between hepatic glutathione levels and the formation of aflatoxin B_1-DNA adducts as influenced by dietary protein intake. Fed. Proc. 36:1116.

Alpert, M.E., Hutt, M.S.R., Wogan, G.N. and Davidson, C.S. 1971. Association between aflatoxin content and hepatoma frequency in Uganda. Cancer 28:253.

Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. 1973. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. U.S.A. 70:2281.

Amla, I., Kamala, C.S., Goplakrishna, G.S., Jayaraj, A.P., Sreenivasamurthy, V. and Parpia, H.A.B. 1971. Cirrhosis in children from a peanutmeal contaminated with aflatoxin. Am. J. Clin. Nutr. 24:609.

Anonymous. 1982. FDA raises aflatoxin action level for cottonseed meal. Food Chem. News Aug. 2, pp. 40-43.

Anonymous. 1984. Validity of aflatoxin link to human cancer questioned. Food Chem. News Jan. 30, pp. 32-33.

Applebaum, R.S., Bracket, R.E., Wiseman, D.W. and Marth, E.H. 1982. Aflatoxin: Toxicity to dairy cattle and occurrence in milk and milk products - A review. J. Food Protection 45:752.

Applebaum, R.S. and Marth, E.H. 1982. Fate of aflatoxin M_1 in cottage cheese. J. Food Protection. 45:903.

Armbrecht, B.H. 1971. Aflatoxin residues in food and feed derived from plant and animal sources. Residue Rev. 41:13.

Armbrecht, B.H., Wiseman, H.G., Shalkip, W.P. and Geleta, J. N. 1971. Swine aflatoxicosis. I. An assessment of growth efficiency and other response in growing pigs fed aflatoxin. Environm. Physiol. 1:198.

Asao, T., Büchi, G. , Abdel-Kader, M.M., Chang, S.B., Wick, E.L. and Wogan, G.N. 1965. The structures of aflatoxins B_1 and G_1. J. Am. Chem. Soc. 87:882.

Ashoor, S.H. and Chu, F.S. 1975. Interaction of aflatoxin B_{2a} with amino acids and proteins. Biochem. Pharmacol. 24:1799.

Asplin, F.D. and Carnaghan, R.B.A. 1961. The toxicity of certain groundnut meals for poultry with special reference to their effect on ducklings and chickens. Vet. Rec. 73:1215.

Association of Official Analytical Chemists. 1975a. Changes in Official Methods of Analysis made at the 88th Annual Meeting, Oct. 14-17, 1974. J. Ass. Off. Anal. Chem. 58:393.

Association of Official Analytical Chemists. 1975b. Official Methods of Analysis. 12th ed. Washington, D.C.

Association of Official Analytical Chemists. 1980. Official Methods of Analysis. 13th ed. Washington, D.C.

Association of Official Analytical Chemists. 1965. Official Methods of Analysis. 10th ed. Washington, D.C.

Babubunmin, E.A. and Bassir, O. 1972. Species differences in the anticoagulation activities of aflatoxin B_1 and 4-hydroxy coumarin. Afr. J. Med. Sci. 3:97.

Bassir, O. and Adekunle, A. 1970. Teratogenic action of aflatoxin B_o, palmotoxin B_o and palmotoxin G_o on the chick embryo. J. Pathol. 102:49.

Bassir, O. and Osiyemi, F. 1967. Biliary excretion of aflatoxin in the rat after a single dose. Nature (London) 215:882.

Becroft, D.M. and Webster, D.R. 1972. Aflatoxins and Reye's disease. Br. Med. J. 4:117.

Blount, W.P. 1961. Turkey "X" disease. J. Br. Turkey Fed. 9:52.

Blumberg, W.E. 1978. Enzymatic modification of environmental intoxicants: The role of cytochrome P-450. Quarterly Rev. Biophys. 11:481.

Bossard, E.H. and Combs, G.F. 1970. Lipotropic agents in broiler breeder rations. Poultry Sci. 49:599.

Bourgeois, C.H., Keschamras, N., Comer, D.S., Harikul, S., Evans, H., Olson, C., Smith, T. and Beck, M.R. 1969. Udon encephalopathy: Fatal cerebral edema and fatty degeneration of the viscera in Thai children. J. Med. Ass. Thailand 52:553.

Brackett, R.E. and Marth, E.H. 1982. Fate of aflatoxin M_1 in Parmesan and Mozzarella cheese. J. Food Protection 45:597.

Brekke, O.L., Sinnhuber, R.O., Peplinski, A.J., Wales, J.H., Putnam, G.B., Lee, D.L. and Ciegler, A. 1977. Aflatoxin in corn: Ammonia inactivation and bioassay with rainbow trout. J. Appl. Environ. Microbiol. 34:34.

Brown, J.M.M. and Abrams, L. 1965. Biochemical studies on aflatoxicosis. Onderstepoort J. Vet. Res. 32:119.

Büchi, G., Foulkes, D.M., Kurono, M., Mitchell, G.F. and Schneider, R.S. 1967. Total synthesis of racemic aflatoxin B_1. J. Am. Chem. Soc. 89:6745.

Büchi, G., Muller, P.M., Roebuck, B.D. and Wogan, G.N. 1974. Aflatoxin Q_1: A major metabolite of aflatoxin B_1 produced by human liver. Res. Commun, Chem. Pathol. Pharmacol. 8:585.

Busby, W.F.Jr. and Wogan, G.N. 1981. Aflatoxins. In: Mycotoxins and N-nitroso Compounds: Environmental Risks Volume II. R. Shank (Ed.) CRC Press Inc. Boca Raton, Florida pp. 4-27.

Butler, W.H. 1964. Acute toxicity of aflatoxin B_1 in rats. Brit. J. Cancer. 18:756.

Butler, W.H. 1966. Acute toxicity of aflatoxin B_1 in guinea pigs. J. Pathol. Bacteriol. 91:277.

Butler, W.H. and Clifford, J.I. 1965. Extraction of aflatoxin from rat liver. Nature (London) 206:1045.

Butler, W.H. and Lijinsky, W. 1970. Acute toxicity of aflatoxin G_1 to the rat. J. Pathol. 102:209.

Campbell, T.C., Caedo, J.P., Bulatao-Jayme, J., Salamat, L. and Eugel, R.W. 1970. Aflatoxin M_1 in human urine. Nature (London) 227:403.

Campbell, T.C. and Hayes, J.R. 1976. The role of aflatoxin metabolism in its toxic lesion. Toxicol. Appl. Pharmacol. 35:199.

