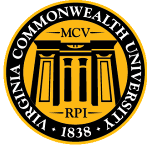

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Complies with the University regulations and that it meets the accepted standards of the Graduate School with respect to originality and quality

For the degree of : Master of Science

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June 7 1973

Professor in charge of the thesis

THE EFFECT OF AFLATOXIN B_1 ON GROWTH RATE AND IRON METABOLISM IN JUVENILE MONGOLIAN GERBILS (*Meriones unguiculatus*)

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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Richmond, Virginia

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1973

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LIST OF ABBREVIATIONS

ppb	parts per billion
ppm	parts per million
kg	kilograms
cc	cubic centimeters
g	grams
R_f	Ratio of distance of solvent front compared to distance that solute moved
ug	micrograms
ml	milliliters
mg	milligrams
cm	centimeters
pH	Hydrogen Ion Concentration (negative logarithm of)
uCi	microcuries
Ci	Curies
cpm	counts per minute
dpm	disintegrations per minute
ng	nanograms
S_1	Standard error of the control group mean
S_2	Standard error of the aflatoxin group mean
S	Standard error of the difference of the means
SD	Standard Deviation
n	number of samples

ABSTRACT

Hastings, William Stanley, Jr., M.S., Virginia Commonwealth University, May 1973. The Effect of Aflatoxin B_1 on Growth Rate and Iron Metabolism in Juvenile Mongolian Gerbils (Meriones unguiculatus). Major Professor: Gerald C. Llewellyn.

The metabolic secretions from Aspergillus flavus, aflatoxins, are often found as contaminants of agricultural products used for human and animal consumption. Aflatoxin B_1 is the most toxic and carcinogenic of the aflatoxins. A reduction in weight gain has been observed when juvenile animals are fed aflatoxin B_1 over an extended period of time. This reduction in weight gain may possibly result from an anemic condition induced by an alteration of iron absorption and/or distribution. The effect of chronic sublethal levels of this hepatocarcinogen on weight gain and the uptake and distribution of $^{59}Fe^{++}$ were studied in this investigation.

An experimental group consisting of 12 male gerbils was fed an agar-based diet containing 10 ppm aflatoxin B_1 and a control group consisting of 6 male gerbils was fed an identical diet which did not contain aflatoxin B_1 for a 75-day period. The total mean aflatoxin B_1 consumption of the experimental group amounted to 0.170 mg aflatoxin B_1/kg of body weight. At the termination of the investigation the mean percent iron absorption of the control and experimental groups was determined. Values of 6.25 and 6.89, respectively,

were calculated. The difference between these means was not determined to be significant.

At the termination of the investigation the mean weight gain of the control group animals was 36.9 g as compared to 29.8 g for the aflatoxin group. The difference indicated that a significant reduction in weight gain was observed in the experimental group. It was apparent that neither the physical activity nor diet consumption measured in the investigation affected the weight gain. Pathological analysis indicated that one-third of the experimental animals had liver injury and two of these had toxic hepatitis.

The difference in the iron absorption between the aflatoxin group and the control group was not found to be significant but certain trends were observed. Animal number 13, the animal with the highest percent absorption, also had toxic hepatitis. However, contradictory evidence was also present with animal number 16 having toxic hepatitis and a very low percent iron absorption.

INTRODUCTION

The study of environmental carcinogens was initiated by Sir Percival Pott in 1775 when he observed that soot caused a higher incidence of scrotal cancer in London chimney sweeps than in men having other occupations in London (Stewart, 1959). In 1918 Yamagiwa was able to induce skin cancers at will on the ears of rabbits using coal tar (Boyd, 1950). The concept that environmental chemicals, either industrial or naturally occurring, could induce carcinogenesis was well established after Yamagiwa's results were published.

During the last 50 years many chemical carcinogens present in industrial operations, air pollution, and naturally occurring in the environment have been discovered. As industrial expansion continues with its accompanying pollution many more environmental carcinogens will inevitably contaminate the air we breathe as well as exposing workers coming into direct contact with these chemicals in industry. Also investigators will undoubtedly continue to locate more naturally occurring environmental carcinogens. Hopefully enough will become known about all environmental carcinogens so that the proper precautions can be taken to prevent exposure.

Aflatoxin B_1 is an extremely potent environmental carcinogen which is a metabolite of the fungus *Aspergillus flavus* (Schoental, 1967). Human beings and animals are exposed to

aflatoxin B_1 by consuming stored grain products contaminated with the fungus (Wogan, 1968).

Rationale For This Investigation

Nothing is known concerning the specific effect from the consumption of a diet containing aflatoxin B_1 on the growth rate or the absorption and distribution of iron in *Meriones unguiculatus*. Juvenile Syrian hamsters, *Mesocricetus auratus*, fed a diet containing aflatoxin B_1 at 1 ppm over a 250-day feeding period (Llewellyn, 1970) had a lower rate of weight gain than animals fed control diet indicating a reduction in growth rate. The reduction in growth rate may possibly result from an anemic condition induced by alteration of iron absorption and/or distribution. This speculation is based on research conducted by Theron (1967) which demonstrated that the red blood cells transport aflatoxin in ducklings. The purpose of the present investigation is to ascertain whether the dietary consumption of 10 ppm aflatoxin B_1 by *M. unguiculatus* affects changes in growth rate and in the absorption and distribution of iron.

