A STUDY OF AFLATOXICOSIS IN LAYING HENS TO DETERMINE THE ABSENCE OR PRESENCE OF AFLATOXINS IN EGGS

by 45

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INTRODUCTION

Since the discovery of aflatoxins as the etiology of Turkey X disease at the beginning of this decade (Sargeant et al., 1961b), numerous researchers have oriented their investigations at this fungal metabolite.

Much work has been done in the areas concerning their physical, chemical, and toxic properties and methods of extraction and identification. Still, however, as Hesseltine et al. (1966) reported, "The question regarding the fate of aflatoxin ingested by farm animals in milk, meat and eggs has been inadequately explored. . . . The fate of each of the aflatoxins needs to be studied in animals and their tissues, particularly those destined for human consumption."

Zuckerman and Fulton (1966) reported, "There is strong suggestive evidence of hepatotoxins in the aetiology of human liver disease, such as veno-occlusive disease, cirrhosis, and primary carcinoma of the liver, ..." Since aflatoxins are among the most hepatotoxic and carcinogenic toxins known, their presence in human foods could be of public health significance.

This study was undertaken to determine if laying hens administered aflatoxins in known quantities would pass the aflatoxins to their eggs and thus pose a possible public health hazard.

In this study, two methods of oral administration with three comparable dosage levels were used. Aflatoxins were administered for 16 days. Eggs were assayed on the 8th, 13th, and 17th days for the presence of aflatoxins or their fluorescing metabolites.

REVIEW OF LITERATURE

Discovery of Aflatoxins

The initial fundamental discoveries of Aflatoxins were made in Great Britain (Allcroft et al., 1961; Sargeant et al., 1961b; and Lancaster et al., 1961). These came after the loss of 100,000 turkey poults from liver damage or turkey "X" disease (Blount, 1961), followed within a short time by similar incidents in ducklings and chickens (Asplin and Carnaghan, 1961), swine (Loosmore and Harding, 1961), and calves (Loosmore and Markson, 1961). The common factor in these incidents was a shipment of Brazilian peanut meal used in the animal feeds.

It was found that the toxic principle(s) could be extracted with chloroform from toxic meals and the extracts reproduce the toxicity in susceptible species (Allcroft et al., 1961; Lancaster et al., 1961; Sargeant et al., 1961a; Sargeant et al., 1961b). The toxic principles were recognized as metabolites derived from the fungus Aspergillus flavus Link ex Fries and not the groundnuts (Sargeant et al., 1961b). An Interdepartmental Working Party on Groundnut Toxicity Research in 1962 named these metabolites as aflatoxins, deriving the name from Aspergillus FLAvus TOXINS.

De Iongh et al. (1962) concluded that toxic extracts contained at least 2 toxic substances. Further work on the purification and characterization of the toxin was done by Nesbitt et al. (1962). For convenience, they referred to the 2 toxic fractions as aflatoxin 'B' and aflatoxin 'G' because of their

blue-violet and green fluorescence. In 1963, Hartley et al. reported a total of 4 closely related toxins. The 2 fractions previously reported were called B_1 and G_1. Their dihydro-derivatives B_2 and G_2 had not been reported previously. Van der Zijden et al. (1962) isolated in crystalline form, aflatoxin B_1, and gave an account of some of its physico-chemical properties.

The elucidation of the chemical structures of the two major toxic metabolites, aflatoxin B_1 and G_1, was reported by Asao et al. in 1963 and in greater detail in 1965. Chang et al. (1963) described the chemical structure of aflatoxin B_2 as a dihydro-aflatoxin B . Hartley et al. (1963) converted aflatoxin B_1 to B_2 and G_1 to G_2 by hydrogenation. The chemical structures assigned to the aflatoxins received strong support from X-ray crystallographic studies by Cheung and Sim (1964).

Allcroft and Carnaghan (1963a) reported that milk from cows fed toxic groundnuts was toxic to day-old ducklings. De Iongh et al. (1964b) extracted the toxic milk and demonstrated the presence of a slow running blue-violet fluorescent compound. A similar result was obtained from rat milk and was called "milk toxin" and later "aflatoxin M." Butler and Clifford (1965) extracted from rat liver a comparable blue-violet spot R_f 0.2 after giving a single LD_{50} dose of 7 mg./kg. aflatoxin B_1. Aflatoxin M was concluded to be a metabolite of aflatoxin B. Holzapfel et al. (1966) isolated and resolved 2 components, M_1 and M_2 (dihydroderivative of M_1).

Aflatoxin M_1 is almost as toxic as aflatoxin B_1, while aflatoxin G_1 is only about one half as toxic. The dihydro aflatoxins B_2, G_2, and M_2 are about one fourth as active as the respective aflatoxins B_1, G_1, and M_1 (Carnaghan et al., 1963; Chang et al., 1963; Holzapfel et al., 1966).

General Characteristics of Aflatoxins

The aflatoxins are extractable into organic solvents, exhibit intense fluorescence by which traces can be easily detected in ultraviolet light, and have high melting points (Hartley et al., 1963; Asao et al., 1965). The four originally isolated aflatoxins are closely related difurano coumarin compounds. When separated by thin-layer chromatography on silica gel plates developed in chloroform/methanol (97/3, v/v), they are observed to fluoresce under ultraviolet light in descending order as blue (B_1 and B_2) and green (G_1 and G_2) spots. The R_f values are in the order of B_1, 0.56; B_2, 0.53; G_1, 0.48; and G_2, 0.46. Absolute R_f values are poorly reproducible (Asao et al., 1965). Some of the physical properties of aflatoxins are summarized in Table 1.

Of the 4 aflatoxins, B_1 is the most toxic. Relative toxicity may be illustrated by the following single dose LD_{50} in day-old ducklings: B_1, 18.2 ug.; B_2, 84.8 ug.; G_1, 39.2 ug.; and G_2, 172.5 ug. (Carnaghan et al., 1963). Aflatoxin B_1 is usually present in the largest quantity. The amounts and relative proportions of the 4 are variable, depending upon mold strain,

TABLE 1—Physical Properties of Aflatoxins

Aflatoxin	Molecular formula	Molecular weight	Melting point C	Maximum fluorescence emission - mu
B_1	$C_{17}H_{12}O_6$	312	268-269*	425
B_2	$C_{17}H_{14}O_6$	314	286-289*	425
G_1	$C_{17}H_{12}O_7$	328	244-246*	450
G_2	$C_{17}H_{14}O_7$	330	237-240*	450

* Decomposes.

medium composition, and culture conditions, especially temperature (Schindler et al., 1967; Davis et al., 1966). Typically, aflatoxins B_2 and G_2 are present in small relative amounts (Hartley et al., 1963).

Although few systematic studies have been made on the stability of aflatoxins, they are generally considered relatively stable. Some degradation does occur upon standing in methanolic solution and this is accelerated by light and heat. Substantial degradation occurs on chromatograms exposed to air and ultraviolet or visible light (Wogan, 1966). Chlorine gas and 5% NaOCl will destroy aflatoxins (Trager and Stoloff, 1967; Fischbach and Campbell, 1965). Giegler et al. (1966) reported on the microbial detoxification of aflatoxins by Flavobacterium aurantiacum.

Aflatoxins can survive autoclaving and are resistant to a number of chemicals (Trager and Stoloff, 1967). Mann et al. (1967) did find heating at 100 C for 120 minutes with a moisture

content of 20% would reduce the amount of aflatoxins present in cottonseed meal (80% reduction) and peanut meal (34% reduction).

Aflatoxins are produced by some strains of Aspergillus flavus. Hodges et al. (1964) isolated aflatoxins from Penicillium perberulum cultures. Aspergillus parasiticus of the Aspergillus flavus-oryzae group produces aflatoxins also (Codner et al., 1963). Murakami et al. (1967), in an attempt to further classify Aspergillus flavus ATCC 15517, a well known aflatoxin producer, discovered certain properties that lead them to believe this strain belongs to the species A. parasiticus as a new variety.