Campbell, T.C. and Salamat, L. 1971. Aflatoxin ingestion and excretion by humans. In: Mycotoxins in Human Health. I.F.H. Purchase (Ed.) Macmillan Press, London pp. 271-280.

Campbell, T.C. and Stoloff, L. 1974. Implications of mycotoxins for human health. J. Agric. Food Chem. 22:1006.

Carnaghan, R.B.A., Hartley, R.D. and D'Kelly, J. 1963. Toxicity and fluorescence properties of the aflatoxins. Nature (London) 200:1101.

Carnaghan, R.B.A., Lewis, G., Patterson, D.S.P. and Allcroft, R. 1966. Biochemical and pathological aspects of groundnut poisoning in chickens. Pathol. Vet. 3:601.

Chang, S.B., Abdel-Kader, M.M., Wick, E.L. and Wogan, G.N. 1963. Aflatoxin B_2: Chemical identity and biological activity. Science 142:1191.

Chaves-Carballo, E., Elifson, R.D. and Gomez, M.R. 1976. An aflatoxin in the liver of a patient with Reye-Johnson syndrome. Mayo Clin. Proc. 51:48.

Chen, C. 1983. Tissue deposition and clearance of aflatoxins from broiler chickes. Master Thesis. Michigan State University. East Lansing, Michigan.

Chen, C., Pearson, A.M., Coleman, T.H., Gray, J.I., Pestka, J.J. and Aust, S.D. 1984. Tissue deposition and clearance of aflatoxins from broiler chickens fed a contaminated diet. Food Chem. Toxicol. 22:447.

Chipley, J.R., Mabee, M.S., Applegate, K.L. and Dreyfuss, M. S. 1974. Further characterization of tissue distribution and metabolism of ^{14}C-aflatoxin B_1 in chickens. Appl. Microbiol. 28:1027.

Ciegler, A. 1975. Mycotoxins: Occurrence, chemistry, biological activity. Lloydia 38:21.

Ciegler, A. 1978. Fungi that produce mycotoxins: conditions and occurrence. Mycopathologia 65:5.

Coles, B.F., Welch, A.M., Herzog, P.J., Smith, J.R.L. and Garner, R.C. 1980. Biological and chemical studies on 8,9-dihydroxy-8,9-dihydro-aflatoxin B_1 and some of its esters. Carcinogenesis 1:79.

Conney, A.H., Lu, A.Y.H., Levin, W. Somogyi, A., West, S., Jacobson, M., Ryan, D. and Kuntzman, R. 1973. Effect of enzyme inducers on substrate specificity of the cytochrome P-450's. Drug Metab. Dispos. 1:199.

Conway, H.F., Anderson, R.A. and Bagley, E.B. 1978. Detoxification of aflatoxin-contami ted corn by roasting. Cereal Chem. 55:115.

Cottier, G.J., Moore, C.H., Diener, U.L. and Davis, N.D. 1968. Aflatoxin studies with White Rock chickens. Poultry Sci. 47:1663.

Cottier, G.J., Moore, C.H., Diener, U.L. and Davis, N.D. 1969. The effect of feeding four levels of aflatoxin on hatchability and subsequent performance of broilers. Poultry Sci. 48:1797.

Croy, R.G., Essigman, J.M., Reinhold, U.N. and Wogan, G.N. 1978. Identification of the principal aflatoxin B_1-DNA adduct formed in vivo in rat liver. Proc. Natl. Acad. Sci. U.S.A. 75:1745.

Dalezios, J.I. and Wogan, G.N. 1972. Metabolism of aflatoxin B_1 in rhesus monkeys. Cancer Res. 32:2297.

Dalezios, J.I., Wogan, G.N. and Weinbeb, S.M. 1971. Aflatoxin P_1: A new aflatoxin metabolite in monkeys. Science 171:584.

Dann, R.E., Mutscher, L.A. and Couri, D. 1972. The in vivo metabolism of ^{14}C-labeled aflatoxins B_1, B_2 and G_1 in rats. Res. Commun, Chem. Pathol. Pharmacol. 3: 667.

Dashek, W.V., Barker, S.M., Statkiewicz, W.R., Shanks, E.T. and Llewellyn, G.C. 1983. Histochemical analysis of liver cells from short term aflatoxin-dosed and non-dosed Coturnix coturnix japonica. 1. Aflatoxin-sensitive quail. Poultry Sci. 62:2347.

Davis, N.D. and Diener, U.L. 1970. Environmental factors affecting the production of aflatoxin. In: Proceedings of the First US-Japan Conference on "Toxic Microorganisms" M. Herzberg (Ed.) US Government Printing Office Washington, D.C. pp. 43-47.

Degen, G.H. and Neumann, H.G. 1978. The major metabolite of aflatoxin B_1 in the rat is a glutathione conjugate. Chem. Biol. Interactions 22:239.

De Iongh, H., Vles, R.O. and Van Pelt, J.G. 1964. Milk of mammals fed an aflatoxin-containing diet. Nature (London) 202:466.

Detroy, R.W. and Hesseltine, C.W. 1968. Isolation and biological activity of a microbial conversion product of aflatoxin B_1. Nature (London) 219:967.

Diener, U.L. and Davis, N.D. 1966. Aflatoxin production by isolates of Aspergillus flavus. Phytopathology 56:1390.

Di Paolo, J.A., Elis, J. and Erwin, H. 1967. Teratogenic response by hamsters, rats and mice to aflatoxin B_1. Nature (London) 215:638.

Doerr, J.A., Wyatt, R.D. and Hamilton, P.B. 1976. Impairment of coagulation function during aflatoxicosis in young chickens. Toxicol. Appl. Pharmacol. 35:437.

Donaldson, W.E., Tung, H.T. and Hamilton, P.B. 1972. Depression of fatty acid synthesis in chick liver (Gallus domesticus) by aflatoxin. Comp. Biochem. Physiol. 41B:843.

Doyle, M.P., Applebaum, R.S., Brackett, R.E. and Marth, E.H. 1982. Physical, chemical and biological degradation of mycotoxins in foods and agricultural commodities. J. Food Protection 45:964.

Dunnett, C.W. 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Ass. 50:1096.

Dunnett, C.W. 1964. New tables for multiple comparisons with a control. Biometrics 20:482.

Dutton, M.F. and Heathcote, J.G. 1966. Two new hydroxyaflatoxins. Biochem J. 101:21.

Dutton, M.F. and Heathcote, J.G. 1968. Structure, biochemical properties and origins of aflatoxins B_{2a} and G_{2a}. Chem. Ind. 7:418.