Objectives

1. To evaluate the growth rate in *M. unguiculatus* as a result of consuming a diet containing 10 ppm aflatoxin B_1 for a 75-day period.
2. To determine if there is a difference in the absorption and/or distribution of iron between members of this species

consuming a diet containing 10 ppm aflatoxin B_1 and a control diet during the last seven days of the investigation.

3. To evaluate if pathological liver injury resulting from exposure to diet containing 10 ppm aflatoxin B_1 influences iron absorption and/or distribution in this species.

LITERATURE REVIEW

Aflatoxin Literature

Fungi are the major cause of deterioration, spoilage and toxin production in stored crops all over the world. They cause about one percent of the world's supply to be unfit for human consumption every year (Johnson, 1962). The fungus, *A. flavus*, is one of the types of fungi found on these agricultural products. A group of metabolites of *A. flavus*, aflatoxins, are potent hepatotoxins and are extremely carcinogenic with the liver being one of the more sensitive organs. Humans and animals are exposed to aflatoxins by consuming agricultural products that had at some stage been contaminated with the fungus (Wogan, 1968).

Discovery of Toxin

In 1960 more than 100,000 young turkeys on poultry farms in the southern and eastern regions of England died in the course of a few months from an apparently new disease that was termed "Turkey X Disease" (Blount, 1961). On one farm 5,000 partridge and pheasant poults died and on another 14,000 ducklings died (Asplin and Carnaghan, 1961). Reports were also received from Kenya and Uganda of severe losses of ducklings from a similar disease (Asplin and Carnaghan, 1961).

Simultaneous investigations by Blount (1961) and Asplin and Carnaghan (1961) indicated that the common factor in the disease among the English poultry was the presence of contaminated Brazilian groundnut meal in the animals' feed and the occurrence of certain liver lesions. Asplin and Carnaghan (1961) also examined the liver lesions of ducklings sent from Uganda. These animals had not been fed Brazilian groundnut meal but had developed lesions similar to those observed in the English poultry.

After it had been established that groundnut meals contained a toxin and not a virus, the "Turkey X Disease" was investigated more extensively. It was demonstrated that ducklings with symptoms similar to "Turkey X Disease" were very sensitive to toxin-contaminated meal. This provided an excellent biological test for the presence of the toxin. Asplin and Carnaghan (1961) then extracted the toxin from the meal with hot methanol, partitioning off the aqueous suspension of the extract into chloroform, and finally concentrating the residues by distribution in a system composed of methanol: water: petroleum ether (10:1:10) whereupon the toxin passed into the methanol layer. By this means, an increase in concentration of the toxin by 250-fold was achieved.

Sargeant *et al*. (1963) were able to isolate and grow pure cultures of the fungal species present in a toxic sample of peanuts from Uganda. They extracted one of the cultures that had been grown on Czapek's medium with chloroform and found a certain fluorescent material with an R_f value of 0.7 when

chromatographed on paper. The extract was known from their previous work to produce the characteristic symptoms of "Turkey X Disease". The toxin-producing fungus was identified as *A. flavus* and the toxin was given the name of aflatoxin because of its origin. Later it was shown that the fungus secreted a mixture of aflatoxins whose composition depended on the particular strain of *A. flavus* present.

Chemical Structure

Aflatoxins have similar structures and form a unique group of highly oxygenated heterocyclic compounds. Since relatively small amounts of the toxin were available to the researchers when the structural configurations were worked out, the progress was very slow. The methods of modern organic chemistry including interpretation of ultraviolet, infrared, nuclear magnetic resonance and mass spectra data were used to determine the structures (Goldblatt, 1969).

The four major aflatoxin compounds secreted by *A. flavus*, B_1, B_2, G_1 and G_2, are illustrated in Figure 1 and occur in varying proportions according to the environment, the substrate, and the strain of *A. flavus*. In agricultural products aflatoxin B_1 is encountered most frequently with the others present in small amounts or completely absent. However, significant amounts of each of the other toxins, excluding B_1, have been found (Asao *et al*., 1965).

If aflatoxin B_1 or a mixture of aflatoxins is fed to animals, certain related toxins may be recovered from the