General Characteristics of Aspergillus Flavus

The presence of Aspergillus flavus does not necessarily imply the presence of aflatoxins. There is no parallelism between abundant growth of the fungus and high concentrations of these metabolites (Schindler et al., 1967). Optimum temperature for the growth of A. flavus is approximately 30 C, but growth will occur in temperatures from 12 to 47 C. It requires a relative humidity of 70 to 85% and a food moisture content of approximately 18% (Christensen, 1965).

The optimum temperature for maximum production of aflatoxin is approximately 24 C. At 41 C and above and 7 C and below, no aflatoxins are produced (Schindler et al., 1967).

Aspergillus flavus is generally considered a storage fungus. If crops, especially peanuts or seeds of cereal plants, become damaged or are maintained at a temperature and moisture content

that permits storage fungi growth, they may become rapidly invaded by the storage fungi (Christensen, 1965).

Laboratory studies have shown A. flavus can grow on and produce aflatoxins on numerous agricultural commodities. In addition to peanuts and peanut products (Codner et al., 1963), aflatoxins have been produced on cottonseed cake (Loosmore et al., 1964), crushed wheat (Chang et al., 1963), rice (Shotwell et al., and corn steep (Schroeder, 1966).

Morphologically, the A. flavus group differs from other species of the genus in that the conidial heads are globose to columnar, and very light yellow-green, deep yellow-green, olive-brown, or brown. The conidiophores are colorless and usually roughened. The vesicles are fertile over most of their surface. On Czapek's agar, the fungus has a velvety to coarse surface (Raper and Fennel, 1965).

In addition to the aflatoxins, A. flavus produces oxalic acid, kojic acid, a tremorgenic substance (non-fluorescent), aspergillic acid and related compounds, beta nitropropionic acid, and A. flavus endotoxin (Wilson, 1966).

Susceptibility of Various Species to Aflatoxins

Aflatoxin-containing meals have been shown to be hepatotoxic to many species of mammals, birds, and fish (see below). Susceptibility to the toxic effects of aflatoxin vary greatly with age, sex, nutritional status, and especially with the species. Breed differences in chickens have been reported (Brown and

Abrams, 1965). The very young are more susceptible than adults, and mature males are more susceptible than mature females, except at certain stages of pregnancy when the latter appear to become more susceptible (Butler and Wigglesworth, 1966).

Low protein (4%) diet has been found to increase the susceptibility of rats to aflatoxin (Madhavan and Gopalan, 1965). Rats given a diet devoid of protein are even more susceptible.

Depending upon their susceptibility to aflatoxins, the animal species tested can be roughly divided into:

1. Very susceptible - for which the LD_{50} of aflatoxin B_1 is in the order of 1 mg./kg. bodyweight or less: duckling (Khaki-Campbell or Pekin) (Armbrecht and Fitzhugh, 1964; Armbrecht et al., 1965; Butler, 1964a; Carnaghan et al., 1963; Nesbitt et al., 1962); rainbow trout (Halver et al., 1966); guinea pig (Butler and Barnes, 1963; Paterson et al., 1962); rabbit (Wogan, 1966); new-borne rat (Wogan, 1966); turkey poult (Asplin and Carnaghan, 1961); dog (Wogan, 1966).

2. Susceptible - but which require up to 10 times higher doses: pig (Harding et al., 1963; Hintz et al., 1967); rat (Butler, 1964b; Wogan, 1966); monkey (Madhavan et al., 1965; Tulpule et al., 1964); calf (Allcroft and Carnaghan, 1963a; Allcroft and Lewis, 1963); pheasant (Abrams, 1965); chick (Asplin and Carnaghan, 1961); ferret (Abrams, 1965); hamster (Wogan, 1966); cow (Allcroft and Lewis, 1963); quail (Coturnix) (Armbrecht et al., 1965); Coho salmon (Halver et al., 1966); and certain breeds of chicken (Brown and Abrams, 1965; Gardiner and Oldroyd, 1965).

3. Resistant - which can tolerate relatively large doses of aflatoxin without ill effects: mice (Platonow, 1964); sheep (Allcroft and Carnaghan, 1963b).

Macroscopic Lesions

Liver damage is the primary pathological lesion observed in all species. The liver may have a loss of color to a yellow or putty color and mottling. This may be accompanied with some degree of ascites and edema. Pigs are the only specie that show generalized jaundice (Loosmore and Harding, 1961). Carnaghan et al. (1966), in their research with chickens, found in the first 3 weeks the livers were enlarged and putty-colored with a reticulated network. The consistency was soft, and petechial hemorrhages frequently were present. After 3 weeks, there was an apparent reduction in liver size with an increasing firmness of texture until the seventh week when well defined, raised, nodular areas were seen on the surface. Diffused, white, pinhead-sized foci were visible from the sixth week. Throughout their experiment period, the livers remained a pale yellow ochre color and petechial hemorrhages were observed in the livers of the majority.

Microscopic Lesions

Hepatic cell necrosis, initially with subsequent bile duct proliferation, is typically present in affected animals. In calves and pigs, severe fibrosis is usual, but this is not so well defined in the avian liver (Allcroft, 1965). Clegg and

Bryson (1962) and Hill (1963) reported a veno-occlusive phenomenon in cattle which has not been described for other species.

In young chickens, retrogressive and regenerative parenchymal changes in the liver may occur and persist until the birds are between 3 and 4 months of age. Lymphoid hyperplasia occurs particularly in those areas where regenerative cells are active. By 4 months, no evidence of parenchymal cell regeneration is seen, but multiple circumscribed areas of lymphoid hyperplasia are found. The renal changes affecting the glomeruli are not observed in chickens as they are in turkeys (Asplin and Carnaghan, 1961).

Carnaghan et al. (1966) found in livers of chickens after 3 1/2 days feeding on toxic meal, there was marked vacuolation of the cytoplasm of the parenchymal cells due to intracellular fat globules and slight enlargement of the nuclei. The degree of fatty change was more pronounced in those cells proximal to the portal tract. Slight proliferation of the bile ductule epithelial cells was present in the periportal areas. After one week, the fatty change was more marked, the cells being enlarged with swollen nuclei and prominent nucleolus; proliferation of ductular cells increased, taking the form of chains radiating from the portal tract. At 2 weeks, there was marked ballooning of the parenchymal cells with fat. A few scattered hemorrhages were present throughout the liver. After 3 weeks, fatty change of the parenchymal cells was even more pronounced. Scattered hemorrhages of varying sizes were seen. Regeneration of the parenchymal cells began at 4 weeks, and during the 6th week, the lymphoid hyperplasia was observed.

Carcinogenic Properties of Aflatoxins

Lancaster et al. (1961) first demonstrated that toxic groundnut meal contained a carcinogenic factor for rats. They discovered, after feeding diets containing 20% peanut meal (aflatoxin content unknown) for 6 months, 9 of 11 rats developed multiple liver tumors, and 2 of these developed lung metastases. Subsequent work has amply confirmed the carcinogenicity of aflatoxins for rats (Barnes and Butler, 1964; Butler and Barnes, 1963; Dickens and Jones, 1963; Salmon and Newberne, 1963).

Newberne (1965) reported good correlation between liver tumor incidence in rats and dietary aflatoxin content over the range of 0.06 to 1.8 ppm of aflatoxin. The highest level resulted in more than 90% tumor incidence when fed over a period of 370 days. The lowest level of aflatoxins detected (0.005 ppm) failed to induce liver tumors when fed 385 days.

Barnes and Butler (1964) fed rats 1.75 ppm aflatoxin in the diet for 89 days and then returned the rats to an aflatoxin-free diet. All 3 treated animals developed liver cancer after more than 300 days following withdrawal. Wogan (1966) administered partially purified aflatoxins to rats by stomach tube daily for 30 days and then withheld further treatment. Animals receiving the highest dose (150 ug./day) developed liver tumors in 5 months. Animals receiving the lowest dose (15 ug./day) showed significant incidence of precancerous lesions at 5 months which probably would have progressed to tumors over a longer period of time.