Dvorackova, I., Kusak, V., Veseoi, D., Vesela, J. and Nesnidal, P. 1977. Aflatoxin and encephalopathy with fatty degeneration of viscera. Ann. Nutr. Alim. 31: 977.

Edds, G.T. 1979. Aflatoxins. Overview. In: Conference on Mycotoxins in Animal Feeds and Grains Related to Animal Health. Bureau of Veterinary Medicine, FDA. Rockville, Maryland pp. 80-84.

Elis, J. and Di Paolo, J.A. 1967. Aflatoxin B_1: induction of malformations. Arch. Pathol. 83:53.

Epstein, S.M., Bartus, B. and Farber, E. 1969. Renal epithelial neoplasms induced in male Wistar rats by oral aflatoxin B_1. Cancer Res. 29:1045.

Essigman, J.M., Croy, R.G., Nadzan, A.M., Busby, W.F. Jr. Reninhold, U.N., Büchi, G. and Wogan, G.N. 1977. Structural identification of the major DNA adduct formed by aflatoxin B_1 in vitro. Proc. Natl. Acad. Sci. U.S.A. 74:1870.

Exarchos, C.C. and Gentry , R.F. 1982 . Effect of aflatoxin B_1 on egg production. Avian Diseases 26:191.

Falk, H.L., Thompson, S.J. and Kotin, P. 1965. Metabolism of aflatoxin B_1 in the rat. Proc. Am. Ass. Cancer Res. 6:18.

Forgacs, J. and Carll, W.T. 1962. Mycotoxicoses. Adv. Vet. Sci. 7:273.

Frazier, W.C. and Westhoff, D.C. 1978. Food Microbiology 3rd. ed. McGraw Hill Book Co. New York, N.Y. pp. 3-16.

Furtado, R.M. 1980. The effects of withdrawal and processing upon the levels of aflatoxins in the tissues of pigs fed a contaminated ration. Ph.D. Dissertation. Michigan State University, East Lansing, MI.

Furtado, R.M., Pearson, A.M., Gray, J.I., Hogberg, M.G. and Miller, E.R. 1981. Effects of cooking and/or processing upon levels of aflatoxins in meat from pigs fed a contaminated diet. J. Food Sci. 46:1306.

Furtado, R.M., Pearson, A.M., Hogberg, M.G. and Miller, E.R. 1979. Aflatoxin residues in the tissues of pigs fed a contaminated diet. J. Agric. Food Chem. 27:1351.

Furtado, R.M., Pearson, A.M., Hogberg, M.G., Miller, E.R., Aust, S.D. 1982. Withdrawal time required for clearance of aflatoxins from pig tissues. J. Agric. Food Chem. 30:101.

Gänshirt, H. 1965. Documentation of thin layer chromatograms. In: Thin Layer Chromatography. A Laboratory Handbook. E. Stahl (Ed.) Springer-Verlag, Berlin pp. 40-44.

Garlich, J.D., Tung, H.T. and Hamilton, P.B. 1973. The effects of short term feeding of aflatoxin on egg production and some plasma constituents of the laying hen. Poultry Sci. 52:2206.

Garner, R.C. and Hanson, R.S. 1971. Formation of a factor lethal for S. typhimurium TA1530 and TA1531 on incubation of aflatoxin B_1 with rat liver microsomes. Biochem. Biophys. Res. Commun. 45:774.

Garner, R.C., Miller, E.C. and Miller, J.A. 1972. Liver microsomal metabolism of aflatoxin B_1 to a reactive derivative toxic to Salmonella typhimurium TA1530. Cancer Res. 32:2058.

Gillette, J.R. 1966. Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. Advan. Pharmacol. 4:219.

Gillette, J.R. 1979. Effects of induction of cytochrome P-450 enzymes on the concentration of foreign compounds and their metabolites and on the toxicological effects of these compounds. Drug Metab. Rev. 10:59.

Goldblatt, L.A. 1971. Control and removal of aflatoxin. J. Amer. Oil Chem. Soc. 48:605.

Green, C.E., Rice, D.W., Hsieh, D.P.H. and Byard, J.L. 1982. The comparative metabolism and toxic potency of aflatoxin B_1 and aflatoxin M_1 in primary cultures of adult-rat hepatocytes. Food Chem. Toxicol. 20:53.

Gregory, J.F. III, Golstein, S.L. and Edds, G.T. 1983. Metabolite distribution and rate of tissue clearance in turkeys fed a diet containing aflatoxin B_1. Food Chem. Toxicol. 21:463.

Gregory, J.F. III, and Manley, D. 1981. High performance liquid chromatographic determination of aflatoxins in animal tissues and products. J. Ass. Off. Anal. Chem. 64:144.

Guengerich, F.P. 1977. Separation and purification of multiple forms of microsomal P-450. J. Biol. Chem. 252: 3970.

Gumbmann, M.R., Williams, S.N., Booth, A.N., Vohra, P., Ernst, R.A., Bethard, M. 1970. Aflatoxin susceptibility in various breeds of poultry. Proc. Soc. Exptl. Biol. Med. 134:683.

Gurtoo, H.L. and Campbell, T.C. 1974. Metabolism of aflatoxin B_1 and its metabolism-dependent and independent binding to rat hepatic microsomes. Molec. Pharmacol. 10:776.

Gurtoo, H.L. and Dahms, R. 1974. On the nature of the binding of aflatoxin B_{2a} to rat hepatic microsomes. Res. Commun. Chem. Pathol. Pharmacol. 9:107.

Hamilton, P.B. 1971. A natural and extremely severe occurrence of aflatoxicosis in laying hens. Poultry Sci. 50:1880.

Hamilton, P.B. 1977. Interrelationships of mycotoxins with nutrition. Fed. Proc. 36:1899.

Hamilton, P.B. and Garlich, J.D. 1971. Aflatoxin as a possible cause of fatty liver syndrome in laying hens. Poultry Sci. 50:800.

Hamilton, P.B. and Garlich, J.D. 1972. Failure of vitamin supplementation to alter the fatty liver syndrome caused by aflatoxin. Poultry Sci. 51:688.

Harland, E.C. and Cardeilhac, P.T. 1975. Excretion of carbon-14-labeled aflatoxin B_1 in bile, urine and intestinal contents of the chicken. Am. J. Vet. Res. 36:909.

Hartley, R.D., Nesbitt, B.F. and O'Kelly, J. 1963. Toxic metabolites of Aspergillus flavus. Nature (London) 198:1056.

Hayes, W.J. 1975. Toxicology of Pesticides. Williams and Wilkins, Co. Baltimore, Maryland.

Hayes, A. W. 1978. Biological activities of mycotoxins. Mycopathologia. 65:29.