These data indicate that continuous exposure to the aflatoxins is not required for liver tumor induction.

Other studies (Ashley et al., 1964; Ashley et al., 1965; Sinnhuber et al., 1965) have shown that rainbow trout develop liver tumors at significant incidence rates when fed diets containing only 0.5 to 2.0 ug. of aflatoxin B_1 per kilogram (i.e., 0.5 to 2.0 ppb).

The carcinogenicity of aflatoxins in peanut meal for mice and ducks has been reported (Newberne, 1965). Svoboda et al. (1966) reported chronic doses of aflatoxin B_1 in monkeys caused hepatocellular carcinoma. Butler (1965), in comparing the dosages of aflatoxins to that of several well-known carcinogens, reported aflatoxins are the most active hepatocarcinogens known.

Other Biological and Biochemical Effects

Fetal liver extracts from rats given aflatoxins orally at various stages of pregnancy have shown that aflatoxins crossed the placenta (Butler and Wigglesworth, 1966). Ellis and DiPaolo (1967) have shown in hamsters that aflatoxin B_1 produced severe toxic effects in the liver of both mother and offspring. Malformed fetuses, ranging in severity from umbilical hernias to anencephaly, occurred.

Clifford and Rees (1967) detected aflatoxin B_1 in the nuclei of liver cells from affected rats and found in vitro all of the aflatoxins became weakly bound to DNA which inhibited the production of nuclear RNA. They suggested that the interaction of the

toxin with DNA gave rise to the aflatoxins inhibitory action on mitosis and its necrogenic action.

Brown (1965) found, in the livers of affected chickens, a marked decrease in the activity of certain mitochondrial dehydrogenases and enzymes of the electron transfer chains. Affected birds had mild to moderate anemia, severe hypoproteinaemia, and grossly abnormal plasma protein electro-phoretograms. Brown believed suppression of protein synthesis was due to a lowered rate of ATP synthesis consequent on mitochondrial injury.

Zuckerman and Fulton (1966), in their studies of the toxic effects of aflatoxin on human embryonic liver cells in culture, noted microscopical changes after 3 to 5 hours exposure to 10 ppm aflatoxin B_1. These consisted of a change in the normal granular appearance of RNA in the cytoplasm and loss of definition of the nucleoli. After 16 hours, there was complete loss of RNA and reduction in size of the hepatic cells. The nucleoli could no longer be identified and there was loss of chromatin from the nucleus. With 5 ppm, no obvious changes occurred until 20 to 24 hours after exposure.

Metabolism of Aflatoxins by Animals

Allcroft and Carnaghan (1963a) observed that cows fed aflatoxin - containing peanut meals - excreted in their milk a compound toxic to ducklings. Subsequent findings by De Iongh et al. (1964b) demonstrated lactating rats also excreted in their milk a toxic compound. Little, if any, aflatoxin B_1 was detected.

The toxic compound had greatly altered chromatographic properties and was determined to be a metabolite of aflatoxin B_1.

Shank and Wogan (1965) studied the distribution and excretion of C^{14} during the 24-hour period following intraperitoneal administration of labeled aflatoxins to rats. When the methoxy-labeled aflatoxin was used, 25 to 30% of C^{14} was recovered in CO_2, 25% in urine, 25% in feces and intestinal content, and the liver contained 6 to 9%. When ring-labeled aflatoxin was administered, about 20% C^{14} was recovered in the urine, 60% in the feces and intestinal contents, and the liver contained about 9%. No C^{14} was found in the CO_2.

Nabney et al. (1967), in their studies of the metabolism of aflatoxin in lactating ewes, recovered only 8.1% of the total dose given. Ninety percent of the recovered aflatoxin occurred in the first 48 hours. None was detected in milk after 6 days and none in the urine and feces after 8 and 9 days, respectively. Of the 8.1% recovered, 0.1% was in the milk, 6.4% in the urine, and 1.6% in the feces.

Investigations of Animal Tissues and Food of Animal Origin for Aflatoxins and Its Metabolites

Allcroft and Carnaghan (1963a), De Iongh et al. (1964b), and van der Linde et al. (1965) have found the milk from cows receiving aflatoxon-contaminated feeds was toxic to ducklings and produced biological effects similar to those produced by aflatoxin. Similar findings of the "milk toxin" were reported by Purchase

(1966) in milk of goats and cows receiving 10 and 12 mg. aflatoxin per day, respectively.

Brown and Abrams (1965) reported neither aflatoxins nor their metabolites were present in the milk of cows fed on highly toxic rations, and felt the reason for their difference in findings from the above workers was due to breed differences. They also reported that the eggs from hens fed a ration containing 0.5 ppm aflatoxin for 6 weeks were not toxic to a group of test ducklings. Aflatoxins were not found to be present in the eggs of hens maintained on rations containing 0.75 ppm, nor did they have any effect on fertility and hatchability of eggs (Abrams, 1965). These results, demonstrating the absence of aflatoxins in the eggs from hens receiving aflatoxins, are in agreement with those reported previously by Allcroft and Carnaghan (1963a). In addition to not being able to detect toxic effects of eggs from hens fed aflatoxins, Allcroft and Carnaghan (1963a) were not able to detect any toxicity in the liver from a cow or a pig, or clotted blood and serum from a cow from animals fed rations containing toxic groundnut meal. Platonow (1965) was unable to extract aflatoxin or its fluorescing metabolites in breast meat, leg meat, or liver from chickens fed a ration containing 3.1 ppm aflatoxin.

Butler and Clifford (1965) extracted livers, portal blood, and systemic blood from rats which had received a single LD_{50} dose of aflatoxin B_1 either orally or intraperitoneally. They found "milk toxin" in extracts one-half hour after dosing up to 24 hours after dosing.

Laboratory Production of Aflatoxins

Since aflatoxins are not available commercially and would be expensive if they were, most investigators are obliged to produce them.

Using a medium of yeast extract and sucrose in distilled water, Davis et al. (1966) reported yields of B_1 and G_1 up to 63 mg./100 ml. medium in a stationary culture. Hayes et al. (1966) reported lower yields with the same strain of mold in the same medium when the culture was aerated. Mateles and Ayde (1965) had similar results in that yields were greater in shaken cultures, but less in aerated fermentors.

Hesseltine et al. (1966) reviewed a number of methods of aflatoxin production in which various cereal grains were used as the substrate. Production of aflatoxins is usually maximal in 4 to 7 days at 24 to 28 C, and does not parallel maximum mycelial growth (Davis et al., 1965; Hesseltine et al., 1966; Schindler et al., 1967; Stubblefield et al., 1967). After this time, the aflatoxins decrease while mycelial growth increases.

Extraction and Assay Procedures

Most methods extract for aflatoxins with chloroform, acetone, or methyl alcohol or some combination using these solvents. Extraction is followed by a cleanup process. Separation for purification and identification is usually done by thin-layer chromatography.

Trager et al. (1964) compared a number of analytical procedures for aflatoxin assays for their efficacy in detection and quantitative estimation of aflatoxins. All but one employed aluminum-oxide as the TLC absorbent. The one method employing silica gel TLC was shown to be superior to the aluminum-oxide techniques. When silica gel was used in each procedure instead of aluminum-oxide, all of the procedures were found to be approximately equal.

A simple, rapid, and reliable method was developed by Eppley (1966). Fifty grams or 1-kg. samples can be assayed. The techniques have now been adopted as the current A.O.A.C. official first action method.[1]

In all of the above methods, quantitative measurements are based on visual comparison of intensity of fluorescence of the unknown sample spot to that of a known aflatoxin standard on TLC plates when viewed under ultraviolet light.

Bioassays for the presence of toxicity include the day-old duckling test, chick embryo test (Verrett et al., 1964), tissue cultures using human embryonic liver cells (Zuckerman and Fulton, 1966), and human embryonic lung cells (Legator, 1966).

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[1] "Changes in Methods," J.A.O.A.C., 49, (1, 1966): 229-231.

MATERIALS AND METHODS

Aflatoxin Production

Aflatoxins were produced by slight modification of the method described by Davis et al. (1966). Inoculations of Aspergillus flavus ATCC 15517[1] were made on 37 slants of Czapek's solution agar[2] modified with 7 grams of yeast extract and 30 percent sucrose.

These cultures were incubated at 25 C for 7 days. To each slant, 10 ml. of sterile YES medium (2% yeast extract and 20% sucrose in distilled water) was added. The spores were gently agitated by a sterile loop to form a suspension. The spore suspensions were transferred to 33 Roux flasks containing 100 ml. of sterile YES medium by a 20-cc. syringe with a 6-inch 16-gauge needle. Each Roux flask was inoculated with the spore suspension from one slant only.

The Roux flasks were gently agitated to mix the spore suspension and 100 ml. YES medium and placed in an incubator at 25 C for 7 days. At the end of 7 days, the liquid YES medium was drained from the Roux flasks. The flasks were placed slanting down on an incline for one hour to facilitate drainage. The toxic YES medium was filtered 4 times through filter paper[3] in an

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[1] American Type Culture Collection, Rockville, Maryland.

[2] Difco Laboratories, Detroit, Michigan.

[3] Whatman No. 1, 18.5 cm. supplied by Fisher Scientific Co., Pittsburgh, Pennsylvania.

18-cm. Buckner funnel under negative pressure. The filtered toxic YES medium was mechanically[1] stirred for one hour with intermittent manual shaking to insure uniformity, and then poured into sterile 500-ml. bottles for storage at 0 C. The bottles were tightly wrapped in aluminum foil. Asepsis was maintained throughout the entire procedure. All Aspergillus flavus cultures were handled in an enclosed hood.[2]

A sufficient quantity of the liquid YES medium was prepared at one time to suspend the spores, place in the Roux flasks, and have 675 ml. remaining for control purposes. The control YES medium was autoclaved, incubated, and otherwise handled as the toxic YES medium.

Aflatoxin Assays of the YES Medium

Extraction. One milliliter of the toxic YES medium was shaken for one hour with 100 ml. of chloroform in a separatory funnel on a mechanical wrist action shaker.[3] Twenty-five milliliters of the chloroform phase were collected in a 50-ml. beaker and reduced to near dryness under a gentle stream of nitrogen in a steam bath. The residue was quantitatively transferred by 3 chloroform washings to a 4-dram screw cap vial and reduced to dryness under a gentle nitrogen stream. The vial was closed and

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[1] Thermix, Stirring - Hot Plate, Fisher Scientific Co., St. Louis, Missouri.

[2] Isolator-Lab., Fisher Scientific Co., Pittsburgh, Pa.

[3] Modified Eimer and Amend, Fisher Scientific Co., Pittsburgh, Pennsylvania.

stored at 0 C until quantitative assay could be performed on TLC plates. The extraction procedure was triplicated.

Preparation of TLC Plates. Three, 20 by 20 cm., glass plates were placed in a line on a flat surface and secured by Scotch filament tape along the outside 2 edges to the flat surface. The glass surface was wiped with chloroform. Twenty grams of MN Silica Gel G-HR[1] in 50 ml. distilled water were vigorously shaken for 30 seconds in a 500-ml. ground glass stoppered flask, immediately poured on the plates, and spread evenly by a 15-inch by 1-cm. glass rod. The taped outside edges of the plates provided approximately a 250-u space between the glass rod and the glass plate surface, preventing their contact. This resulted in an approximate 250-u thickness of silica gel surface over the plates between the taped outside edges. The silica gel was allowed to dry for 15 to 20 minutes and the tape then removed. The plates were allowed to dry for another 10 to 15 minutes before moving to a storage rack.[2] Prior to use, the plates were activated by baking in an oven at 250 F for one hour.

Thin Layer Chromatography. One milliliter of chloroform was pipetted into the vials containing the extract material. With a 10-microliter syringe[3] and a spotting template,[4] 1.0, 3.0, 5.0,

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[1] Brinkman Instruments, Westbury, New York.

[2] Warner-Chilcott Model 200-3, Scientific Products, North Kansas City, Missouri.

[3] Model 701-N, Hamilton Company, Inc., Whittier, California.

[4] Spotting Template, #5-730-12, Fisher Scientific Co., St. Louis, Missouri.

7.0, and 10.0 ul. of the chloroform solution were spotted on the silica gel layered glass plates 4 cm. from the bottom. On the same plate, 1.0, 3.0, 5.0, 7.0, and 10.0 ul. of quantitative and 5.0 ul. qualitative standards were spotted. When the extract material was found to contain aflatoxin in much greater concentration than the standards, the extract material was diluted 10 fold (9/1, v/v) with chloroform and replated.

Plates were developed in an unlined unequilibrated glass tank[1] with a glass trough containing 50 ml. chloroform/acetone (9/1, v/v) as the developing solvent. A glass top taped to the tank sealed the tank. The plate was developed at room temperature for 40 to 50 minutes, allowing the solvent front to rise to 4 cm. from the top edge of the plate and approximately 13 cm. from the application points. The plates were removed from the tank and the solvent allowed to evaporate. The dry plates were illuminated from below by placing the plate silica gel side up on a long-wave ultraviolet light[2] in a darkened room. Protective contrast goggles[3] were worn while viewing the plates.

Quantitation of aflatoxin B_1 was made by visually comparing the intensity of fluorescence of the unknown to that of the standards. Quantitation for aflatoxin G_1 was done in a like manner. All assays were triplicated.

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[1] Model K4097, Kensington Scientific Company, Berkeley, Calif.

[2] Blak-Ray Model XX15, Ultraviolet Products Inc., San Gabriel, California

[3] Blak-Ray Model UVC 303 Contrast Control Goggle, ibid.

The quantitative aflatoxin standard, SA 3-6,[1] was prepared from pure crystalline aflatoxins B_1 and G_1 dissolved in chloroform in a concentration of 3.0 ug./ml. of B_1 and 2.0 ug./ml. of G_1. By diluting 2 ml. of the stock standard solution to 10 ml. volume with chloroform, the diluted standard contained 0.0006 ug./ul. aflatoxin B_1 and 0.0004 ug./ul. aflatoxin G_1.

The qualitative aflatoxin standard, SA 5-5,[2] was prepared from pure crystalline aflatoxins B_1, B_2, G_1, and G_2. It was used primarily for facilitation of their identification on TLC analysis. The stock standard solution, dissolved in chloroform, contained 5.0 ug. of B_1, 1.5 ug. of B_2, 5.0 ug. of G_1, and 1.5 ug. of G_2 per ml. By dilution of 2 ml. of the stock standard solution to 10 ml. volume with chloroform, the diluted standard contained 0.001 ug./ul. of B_1; 0.0003 ug./ul. of B_2; 0.001 ug./ul. of G_1, and 0.0003 ug./ul. of G_2.

The diluted and undiluted qualitative and quantitative standards were contained in sealed 4-dram vials inside tightly sealed covered jars containing chloroform to minimize evaporation. The jars were tightly wrapped with aluminum foil and stored at 0 C. Standards were allowed to equilibrate with room temperature in the unopened jars prior to use to prevent moisture condensation with resulting loss of accuracy.

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[1] Supplied by Dr. Leo A. Goldblatt, Southern Utilization Research and Development Division, A.R.S., USDA, New Orleans, La.

[2] Supplied by Dr. Leo A. Goldblatt, ibid.