Hayes, A.W. 1980. Mycotoxins: A review of biological effects and their role in human diseases. Clin. Toxicol. 17:45.

Hayes, J.H., Polan, C.E. and Campbell, T.C. 1977. Bovine liver metabolism and tissue distribution of aflatoxin B_1. J. Agric. Food Chem. 25:1189.

Hesseltine, C.W. 1979. Introduction, definition and history of mycotoxins of importance to animal production. In: Interactions of Mycotoxins in Animal Production. Proceedings of a Symposium. July 13, 1978. National Academy of Sciences, Washington, D.C. pp. 3-18.

Holzapfel, C.W., Steyn, P.S. and Purchase, I.F.H. 1966. Isolation and structure of aflatoxins M_1 and M_2. Tetrahedron Lett. 25:2799.

Howarth, B. Jr. and Wyatt, R.D. 1976. Effect of dietary aflatoxin on fertility, hatchability and progeny performance of nroiler breeder hens. Appl. Environ. Microbiol. 31:680.

Hsieh, D.P.H., Salhab, A.S., Wong, J.J. and Yang, S.L. 1974. Toxicity of aflatoxin Q_1 as evaluated with the chicken embryo and bacterial auxotrophs. Toxicol. Appl. Pharmacol. 30:237.

Hsieh, D.P.H., Wong, Z.A., Wong, J.J., Michas, C. and Ruebber, B.H. 1977. Comparative metabolism of aflatoxin. In: Mycotoxins in Human and Animal Health. J.V. Rodricks, C.W. Hesseltine and Mahlman, M.A. (Eds.) Pathotox Publishers, Inc. Park Forest South, Illinois pp. 37-50.

Huff, W.E., Wyatt, R.D. and Hamilton, P.B. 1975. Effects of dietary aflatoxin on certain egg yolk parameters. Poultry Sci. 54:2014.

Hughes, B.L., Barnett, B.D., Jones, J.E. and Dick, J.W. 1979. Safety of feeding aflatoxin-inactivated corn to White Leghorn-Layer breeders. Poultry Sci. 58:1202.

Hughes, B.L. and Jones, J.E. 1979. Hematology of single comb White Leghorn pullets fed aflatoxin-contaminated and ammonia-treated corn. Poultry Sci. 58:981.

Jacobson, W.C., Harmeyer, W.C. and Wiseman, H.G. 1971. Determination of aflatoxins B_1 and M_1 in milk. J. Dairy Sci. 54:21.

Jacobson, W.C. and Wiseman, H.G. 1974. The transmission of aflatoxin B_1 into eggs. Poultry Sci. 53:1743.

Jaggi, W., Lutz, W.K., Lüthy, J., Zweifel, U. and Schlatter, Ch. 1980. In vivo covalent binding of aflatoxin metabolites isolated from animal tissue to rat-liver DNA. Food Cosmet. Toxicol. 18:257.

Jarvis, B. 1975. Mycotoxins in foods - Their occurrence and significance. Intern. J. Environ. Studies. 8:187.

Jensen, A.H., Brekke, O.L., Frank, R. and Peplinski, A.J. 1977. Acceptance and utilization by swine of aflatoxin-contaminated corn treated with aqueous or gaseous ammonia. J. Anim. Sci. 45:8.

Jones, B.D. 1978. Chemistry of aflatoxin and related compounds. In: Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. Vol. I. T.D. Wylie and L.G. Morehouse (Eds). Marcel Dekker, Inc. New York, N.Y. pp. 136-143.

Keyl, A.C. and Booth, A.N. 1971. Aflatoxin effects in livestock. J. Amer. Oil Chem. Soc. 48:599.

Kiermeier, F. 1977. Significance of aflatoxins in the dairy industry. Annual Bulletin International Dairy Federation. Document 98.

Kiermeier, F. and Mashaley, R. 1977. Einfluss der molkereitechnischen Behandlung der Rohmilch auf den Aflatoxin-M_1 Gehalt der daraus hergestellten Produkte. Z. Lebensm. Unters. Forsch. 164:183.

Kiermeier, F., Weiss, G., Behringer, G. and Miller, M. 1977a. Ueber das Vorkommen und den Gehalt von Aflatoxin M_1 in Käsen des Handels. Z. Lebensm. Unters. Forsch. 163:268.

Kiermeier, F.G., Weiss, G., Behringer, G., Miller, M. and Ranfft, K. 1977b. Vorkommen und Gehalt an Aflatoxin M_1 in molkereianlieferungs Milch. Z. Lebensm. Unters. Forsch. 163:171.

Klaassen, C.D. 1980. Absorption, distribution and excretion of toxicants. In: Toxicology. 2nd. ed. Chapter 3. Doul, J., Klaassen, C.D. and Amdur, M.O. (Eds.) Macmillan Publishing Co. Inc. London. pp. 28-55.

Kratzer, F.H., Bandy, D., Wiley, M. and Booth, A.N. 1969. Aflatoxin effects in poultry. Proc. Soc. Exptl. Biol. Med. 131:1281.

Krishnamachari, V.R., Nagarjan, R.V., Bhat, R.V. and Telak, T.B.G. 1975. Hepatitis due to aflatoxicosis: An outbreak in western India. Lancet (May 10, 1975):1061.

Krogh, P., Hald, B., Hasselager, E., Madsen, A., Montensen, H.P., Larsen, A.E. and Campbell A.D. 1973. Aflatoxin residues in bacon pigs. Pure Appl. Chem. 35:275.

Kumagai, S., Nakano, N. and Aibara, K. 1983. Interactions of aflatoxin B_1 and blood components of various species in vitro: Interconversion of aflatoxin B_1 and aflatoxicol in blood. Toxicol. Appl. Pharmacol. 67:292.

Lancaster, C.M., Jenkins, F.B. and Philip, J.M. 1961. Toxicity associated with certain samples of groundnuts. Nature (London) 192:1095.

Lee, L.S., Cucullu, A.F., Franz, A.D. Jr. and Pons, N.A. Jr. 1969. Destruction of aflatoxins in peanuts during dry and oil roasting. J. Agric. Food Chem. 17:451.

Lilly, L.J. 1965. Induction of chromosome aberrations by aflatoxin. Nature (London) 207:433.

Lin, J.-K., Kennan, K.A., Miller, E.C. and Miller, J.A. 1978. Reduced nicotinamide adenine dinucleotide phosphate-dependent formation of 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 from aflatoxin B_1 by hepatic microsomes. Cancer Res. 38:2424.