Aflatoxin-Feed Mixture Preparation

From a partially purified sample of aflatoxin,[1] containing 49.0% aflatoxin B_1, 7.5% B_2, 7.5% G_1, and 0.6% G_2, 0.408 gram was accurately weighed and dissolved in 1,000 ml. acetone. One milliliter of the acetone-aflatoxin solution was taken for analysis. The acetone-aflatoxin solution was mixed with 2,500 grams of the laying feed[2] used during this study. An additional 1,000 ml. acetone was added to facilitate thorough mixing. The acetone was allowed to evaporate from the feed. Evaporation was hastened by the use of a fan and occasional stirring. The 2,500 grams of toxic feed was thoroughly mixed with 22,500 grams of the same feed, resulting in 25,000 grams of feed containing aflatoxin in the order of 8 mg. aflatoxin per kilogram of feed or 8 ppm aflatoxin. From the 25,000 grams, 9,000 grams were taken and mixed with 9,000 grams of non-toxic feed to make 18,000 grams of 4 ppm aflatoxin mixture. Six thousand grams of this feed was mixed with 6,000 grams of non-toxic feed to make 12,000 grams of feed mixture containing 2 ppm aflatoxin. The 3 mixture levels were stored in new 10-gallon capacity galvanized cans with tight-fitting covers.

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[1] Supplied by Mr. Herbert G. Wiseman, Agricultural Research Center, FDA, Beltsville, Maryland.

[2] Laying feed, code P-36, Department of Grain Science and Industry, Kansas State University, Manhattan, Kansas.

Aflatoxin Assays of the Aflatoxin-Feed Mixtures

The 1 ml. of acetone-aflatoxin solution taken from the 1,000 ml. prepared for the aflatoxin-feed mixture was used for assay. This was extracted, concentrated, plated, and quantitated in the same manner as the toxic YES medium. With verification of the aflatoxin B_1 quantity, the other less toxic aflatoxin portions were not verified quantitatively although they were qualitatively.

Experimental Animals

For this study, 55 laying DeKalb hens[1] were selected, based on production records from the 2 months previous to the experiment. All non-layers were culled prior to selection. The hens came into production 10 months prior to the start of this study.

The hens were placed in individual wire laying cages arranged in 3 parallel rows. In the first row, 3 groups (6 hens per group) received the 3 levels of feed and aflatoxin mixtures ad libitum. The feed mixtures of 2, 4, and 8 ppm aflatoxin were placed in a trough running parallel to the front of the cages. Partitions prevented mixing of the feed of the 3 mixture levels.

Hens in the middle row received daily the toxic YES medium by pipette into their crops. Dosage was comparable to that of the hens receiving aflatoxins mixed in their feed. Basis for

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[1] Purchased from the Department of Dairy and Poultry Science, Kansas State University, Manhattan, Kansas.

dosage was calculated by using the average hen weight (2,000 grams) and average daily feed consumption (approximately 120 grams). Dosage was adjusted according to each hen's variation in weight from the average weight. With the toxic YES medium, containing 120 ug./ml. aflatoxin B_1, 1 ml. was figured to be comparable to 1 ppm aflatoxin B_1 in the feed for a hen weighing 2,000 grams. After 10 days of daily administrations of toxic medium, the hens were weighed again and dosages refigured according to the later weight.

The control hens were caged in the third row. Ten hens served as environmental controls. Three groups of hens, 3 hens per group, received the control YES medium. The dosage for these groups was calculated in the same manner as for those receiving the toxic YES medium.

Individual laying production records and egg weight were tabulated daily.

Toxic medium and control medium were administered daily for 16 days. Toxic feed was fed ad libitum for 16 days. No additional aflatoxins were given after this time.

The hens were weighed periodically during the experiment and at the time of necropsy to determine any weight losses or gains. Hens receiving the toxic feed and medium were necropsied 1, 4, 8, 15, and 29 days after administration of aflatoxin was discontinued. Hens receiving control medium were necropsied 1, 8, and 27 days after administration of aflatoxin was discontinued. Gross lesions were recorded and tissues taken for histopathologic

examination and confirmation of aflatoxicosis. Sections of the liver, kidney, and spleen were fixed in 10% buffered neutral formalin, processed in paraffin, cut at 6 u, and stained by hematoxylin and eosin and periodic acid-Schiff. Frozen sections were cut at 10 u and stained with oil-red O for neutral fats. Livers from the hens necropsied on the first day after discontinuing aflatoxin administration were saved for analysis for aflatoxins.

Assay of Eggs for Aflatoxins

Each egg was numbered and dated, corresponding to the hen's number and the date laid. The eggs were separated according to date laid and group. These eggs were slowly blended[1,2] and 50-gram samples analyzed for aflatoxin content.

Extraction Method.[3] The 50 grams of eggs were blended for 2 minutes in 400 ml. of methyl alcohol. Approximately 20 grams of Celite 545[4] was added during the last minute of blending. The blended material was filtered through a shallow layer of Celite over a circle of filter paper[5] in a 9-cm. Buchner funnel aided

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[1] Waring Blender, Fisher Scientific Co., St. Louis, Missouri.

[2] Powerstat-Variable Transformer, #9-521V2, ibid.

[3] Method described and used by Mr. Herbert G. Wiseman, chemist, Agricultural Research Center, FDA, Beltsville, Maryland (Personal communication).

[4] Filter Aid, Fisher Scientific Co., St. Louis, Missouri.

[5] S & S, No. 589, Black Ribbon, supplied by Fisher Scientific Co., ibid.

by vacuum. The Celite layer was covered with methyl alcohol after the blended egg material had passed, to wash any remaining methyl alcohol soluble material through. The filtrate was adjusted to 50% methyl alcohol by the addition of 4% NaCL solution and transferred to a 1,000-ml. separatory funnel. Four 100-ml. extractions with hexane removed fats and pigments. Four subsequent extractions with 100-ml. volumes of chloroform followed to remove any aflatoxins present. The chloroform solution was washed with a 250-ml. volume of 4% NaCl to remove any methyl alcohol from the chloroform solution.

To a 500-ml. Erlenmeyer flask, containing 10 grams of cupric carbonate (Wiseman et al., 1967) and 4 ml. ethyl alcohol, the chloroform solution was added and stirred for 1 minute. The copper carbonate was allowed to settle for approximately 5 minutes. The chloroform solution was filtered through 32-cm. fluted filter paper.[1] The copper carbonate was mixed with 10 ml. of chloroform and filtered to remove any remaining chloroform soluble material. The filtrates were combined and concentrated under vacuum to near dryness or a convenient volume for chromatographic column cleanup.

Column Chromatography. To further clean the extracted material of other fluorescing or masking materials prior to thin-layer chromatography, 1 of 2 different methods of column chromatography was used. In both, the dried extract was redissolved

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[1] J. Green; Green's 488½ Hand folded filter paper. England. Purchased from the Department of Chemistry, Kansas State University, Manhattan, Kansas.

with chloroform and transferred to the prepared columns. This rinsing was repeated 3 or 4 times. The washings were combined to make a volume of approximately 50 ml.

One method employed was basically as described by Eppley (1966). A ball of pyrex glass wool was placed in the bottom of the column[1] to provide a base. Five grams of granular anhydrous sodium sulfate was layered on top of the glass wool ball. Chloroform was added until the column was approximately 3/4 full. The trapped air was removed by tapping and opening the stopcock. Ten grams of silica gel[2] were dispersed in the chloroform by slowly pouring it into the column and vigorously shaking the column as it was added to prevent air entrapment. Time was allowed for the silica gel to settle. Drawing off some chloroform aided settling. Fifteen grams of granular anhydrous sodium sulfate were layered on top of the silica gel. The chloroform was drawn off to the top of the top layer of sodium sulfate. The sample extract was added to the column and eluted at a flow rate of 10 to 20 ml./minute, followed by 150 ml. hexane, followed by 150 ml. anhydrous diethyl ether. For the final elution, 150 ml. methanol/chloroform (3/97, v/v) was used. The fraction, from the time the methanol/chloroform eluent was added, was collected and concentrated to near dryness under vacuum. The eluate was

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[1] Model 274-019, 19 x 300 mm. chromatographic column with Teflon stopcock, Lab Crest, Division of Fisher Porter Company, Maywood, Illinois.

[2] 0.05-0.2 mm., Brinkman Instruments, Westbury, New York.

transferred to a 4-dram vial with screw cap and handled in the same manner as described for the toxic YES medium extracts.

The other chromatographic column method[1] involved the use of of calcium chloride and Celite as an absorbent. This was prepared by adding 40 grams of $CaCl_2$ to 50 ml. distilled water. Seventy grams of Celite were placed in a blender jar and the jar filled 3/4 full with hexane. While running, 21 ml. of the calcium chloride solution was added and blended for approximately one minute. This was transferred to a column.[2] The hexane was drawn off while positive pressure was applied into the column to facilitate packing of the calcium chloride - Celite. The hexane was finally drawn down to the top of the absorbent.

The dry extract was redissolved in 25 ml. benzene. Twenty-five milliliters of hexane were added and the solution transferred to the column and repeated. A final rinse of 50 ml. benzene and 50 ml. hexane was made and added to the column. This and the following fractions were eluted at the rate of 10 to 20 ml./minute. Two hundred milliliters of pure benzene, followed by 200 ml. of 2% acetone-benzene (4/196, v/v) completed the cleanup. The fraction, from the time the acetone-benzene was added, was collected and concentrated under vacuum to near dryness. The extract was transferred to a 4-dram vial and completely dried.

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[1] Method used and described by Mr. Herbert G. Wiseman, chemist, Agricultural Research Center, FDA, Beltsville, Maryland (personal communication).

[2] 1 x 22 inch glass column with one tapered end. Made by Physical Science Department (glass workshop), Kansas State University, Manhattan, Kansas.

The dry extract was redissolved in 0.1 ml. chloroform and plated as discussed previously.

Assay of Livers for Aflatoxins

The livers from the hens necropsied one day after administration of aflatoxin was terminated were assayed. The livers were individually weighed, manually broken down, and added to 250 ml. chloroform in a ground glass stoppered 500-ml. flask. The flask was shaken for one-half to one hour by a wrist action shaker. The samples were filtered through 32-cm. fluted filter paper. Fifty milliliters of the chloroform filtrate were used for assay, according to the method described previously by Eppley (1966).

RESULTS

Aflatoxin Assay of YES Medium

The YES medium on which the Aspergillus flavus was grown contained all 4 aflatoxins. No fluorescing spots were present in chloroform extracts of the control YES medium to cause interference. In that aflatoxins B_2 and G_2 are considerably less toxic than B_1 and G_1 and were present in small quantities, they were not assayed quantitatively. The toxic YES medium was shown to contain 120 ug. B_1 and 80 ug. G_1 per ml.

Aflatoxin Assay of Toxic Feed

The 1 ml. of the aflatoxins in acetone solution from the 1,000 ml. was shown quantitatively to contain 0.2 ug./ul.

aflatoxin B_1. Since this verified previous analysis for B_1, no verification was made for aflatoxins B_2, G_1, and G_2.

Assays of Eggs for Aflatoxins

None of the 4 aflatoxins administered or any of their fluorescing metabolites were observed on TLC of the egg extracts.

Assays of Livers for Aflatoxins

None of the 4 aflatoxins administered or any of their fluorescing metabolites were observed on TLC of the liver extracts.

Effects of Aflatoxins on Experimental Animals

Egg Production. The daily tabulations were consolidated to determine each hen's egg production percentage (Table 2). The production percentage means for each treatment - combination group are given in Table 3.

The data in Tables 2 and 3 were programmed into an IBM 360 model 50 computer for analysis of variance. The variance prior to the administration of aflatoxins was not statistically different between treatment-combination groups in regards to their laying production ability. When aflatoxins were administered, there was a statistically significant decrease in egg production (see Table 4, variables 2 and 3). The decrease in production had a greater variation between methods of administration than between dosage levels, although both were statistically significant at the .05 level of probability.

TABLE 2—Individual Means for Egg Production Percent, Egg Weight Average (grams), and Body Weight Loss (grams).

		Hen No.	Variables*						
			1	2	3	4	5	6	7
Aflatoxin--feed	2 ppm	1	73.0	64.7	60.0	70.8	71.1	71.8	125
		2	80.9	70.6	70.0	65.3	59.6	59.7	150
		3	87.3	88.2	90.0	60.0	59.7	60.8	150
		4	76.2	82.4	80.0	59.6	59.7	60.5	100
		5	73.0	64.7	60.0	60.4	63.8	63.1	25
		6	80.9	64.7	60.0	65.4	67.8	66.7	0
	4 ppm	7	82.5	76.5	70.0	64.7	64.5	64.5	50
		8	84.1	82.4	80.0	58.2	60.3	61.0	0
		9	71.4	76.5	70.0	62.7	64.7	63.7	- 25
		10	76.2	76.5	70.0	58.3	58.7	58.5	25
		11	66.7	70.6	60.0	66.6	68.1	67.7	75
		12	69.8	64.7	60.0	70.8	72.5	73.7	150
	8 ppm	13	69.8	70.6	60.0	59.5	57.1	56.4	100
		14	71.4	64.7	60.0	65.7	66.9	66.2	75
		15	87.3	76.5	70.0	63.9	61.5	61.3	325
		16	93.6	70.6	60.0	61.6	62.9	63.2	225
		17	82.5	76.5	70.0	58.4	58.6	58.6	0
		18	80.9	82.4	70.0	66.2	66.9	66.4	25
Aflatoxin--media	4 ml./kg.	20	71.4	17.6	0.0	66.1	65.6	---	150
		21	87.3	11.8	0.0	61.7	61.7	---	275
		22	73.0	41.2	30.0	61.4	60.5	59.7	25
		23	80.9	5.9	0.0	66.1	70.6	---	225
		24	85.7	41.2	20.0	74.6	73.3	73.4	300
		25	71.4	29.4	0.0	62.2	62.5	---	575
	2 ml./kg.	27	92.1	76.5	70.0	54.5	55.8	55.5	175
		28	76.2	70.6	60.0	63.4	61.4	62.5	0
		29	73.0	76.5	70.0	56.2	58.0	57.3	-100
		30	82.5	70.6	60.0	59.0	58.0	57.8	125
		31	82.5	5.9	0.0	61.4	60.0	---	350
		32	80.9	70.6	70.0	65.4	63.5	62.0	275
	1 ml./kg.	33	80.9	76.5	70.0	64.1	62.2	61.3	100
		34	82.5	41.2	10.0	65.4	64.8	56.5	250
		35	79.4	82.4	80.0	53.9	55.4	55.3	50
		36	57.1	70.6	70.0	61.8	60.7	58.6	300
		37	63.5	47.1	50.0	68.2	62.6	62.9	0
		38	80.9	76.5	80.0	64.7	62.0	63.7	125
Control--media	2	39	76.9	76.5	70.0	67.5	66.8	66.5	0
		40	75.4	64.7	70.0	59.7	58.5	58.2	- 25
		41	78.5	64.7	60.0	63.7	62.9	62.8	100
	4	42	78.5	64.7	60.0	66.8	70.8	71.6	0
		43	64.6	58.8	60.0	71.9	70.9	69.7	125
		44	78.5	76.5	60.0	71.5	69.8	70.3	0
	8	45	80.0	70.6	70.0	58.2	55.9	56.0	175
		46	69.2	70.6	70.0	58.6	59.2	59.7	0
		47	80.0	47.1	40.0	66.5	67.8	67.3	- 75

* For variables code, see bottom of Table 3 or 4.

TABLE 3—Group Means for Egg Production Percent, Egg Weight Average (grams), and Body Weight Loss (grams).