Lin, J.-K., Miller, J.A. and Miller, E.C. 1977. 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1, a major hydrolysis product of aflatoxin B_1-DNA or ribosomal RNA adducts formed in hepatic microsome-mediated reactions and in rat liver in vivo. Cancer Res. 37:4430.

Linsell, C.A. 1977. Aflatoxins. In: Environment and Man Volume Six. The Chemical Environment. J. Lenihan and W.W. Fletcher (Eds.) Academic Press, New York, N.Y. pp. 121-137.

Llewellyn, G.C., Stephenson, G.A. and Hofman, J.W. 1977. Aflatoxin B_1 induced toxicity and teratogenicity in Japanese medaka eggs (Oryzias latipes). Toxicon 15:582.

Loosmore, R.M. and Harding, J.D. 1961. A toxic factor in Brazilian groundnut causing liver damage in pigs. Vet. Rec. 73:1362.

Loosmore, R.M. and Markson, L.M. 1961. Poisoning of cattle by Brazilian groundnut meal. Vet. Rec. 73:813.

Lötzsch, R. and Leistner, L. 1976. Aflatoxin-Rückstände in Hühnereiern und Eiprodukten. Die Fleischwirtschaft 12:1777.

Lötzsch, R. and Leistner, L. 1977. Transmission of aflatoxins into eggs and egg products. Ann. Nutr. Alim. 31: 499.

Lötzsch, R, Leistner, L. and Ghosh, M.K. 1976. Carry-over Effekt von Aflatoxinen bei Eiern japanischer Zwegwachteln (Coturnix coturnix japonica). Die Fleischwirtschaft 12:1773.

Lu, A.Y. and West, S.B. 1980. Multiplicity of mammalian cytochromes P-450. Pharmacol. Rev. 31:277.

Luter, L., Wyslouzil, W. and Kashyap, S.C. 1982. The destruction of aflatoxins in peanuts by microwave roasting. Can. Inst. Food Technol. J. 15:236.

Lynch, G.P. 1972. Mycotoxins in feedstuffs and their effect on dairy cattle. J. Dairy Sci. 55:1243.

Mabee, M.S. and Chipley, J.R. 1973a. Tissue distribution and metabolism of aflatoxin B_1-^{14}C in broiler chickens. Appl. Microbiol. 25:763.

Mabee, M.S. and Chipley, J.R. 1973b. Tissue distribution and metabolism of aflatoxin B_1-^{14}C in layer chickes. J. Food Sci. 38:566.

Mann, G.E., Codifer, L.P., Jr. and Dollear, F.G. 1967. Effects of heat on aflatoxins in oilseed meals. J. Agric. Food Chem. 15:1090.

Marks, H.L. and Wyatt, R.D. 1979. Genetic resistance to aflatoxin in japanese quail. Science 206:1329.

Marth, E.H. and Doyle, M.P. 1979. Update on molds: degradation of aflatoxins. Food Technol. 33:81.

Masri, M.S., Booth, A.N., Hsieh, D.P.H. 1974a. Comparative metabolic conversion of aflatoxin B_1 to M_1 and Q_1 by monkey, rat, and chicken liver. Life Sci. 15: 203.

Masri, M.S., Garcia, V.S. and Page, J.R. 1969. Aflatoxin M content of milk from cows fed known amounts of aflatoxin. Vet. Rec. 84:146.

Masri, M.S., Haddon, W.F., Lund, R.E. and Hsieh, D.P. 1974b. Aflatoxin Q_1 - A newly identified major metabolite of aflatoxin B_1 in monkey liver. J. Agric. Food Chem. 22:512.

Mc Cann, J., Choi, E., Yamasaki, E. and Ames, B.N. 1975. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. U.S.A. 72:5135.

McGrew, P.B., Barnhart, H.M., Mertens, D.R. and Wyatt, R.D. 1982. Some effects of phenobarbital dosing of dairy cattle on aflatoxin M_1 and fat in milk. J. Dairy Sci. 65:1227.

McKinney, J.D., Cavanagh, G.C., Bell, J.T., Hoverslund, A.S. Nelson, D.M., Pearson, J. and Selkirk, R.J. 1973. Effects of ammoniation on aflatoxins in rations fed to lactating cows. J. Amer. Oil Chem. Soc. 50:79.

Mgbodile, M.U.K., Holscher, M. and Neal, R.A. 1975. A possible protective role for reduced glutathione in aflatoxin B_1 toxicity: Effect of pretreatment of rats with phenobarbital and 3-methyl cholantrene on aflatoxin B_1 toxicity. Toxicol. Appl. Pharmacol. 34:1281.

Millburn, P., Smith, R.L. ans Williams, R.T. 1967. Biliary excretion of foreign compounds, biphenyl, stilbestrol and phenolphtalein in rat: Molecular weight, polarity and metabolism as factors in biliary excretion. Biochem. J. 104:1275.

Miller, D.M., Wilson, D.M., Wyatt, R.D., McKinney, J.K., Crowell, W.A. and Stuart, B.P. 1982. Mycotoxins: High performance liquid chromatographic determination and clearance time of aflatoxin residues in swine tissues. J. Ass. Off. Anal. Chem. 65:1.

Murthy, T.R.K., Jemmali, M. and Rosset, R. 1975. Studies of recovery of aflatoxin B_1 injected in frozen beef at different intervals of storage. Z. Lebensm. Unters. Forsch. 158:5.

Neal, G.E., Judah, D.J., Stirpe, F. and Patterson, D.S.P. 1981. The formation of 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 by the metabolism of aflatoxin B_1 by liver microsomes isolated from certain avian and mammalian species and the possible role of this metabolite in the acute toxicity of aflatoxin B_1. Toxicol. Appl. Pharmacol. 58:431.

Nesbitt, B.F., O'Kelly, J., Sargeant, K. and Sheridan, A. 1962. Toxic metabolites of Aspergillus flavus. Nature (London) 195:1062.

Nesheim, M.C., Austic, R.E. and Card, L.E. 1979. Poultry Production. 12th. ed. Lea & Febiger, Philadelphia, Pennsylvania. pp. 16-57.

Newberne, P.M. and Butler, W.H. 1969. Acute and chronic effects of aflatoxin in the liver of domestic and laboratory animals: A review. Cancer Res. 29:236.

Norred, W.P. 1982. Use of ammonia treatment to destroy aflatoxins in corn. J. Food Protection 45:972.

North, M.O. 1978. Commercial Chicken Production Manual. 2nd ed. Avi Publishing Co. Westport, Connecticut.

Obidoa, O. and Siddiqui, H.T. 1978. Aflatoxin inhibition of avian hepatic mitochondria. Biochem. Pharmacol. 27: 547.