	Variable*	Afs-feed	Afs-media	Control-media	Ppm mean
2 ppm or 1 ml./kg.	1	78.6	74.1	76.9	76.4
	2	72.6	65.7	68.6	69.0
	3	70.0	60.0	66.7	65.3
	4	63.6	63.0	63.6	63.4
	5	63.6	61.3	62.7	62.5
	6	63.8	59.7	62.5	61.9
	7	91.7	137.5	25.0	96.7
4 ppm or 2 ml./kg.	1	75.1	81.2	73.9	77.3
	2	74.5	61.8	66.7	67.9
	3	68.3	55.0	60.0	61.3
	4	63.6	60.0	70.1	63.4
	5	64.8	59.5	70.5	63.8
	6	64.9	59.1	70.5	64.8
	7	45.8	137.5	41.7	81.7
8 ppm or 4 ml./kg.	1	80.9	78.3	76.4	79.0
	2	73.6	24.5	62.8	51.8
	3	65.0	8.3	60.0	41.3
	4	62.6	65.4	61.1	63.4
	5	62.3	65.7	61.0	63.4
	6	62.0	66.5	61.0	63.2
	7	125.0	258.3	160.0	160.0
Method mean (overall means)	1	78.2	77.8	75.7	77.6
	2	73.5	50.7	66.0	62.9
	3	67.8	41.1	62.2	56.0
	4	63.2	62.8	64.9	63.4
	5	63.6	62.1	64.7	63.2
	6	63.5	61.8	64.7	62.8
	7	87.5	177.8	33.3	112.8

* Variables code:

1 Egg production (percent) during pre-treatment period.
2 Egg production (percent) during treatment period.
3 Egg production (percent) during treatment period minus first week.
4 Egg weight average (grams) during pre-treatment period.
5 Egg weight average (grams) during treatment period.
6 Egg weight average (grams) during treatment period minus first week.
7 Loss of weight from start of treatment period to time of necropsy.

Egg Weight. The daily tabulations were consolidated to determine individual egg weight averages (Table 2). The group means (Table 3) show the egg weight averages changed very little within the different treatment-combination groups.

Analysis of variance of the data in Tables 2 and 3 was made. The variance was statistically significant (at the .05 level of probability) in egg weight averages during the pre-treatment period as well as during the treatment period when the method by dose variance was tested (see Table 4, variables 4, 5, and 6, method by dose line).

Body Weights. The differences in body weights of each hen from the time the aflatoxins were first administered to the body weights recorded at necropsy, were programmed into a computer for analysis of variance. The results of this analysis showed a statistically significant variance in loss of body weight due to the different methods of administration. The aflatoxin-medium caused a greater weight loss. No significant difference in weight loss was noted between the 3 dose level groups having the same method of administration (see Table 4).

Pathological Changes. Gross pathological changes were observed in only the liver. The livers varied from normal size to greatly enlarged (2 to 3 times normal) sizes. Color ranged from normal to pale yellow. Petechial and larger hemorrhagic areas were frequently observed. The only significant histopathological finding was bile ductule hyperplasia. This was observed in the two groups receiving the highest level of aflatoxins (8 ppm

TABLE 4—Analysis of Variance.

Source of variation	Degrees of freedom	Mean squares of variables						
		1	2	3	4	5	6	7
Method of administration	2	19	2409*	3418*	14	22	55	72,177*
Dose level	2	25	1394*	2480*	0	7	16	25,931
Method x dose	4	56	870*	1238*	55*	71*	59*	6,479
Error	36	59	221	281	19	19	20**	15,240

* Mean square is significant at .05 level of probability.

** Error calculated with 31 degrees of freedom.

Variables code:

1 Egg production (percent) during pre-treatment period.
2 Egg production (percent) during treatment period.
3 Egg production (percent) during treatment period minus first week.
4 Egg weight average (grams) during pre-treatment period.
5 Egg weight average (grams) during treatment period.
6 Egg weight average (grams) during treatment period minus first week.
7 Loss of weight from start of treatment period to time of necropsy.

aflatoxin in the feed and 4 ml. of aflatoxin medium per kg. body weight). The pathological changes observed are summarized in Tables 5, 6, 7, and 8.

DISCUSSION

The negative results of this study concerning the presence or absence of aflatoxins or their fluorescing metabolites in eggs, by confirming the findings of some (Allcroft and Carnaghan, 1963a; Abrams, 1965) and contradicting others (Wiseman et al., 1968)[1] further emphasize the need for additional research in the field of mycotoxins.

As was pointed out in the literature review, chicken breeds are variable in the resistance or susceptibility to aflatoxins (Brown and Abrams, 1965). Another example of what was believed to be specie variation was reported by Brown and Abrams (1965) when they were not able to detect toxicity in milk from cows fed aflatoxin-contaminated feeds. Several investigators had published papers previously with findings of toxicity in cows' milk and even milk from ewes, goats, and rats.

Platonow (1965) was unable to detect aflatoxins or their fluorescing metabolites in breast meat, leg meat, or livers of broilers fed aflatoxins for 6 weeks. Van Zytveld (1967)[2]

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[1] Herbert G. Wiseman, Agricultural Research Center, FDA, Beltsville, Maryland (Personal communication). 1968.

[2] Wm. A. Van Zytveld. Master's Thesis. Aflatoxicosis: The Presence of Aflatoxins or Their Metabolites in Livers and Skeletal Muscle of Chickens. College of Veterinary Medicine, Kansas State University, Manhattan, Kansas.

TABLE 5—Gross and Microscopic Observations of Liver Lesions in Hens Receiving Aflatoxin Medium.

	Hen No.	Day D +	Liver size	Pete-chia	Fat amount gross	Fat amount micro	Fat distr.	Hyaline masses	Necrosis	Lymph nodules	Bile ductule hyperplasia
1 ml./kg.bwt.	34	1	1-2X	0	++	++	diffuse	0	0	+	0
	33	1	2X	++	++	+++	diffuse	0	0	0	0
	36	4	normal	0	++	+++	diffuse	0	0	0	0
	37	8	1-2X	0	+	++	diffuse	0	0	+	0
	35	15	1-2X	+	++	+++	diffuse	+	0	+	0
	38	29	normal	0	0	++	diffuse	+	0	+	0
2 ml./kg.bwt.	32	1	2X	+	++	+++	diffuse	0	0	+	0
	31	1	1-2X	0	+	+	diffuse	+	0	++	0
	28	4	2X	+	++	+++	diffuse	0	0	++	0
	29	8	3X	+	+++	+++	periportal	+	0	+	0
	27	15	1-2X	+	+	+	diffuse	0	0	++	0
	30	29	normal	0	0	+	diffuse	0	0	+	+
4 ml./kg.bwt.	21	1	1-2X	0	+++	+++	diffuse	0	0	++	0
	20	1	1-2X	0	+++	+++	diffuse	0	0	++	0
	23	4	normal	0	+	++	diffuse	0	0	++	+
	24	8	2X	0	++	++	diffuse	0	0	+	++
	22	15	2X	0	++	+++	diffuse	0	0	+	++
	25	29	normal	0	0	++	periportal	0	0	++	+

TABLE 6—Gross and Microscopic Observations of Liver Lesions in Hens Receiving Aflatoxin-Feed Mixtures.