Oettle, A.G. 1964. Cancer in Africa, especially in regions south of the Sahara. J. Nat. Cancer Inst. 33:383.

Olson, L.C., Bourgeois, C.H., Jr., Kreshamras, N., Harikul, S., Sanyakorn, C.K. and Grossman, R.A. 1970. Encephalopathy and fatty degeneration of the viscera in Thai children. Amer. J. Dis. Child. 120:1.

Ong, T.M. 1975. Aflatoxin mutagenesis. Mutation Res. 32: 35.

Park, B.K. 1982. Assessment of the drug metabolizing capacity of the liver. Br. J. Clin. Pharmacol. 14:631.

Patterson, D.S.P. 1977. Metabolism of aflatoxin and other mycotoxins in relation to their toxicity and the accumulation of residues in animal tissues. Pure Appl. Chem. 49:1723.

Patterson, D.S.P. and Allcroft, R. 1970. Metabolism of aflatoxin in susceptible and resistant animal species. Food Cosmet. Toxicol. 8:43.

Patterson, D.S.P., Glancy, E.M. and Roberts, B.A. 1980. The carry-over of aflatoxin M_1 into the milk of cows fed rations containing a low concentration of aflatoxin B_1. Food Cosmet. Toxicol. 18:35.

Patterson, D.S.P. and Roberts, B.A. 1970. The formation of aflatoxins B_{2a} and G_{2a} and their degradation products in vitro detoxification of aflatoxin by levers of certain avian and mammalian species. Food Cosmet. Toxicol. 8:527.

Patterson, D.S.P. and Roberts, B.A. 1971. The in vitro reduction of aflatoxins B_1 and B_2 by soluble avian liver enzymes. Food Cosmet. Toxicol. 9:829.

Patterson, D.S.P. and Roberts, B.A. 1972. Aflatoxin metabolism in duck-liver homogenates. The relative importance of reversible cyclopentenone reduction and hemiacetal formation. Food Cosmet. Toxicol. 10:501.

Payet, M., Cross, J., Quenum,C., Sankale, M. and Moulanier, M. 1966. Deus observations d'enfants ayart consumme de facon prolongee des farines souillees par Aspergillus flavus. Presse Med. 74:649.

Peers, F.G., Gilman, C.A. and Linsell, C.A. 1976. Dietary aflatoxins and human liver cancer, a study in Swaziland. Int. J. Cancer 17:167.

Peers, F.G. and Linsell, C.A. 1973. Dietary aflatoxins and liver cancer- A population based study in Kenya. Br. J. Cancer 27:473

Peers, P.G. and Linsell, C.A. 1975. Aflatoxin contamination and its heat stability in Indian cooking oils. Trop. Sci. 17:229.

Pier A.C. 1981. Mycotoxins and animal health. Adv. Vet. Sci. Comp. Med. 25:185.

Pohland, A.E., Cushmac, M.E. and Andrellos, P.J. 1968. Aflatoxin B_1. J. Ass. Off. Anal. Chem. 51:907.

Polan, C.E., Hayes, J.R. and Campbell, T.C. 1974. Consumption and fate of aflatoxin B_1 by lactating cows. J. Agric. Food Chem. 22:635.

Polzhofer, K. 1977. Aflatoxinbestimmung in Milch und Milchprodukten. Z. Lebensm. Unters. Forsch. 163:175.

Pong, R.S. and Wogan, G.N. 1971. Toxicity and biochemical and fine structural effects of synthetic aflatoxins M_1 and B_1 in rat liver. J. Natl. Cancer Inst. 47:485.

Portman, R.S., Plowman, K.M., and Campbell, T.C. 1968. Aflatoxin metabolism by liver microsomal preparations by two different species. Biochem. Biophys. Res. Commun. 33: 711.

Powrie, W.D. 1976. Characteristics of edible fluids of animal origin: eggs. In: Principles of Food Science Pt. I Food Chemistry. O. R. Fennema (Ed.) Marcel Dekker, Inc. New York, N.Y. pp. 659-675.

Przybylski, W. 1975. Formation of aflatoxin derivatives on thin layer plates. J. Ass. Off. Anal. Chem. 58:163.

Purchase, I.F.H. 1967. Acute toxicity of aflatoxins M1 and M_2 in one-day old ducklings. Food Cosmet. Toxicol. 5: 339.

Purchase, I.F.H. 1972. Aflatoxin residues in food of animal origin. Food Cosmet. Toxicol. 10:531.

Purchase, I.F.H., Steyn, M. and Gifillan, T.C. 1973. Metabolism and acute toxicity of aflatoxin B_1 in rats. Chem. Biol. Interactions 7:283.

Purchase, I.F.H., Steyn, M., Rinsma, R. and Tustin, R.C. 1972. Reduction of the aflatoxin M_1 content of milk by processing. Food Cosmet. Toxicol. 10:383.

Raj, A.J., Santhanam, K., Gupta, R.P. and Venkitasubramanian, T.A. 1975. Oxidative metabolism of aflatoxin B_1: Observations on the formation of epoxide-glutathine conjugate. Chem. Biol. Interactions 11:301.

Reed, J.R., Deacon, L.E., Farr, F. and Couch, J.R. 1968. Inositol and fatty liver syndrome. Proc. Texas Nutr. Conf. 23:204.

Reye, R.D., Morgan, G. and Baral, J. 1963. Encephalopathy and fatty degeneration of the viscera. A disease entity of childhood. Lancet 2:749.

Roberts, J.C., Sheppard, A.H., Knight, J.A. and Ruffey, P. 1968. Studies in mycological chemistry 22. Total synthesis of (+-)-aflatoxin B_2. J. Chem. Soc. (C):22.

Rodricks, J.V. and Stoloff, L. 1977. Aflatoxin residues from contaminated feed in edible tissues of food producing animals. In: Mycotoxins in Human and Animal Health. J.V. Rodricks, C.W. Hesseltine and M.A. Mehlman (Eds.) Pathotox Publishers Inc., Park Forest South, Illinois. pp. 67-79.

Roebuck, B.D., Siegel, W.G. and Wogan, G.N. 1978. In vitro metabolism of aflatoxin B_2 by animal and human liver. Cancer Res. 38:999.

Ryan, N.G., Hogan, G.R., Hayes, A.W., Unger, P.D. and Siraj, M.Y. 1979. Aflatoxin B_1: its role in the etiology of Reye's syndrome. Pediatrics 64:71.

Salhab, A.S., Abramson, F.P., Gelhoed, G.W. and Edwards, G.S. 1977. Aflatoxicol M_1, a new metabolite of aflatoxicol. Xenobiotica 7:401.

Salhab, A.S. and Edwards, G.S. 1977. Comparative in vitro metabolism of aflatoxicol by liver preparations from animals and humans. Cancer Res. 37:1016.

Salhab, A.S. and Hsieh, D.P.H. 1975. Aflatoxin H_1: a major metabolite of aflatoxin B_1 produced by human and Rhesus monkeys livers in vitro. Res. Commun. Pathol. Pharmacol. 10:419.

Sargeant, K., Allcroft, R. and Carnaghan, R.B.A. 1961a. Groundnut toxicity. Vet. Rec. 73:865.

Sargeant, K., O'Kelly, J., Carnaghan, R.B.A. and Allcroft, R. 1961b. The assay of a toxic principle in certain groundnut meals. Vet. Rec. 73:1219.

Sargeant, K., O'Kelly, J., Carnaghan, R.B.A. and Allcroft, R. 1961c. Toxicity associated with certain samples of groundnuts. Nature (London) 192:1096.

Sawhney, D.S., Vadehra, D.V. and Baker, R.C. 1973. The metabolism of ^{14}C-aflatoxins in laying hens. Poultry Sci. 52:1302.

Schabort, J.C. and Steyn, M. 1969. Substrate and phenobarbital inducible aflatoxin 4-hydroxylation and aflatoxin metabolism by rat liver microsomes. Biochem. Pharmacol. 18: 2241.

Schoental, R. 1970. Hepatotoxic activity of retrorsine, senkirkine and hydroxysenkirkine in newborn rats, and the role of epoxides in carcinogenesis by pyrrolizidine alkaloids and aflatoxins. Nature (London) 227:401.

Schroeder, H.W. and Ashworth, L.J. 1966. Aflatoxins: some factors affecting production and location of toxins in Aspergillus flavus-oryzae. J. Stored Prod. Res. I:267.

Schuller, P.L., Verhülsdonk, C.A. and Paulsch, W.E. 1977. Aflatoxin M_1 in liquid and powdered milk. Zesz. probl. Postep. Nauk. roln. 189:255.

Shank, R.C., Bourgeois, C.H., Keschamras, N. and Chandauimol, P. 1971. Aflatoxins in autopsy specimens from Thai children with acute disease of unknown etiology. Food Cosmet. Toxicol. 9:501.

Shank, R.C. and Wogan, G.N. 1965. Distribution and excretion of ^{14}C-labeled aflatoxin B_1 in the rat. Fed. Proc. 24:627.

Shank, R.C., Wogan, G.N., Gibson, J.B. and Nondasuta, A. 1972. Dietary aflatoxins and human liver cancer. II. Aflatoxins in market foods and foodstuffs of Thailand and Hong Kong. Food Cosmet. Toxicol. 10:61.

Sims, W.M., Kelly, D.C. and Sanford, P.E. 1970. A study of aflatoxins in laying hens. Poultry Sci. 49:1082.

Sinnhuber, R.O., Lee, D.J., Wales, J.H., Landers, M.K. and Keyl, A.C. 1970. Aflatoxin M_1- a potent liver carcinogen for rainbow trout. Fed. Proc. 29:568.

Smith, J.W. and Hamilton, P.B. 1970. Aflatoxicosis in the broiler chicken. Poultry Sco. 49:207.

Snedecor G.W. and Cochran, W.G. 1967. Statistical Methods. 6th, ed. Iowa State University Press, Ames, Iowa.

Southern, L.L. and Clawson, A.J. 1980. Ammoniation of corn contaminated with aflatoxin and its effects on growing J. Animal Sci. 50:459.

Stoloff, L. 1977. Aflatoxins-An Overview. In: Mycotoxins in Human and Animal Health. J.V. Rodricks, C.W. Hesseltine and M.A. Mehlman (Eds.) Pathotox Publishers, Inc. Park Forest South, Illinois. pp. 7-27.

Stoloff, L. 1980. Aflatoxin control: Past and present. J. Ass. Off. Anal. Chem. 63:1067.

Stoloff, L. and Trucksess, M.W. 1978. Survey for aflatoxin B_1 in chicken eggs. J. Ass. Off. Anal. Chem. 61:995.

Stoloff, L., Trucksess, M.W., Hardin, N., Francis, O.J., Hayes, J.R., Polan, C.E., Cambell, T.C. 1975. Stability of aflatoxin M_1 in milk. J. Dairy Sci. 58:1789.

Strzelecki, E.L. 1973. Behavior of aflatoxins in some meat products. Acta Microbiol. Pol. Ser. B. 5:171.

Swenson, D.H. 1981. Metabolic activation and detoxication of aflatoxins. In: Reviews in Biochemical Toxicology. E. Hodgson, J.R. Bend and R.M. Philpot (Eds.) Elsevier North Holland, Inc. New York, N.Y. Vol. 3 p. 155.

Swenson, D.H., Lin, J.K., Miller, E.C. and Miller, J.A. 1977. Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA in vivo. Cancer Res. 32:172.

Swenson, D.H., Miller, E.C. and Miller, J.A. 1974. Aflatoxin B_1-2,3-oxide: Evidence of its formation in rat liver in vivo and by human liver microsomes in vitro. Biochem. Biophys. Res. Commun. 60:1036.

Swenson, D.H., Miller, J.A. and Miller, E.C. 1973. 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1: An acid hydrolysis product of an RNA-aflatoxin B_1 adduct formed by hamster and rat liver microsomes in vitro. Biochem. Biophys. Res. Commun. 53:1260.

Swenson, D.H., Miller, J.A. and Miller, E.C. 1975. The reactivity ahd carcinogenicity of aflatoxin B_1-2,3-dichloride, a model for the putative 2,3-oxide metabolite of aflatoxin B_1. Cancer Res. 35:3811.

Swick, R.A. 1984. Hepatic metabolism and bioactivation of mycotoxins and plant toxins. J. Animal Sci. 58:1017.

Trucksess, M.W. and Stoloff, L. 1979. Extraction, cleanup and quantitative determination of aflatoxin B_1 and M_1 in beef liver. J. Ass. Off. Anal. Chem. 62:1080.

Trucksess, M.W. and Stoloff, L. 1984. Determination of aflatoxicol and aflatoxins B_1 and M_1 in eggs. J. Ass. Off. Anal. Chem. 67:317.

Trucksess, M.W., Stoloff, L., Pons, W.A., Cucullu, A.F., Lee, L.S. and Franz, A.O. 1977. Thin layer chromatographic determination of aflatoxin B_1 in eggs. J Ass. Off. Anal. Chem. 60:795.