	Hen No.	Day D +	Liver size	Pete-chia	Fat amount gross	Fat amount micro	Fat distr.	Hyaline masses	Necrosis	Lymph nodules	Bile ductule hyperplasia
2 ppm	1	1	normal	0	0	+	diffuse	0	0	+	0
	6	1	1-2X	0	+	++	diffuse	0	0	++	0
	2	4	2X	++	++	+++	diffuse	+	massive	+	0
	4	8	normal	+	0	+	diffuse	0	0	+	0
	5	15	normal	0	0	+	diffuse	+	0	+	0
	3	29	normal	0	0	++	diffuse	0	0	0	0
4 ppm	11	1	3X	+++	+++	+++	diffuse	+	massive	++	0
	12	1	1-2X	0	++	+++	diffuse	+	focal	++	0
	8	4	3X	+	+++	+++	diffuse	+	0	+	0
	9	8	3X	++	+++	+++	diffuse	0	0	0	0
	10	15	normal	0	0	+	diffuse	+	focal	++	0
	7	29	normal	0	0	+	diffuse	0	0	++	0
8 ppm	13	1	3X	+++	+++	+++	diffuse	0	0	++	+
	14	1	3X	+++	+++	+++	diffuse	+	0	++	+
	17	4	3X	+++	+++	+++	diffuse	+	0	++	0
	16	8	2X	++	++	++	diffuse	+	massive	++	++
	18	15	2X	+	0	++	periportal	+	massive	++	++
	15	29	normal	0	0	+	periportal	0	0	++	++

TABLE 7—Gross and Microscopic Observations of Liver Lesions in Hens Receiving Control Medium.

	Hen No.	Day D +	Liver size	Petechia	Fat amount gross	Fat amount micro	Fat distr.	Hyaline masses	Necrosis	Lymph nodules	Bile ductule hyperplasia
1 ml./kg.	41	1	normal	0	0	+	diffuse	0	0	+	0
	40	8	normal	0	0	+	diffuse	+	0	+	0
	39	27	normal	0	0	+	diffuse	0	0	+	0
2 ml./kg.	44	1	1-2X	0	+	++	diffuse	+	0	+	0
	43	8	1-2X	+	++	+++	diffuse	+	0	+	0
	42	27	normal	0	0	++	diffuse	+	0	+	0
4 ml./kg.	47	1	2X	+++	+++	+++	diffuse	+	massive	++	0
	46	8	normal	0	0	++	diffuse	+	focal	+	0
	45	27	1-2X	+	+	+++	diffuse	+	massive	+	0

TABLE 8—Gross and Microscopic Observations of Liver Lesions in Hens Serving as Environmental Controls.

Hen No.	Day D +	Liver size	Petechia	Fat amount gross	Fat amount micro	Fat distr.	Hyaline masses	Necrosis	Lymph nodules	Bile ductule hyperplasia
48	-	normal	0	0	++	diffuse	+	0	+	0
54	-	normal	0	++	+++	diffuse	+	0	+	0
56	-	normal	0	0	+++	diffuse	+	0	++	0
57	-	normal	0	0	+	diffuse	+	0	+	0

detected aflatoxins and/or metabolites in the breast meat, leg meat, or liver from 15 of 45 broilers fed aflatoxins for 6 weeks.

Since the two previous negative reports for egg toxicity using bioassays were concluded, it was believed the use of more sensitive TLC might detect the presence of aflatoxins or their fluorescing metabolites. During the course of this study, the author was referred to the Bureau of Veterinary Medicine, Agricultural Research Center, FDA, Beltsville, Maryland. Mr. Wiseman, chemist, wrote they had detected the presence of aflatoxin in hen eggs when the hens were fed 0.4 ppm aflatoxin in the feed ad libitum. They used TLC for assays.

The method described for extraction was tried with negative results. A visit to the laboratories of the Agricultural Research Center, FDA, Beltsville, Maryland, was made. Eggs from hens receiving the toxic YES medium were taken for aflatoxin assay. Aflatoxins or their fluorescing metabolites could not be detected. Additional efforts by the author, using the methods described by Eppley (1966) and Mr. Wiseman, did not detect aflatoxins in subsequent assays of eggs receiving toxic YES medium or aflatoxin contaminated feed. Therefore, it is concluded that no aflatoxins or their fluorescing metabolites were transmitted to the eggs of the hens used in this study. A bioassay would be required to determine if non-fluorescing toxic metabolites of the aflatoxins were present.

The statistically significant decrease in egg production and the pathological changes observed in the hens administered aflatoxins are indicative of the toxicity of the material used.

The pathological changes observed both grossly and microscopically in control hens as well as aflatoxin-administered hens were suggestive of an intercurrent disease process. Because of the similarity of lesions between these groups, no significance can be placed on the observed fatty changes, petechial hemorrhages, enlarged liver sizes, and the presence of lymph nodules and hyaline material. The lymphocytic infiltration observed in kidney sections of two control birds is suggestive of leukosis. Salmonella or Pasteurella infection is one possible cause of liver necrosis with granuloma formation. Thus, the etiology of the intercurrent disease process is only speculative at this time. As bile ductule hyperplasia was not observed in any of the control hens, and was seen only in the livers of chickens receiving the highest dose rate, it is likely that this is a significant change due to the aflatoxins.

The greater decrease in egg production and loss of body weight in the groups receiving toxic YES medium could be due to several factors. Handling of the hens was necessary to administer the toxic medium. The toxic medium was given in a matter of seconds rather than over a period of hours. Some degradation of the aflatoxins probably occurred in the feed before it was consumed. Individual hens may have eaten different relative quantities.

The statistically significant method by dose mean squares, involving egg weights (Table 4, variables 4, 5, and 6), are most likely due to the 3 hens of the 4 ppm control medium group laying

exceptionally large eggs. The egg weight averages of the group receiving 4 ppm aflatoxin medium is the lowest of all treatment-combination test groups. It appears the method by dose variation is accounted for primarily by the differences in the aflatoxin medium 4-ppm group and the control medium 4-ppm group for variables 4, 5, and 6. However, since this variation existed in the pre-treatment period as well as during the treatment period, it is felt the variation is due to chance rather than due to the effect of aflatoxins.

CONCLUSION

Aflatoxins or their fluorescing metabolites were not detectable in the eggs of hens receiving aflatoxins mixed in feed at dose levels of 2, 4, and 8 ppm, and eggs from hens receiving *per os* comparable quantities of crude aflatoxins in YES medium.

Assays of livers from hens at each dose level were negative for aflatoxins or fluorescing metabolites.

A statistically significant decrease in egg production due to the administration of aflatoxins was noted, but there was no effect on egg size.

The possibility of a public health hazard existing, due to the consumption of eggs from hens receiving aflatoxins, must be considered minimal for the following reasons: 1. No aflatoxins or fluorescing metabolites were found in the eggs from this breed of hens. 2. If another breed of hens should transmit the toxins to their eggs, the quantity would most likely be very minute.

3. With the present marketing and purchasing methods, there is little possibility of an individual receiving eggs from one source for any length of time.

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A STUDY OF AFLATOXICOSIS IN LAYING HENS TO DETERMINE THE ABSENCE OR PRESENCE OF AFLATOXINS IN EGGS

by

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Aflatoxins were administered to laying hens to determine if the aflatoxins or their fluorescing metabolites would pass in the eggs and thus pose a possible public health hazard.

The aflatoxins were administered to groups of 6 hens, mixed in the feed at levels of 2, 4, and 8 ppm. Dose levels of 240, 480, and 960 ug./2,000 g. body weight of crude aflatoxins produced on liquid yeast extract and sucrose medium by Aspergillus flavus, ATCC 15517, were also administered daily by pipette to 3 other test groups of 6 hens per group. Environmental and control (yeast extract and sucrose) medium test groups were maintained.

Aflatoxins were administered for 16 days. Eggs from the 8th, 13th, and 17th days were assayed by thin layer chromatography. No aflatoxins or fluorescing metabolites were observed.

The hens receiving aflatoxins were necropsied 1, 4, 8, 15, and 29 days after the termination of aflatoxin administration. The livers from the hens necropsied on the first day were negative when assayed for aflatoxins with thin layer chromatography. Histopathological studies of the livers revealed significant liver damage (bile ductule proliferation) was present in the two groups which received the highest dose level of aflatoxins.

There was a statistically significant ($P = 0.05$) decrease in egg production. No statistically significant effect on egg weight means was noted.