Trucksess, M.W., Stoloff, L., Young, K., Wyatt, R.D. and Miller, B.L. 1983. Aflatoxicol and aflatoxins B_1 and M_1 in eggs and tissues of hens consuming aflatoxin-contaminated feed. J. Poultry Sci. 62:2176.

Tung, H.T., Donaldson, W.E. and Hamilton, P.B. 1972. Altered lipid transport during aflatoxicosis. Toxicol. Appl. Pharmacol. 22:97.

Tung, T.C. and Ling, H.C. 1968. Study on aflatoxin of foodstuffs in Taiwan. J. Vitaminol. 14:48.

Ulloa-Sosa, M. and Schroeder, H.W. 1969. Note on aflatoxin decomposition in the process of making tortillas from corn. Cereal Chem. 46:397.

Van Egmond, H.P. 1983. Mycotoxins in dairy products. Food Chem. 11:289.

Van Egmond, H.P., Paulsch, W.E., Veriga, H.A. and Schuller, P.L. 1977. The effects of processing on aflatoxin M_1 content of milk and milk products. Arch. Inst. Pasteur (Tunis) 54:381.

Van der Linde, J.A., Van der Frens, A.M. and Van Esch, G.J. 1965. Experiments with cows fed groundnut meal containing aflatoxin. In: Mycotoxins in Foodstuffs. G.N. Wogan (Ed.) MIT Press, Cambridge, Massachusetts p. 247.

Van der Merwe, K.J., Fourie, L. and Scott, de B. 1963. The structures of aflatoxins. Chem Ind. p. 1660.

Van Rensburg, S.J., Van der Watt, J.J., Purchase, I.F.H., Pereira Coutinho, L. and Markahm, R. 1974. Primary cancer rate and aflatoxin intake in a high cancer area. S. Afr. Med. J. 48:2508a.

Van Zytveld, W.A., Kelley, D.C. and Dennis, S.M. 1970. Aflatoxins or their metabolites in livers and skeletal muscles of chicken. Poultry Sci. 49:1351.

Waltking, A.E. 1971. Fate of aflatoxin during roasting and storage of contaminated peanut products. J. Ass. Off. Anal. Chem. 54:533.

Washburn, K.W. and Wyatt, R.D. 1978. Effects and mechanism of action of aflatoxin on shell quality. Poultry Sci. 57:111.

Wei, C.I., Decad, G.M., Wong, Z.A., Byard, J.L. and Hsieh, D. P.H. 1978. Characterization and mutagenicity of water-soluble conjugates of aflatoxin B_1. Toxicol. Appl. Pharmacol. 45:274.

White, R.E. and Coon, M.J. 1980. Oxygen activation by cytochrome P-450. Ann. Rev. Biochem. 49:315.

Williams, C.M., Colwell, W.M. and Rose, L.P. 1980. Genetic resistance of chickens to aflatoxin assessed with organ culture techniques. Avian Diseases 24:415.

Williams, R.T. 1959. Detoxication Mechanisms. John Wiley, New York, N.Y.

Williams, R.T., Millburn, P. and Smith, R.L. 1965. The influence of enterohepatic circulation on toxicity of drugs. Ann. New York Acad. Sci. 123:110.

Wilson, B.J. and Hayes, A.W. 1973. Microbial toxins. In: Toxicants occurring naturally in foods. Comittee on Food Protection, Food and Nutrition Board. Natl. Res. Council/Natl. Acad. Sci. Washington, D.C.

Wiseman, D.W. and Marth, E.H. 1983a. Behavior of aflatoxin M_1 in yogurt, buttermilk and kefir, J. Food Protection 46:115.

Wiseman, D.W. and Marth, E.H. 1983b. Heat and acid stability of aflatoxin M_1 in naturally and artificially contaminated milk. Milchwissenschaft 38:464.

Wogan, G.N. 1964. Experimental toxicity and carcinogenicity of aflatoxins. In: Mycotoxins in Foodstuffs. G.N. Wogan (Ed.) MIT Press, Cambridge, Massachusetts. p. 163.

Wogan, G.N. 1968. Biochemical responses to aflatoxins. Cancer Res. 28:2282.

Wogan, G.N. 1973. Aflatoxin carcinogenesis. In: Methods in Cancer Research H. Busch (Ed.) Academic Press, New York, N.Y. pp. 309-344.

Wogan, G.N. 1977. Mode of action of aflatoxins. In: Mycotoxins in Human and Animal Health. J.V. Rodricks, C.W. Hesseltine and M.A. Mehlman (Eds.) Pathotox Publishers Inc., Park Forest South, Illinois. p. 29.

Wogan, G.N., Edwards, G.S. and Newberne, P.M. 1971. Structure activity relationship in toxicity and carcinogenicity of aflatoxins and analogs. Cancer Res. 31:1936.

Wogan, G.N. and Newberne, P.M. 1967. Dose-response characteristics of aflatoxin B_1 carcinogenesis in the rat. Cancer Res. 27:2370.

Wogan, G.N. and Paglialunga, S. 1974. Carcinogenicity of synthetic aflatoxin M_1 in rats. Food Cosmet. Toxicol. 12:381.

Wogan, G.N., Paglialunga, S., Newberne, P.M. 1974. Carcinogenic effects of low dietary levels of aflatoxin B_1 in rats. Food Cosmet. Toxicol. 12:381.

Wong, J.J. and Hsieh, D.P.H. 1976. Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. Proc. Natl. Acad. Sci. U.S.A. 73:2241.

Wong, Z.A. and Hsieh, D.P.H. 1978. Aflatoxicol: major aflatoxin B_1 metabolite in rat plasma. Science 200:325.

Wong, Z.A. and Hsieh, D.P.H. 1980. The comparative metabolism and toxicokinetics of aflatoxin B_1 in the monkey, rat and mouse. Toxicol. Appl. Pharmacol. 55:115.

Wong, Z.A., Wei, C.J., Rice, D.W. and Hsieh, D.P.H. 1981. Effects of phenobarbital pretreatment on the metabolism and toxicokinetics of aflatoxin B_1 in the Rhesus monkey. Toxicol. Appl. Pharmacol. 60:387.

World Health Organization (United Nations Environmental Programme). 1979. Mycotoxins. Environmental Health Criteria II. W.H.O. Geneva, Switzerland.

Wyatt, R.D., Lockhart, W.C. and Huston, T.M. 1977. Increased resistance of chickens to acute aflatoxicosis by acclimation to low environmental temperatures. Poultry Sci. 56:1648.