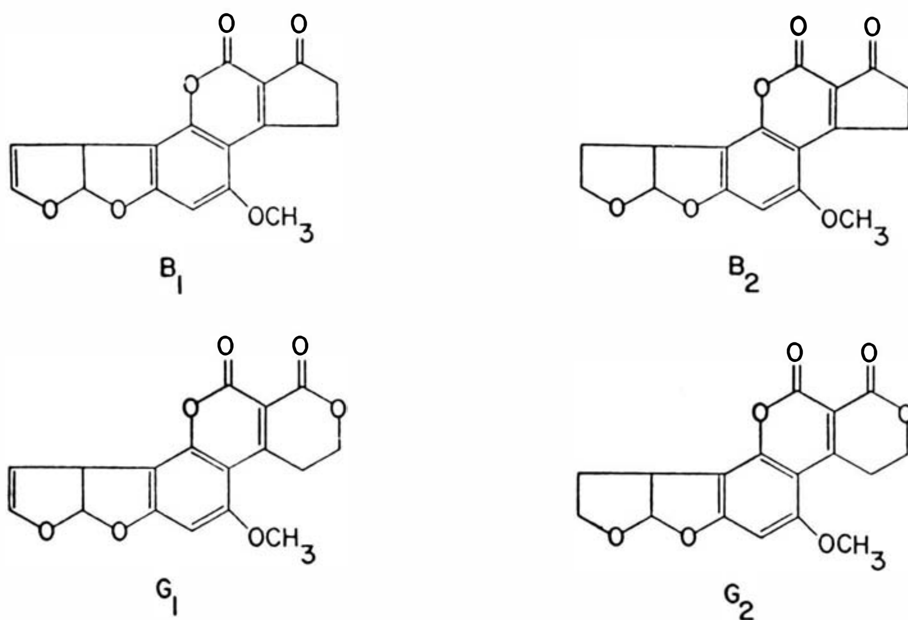

Figure 1. Structures of Aflatoxins B_1, B_2, G_1, and G_2

From: Goldblatt, L.A. Aflatoxin (New York: Academic Press, 1969) p. 5.

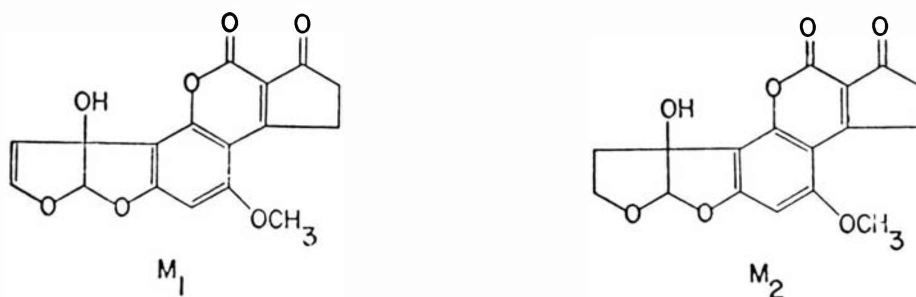

Figure 2. Structures of Aflatoxins M_1 and M_2

From: Goldblatt, L.A. Aflatoxin (New York: Academic Press, 1969) p. 7.

secretions of that animal. Holzapfel *et al.* (1961) reported the presence of aflatoxin M_1 and M_2 from secretion products of various animals and also detected the toxins among the metabolic breakdown products of *A. flavus*. These compounds are depicted in Figure 2.

Dutton and Heathcote (1968) isolated two more aflatoxins from cultures of *A. flavus* and designated them aflatoxin B_{2a} and G_{2a}. The two compounds are illustrated in Figure 3.

Another aflatoxin called aspertoxin was reported by Rodricks *et al.* (1968). The structure of aspertoxin was established simultaneously by Rodricks *et al.* (1968) and Waiss *et al.* (1968) and is illustrated in Figure 4.

Aflatoxin B_1 was totally synthesized by Buchi *et al.* (1966), but the process is so complicated that all aflatoxins used for research are extracted from *A. flavus* cultures.

Metabolic and Biochemical Effects of Aflatoxin B_1

Two aflatoxin B_1 investigations with the Mongolian gerbil have been conducted. In one investigation by Dunkin and Llewellyn (1971) aflatoxin B_1 was fed to adult male gerbils in their drinking water at 10 ug/ml for a 30-day period. The animals did not undergo significant changes in body weight. Oral concentrations of aflatoxin B_1 consumed ranged from 0.79 to 3.61 mg/kg body weight with no lethality. A second but unpublished experiment was conducted by Llewellyn and Novak (1969) where adult male gerbils were fed 1 ppm aflatoxin B_1 for 300 days with no weight changes or pathological damage to

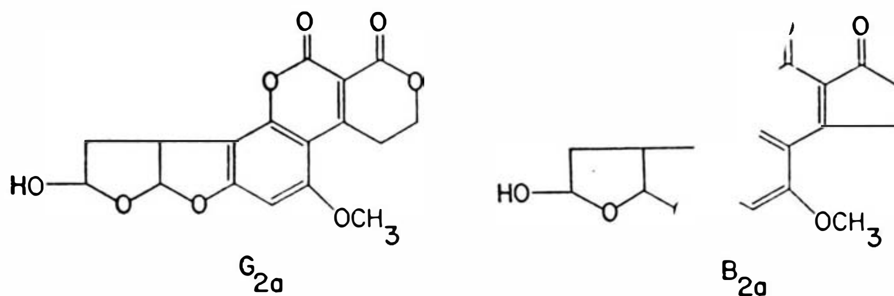

Figure 3. Structures of Aflatoxins B_2 ɪd G_{2a}

From: Goldblatt, L.A. Aflatoxin (Ne ɔrk: Academic Press, 1969) p. 7.

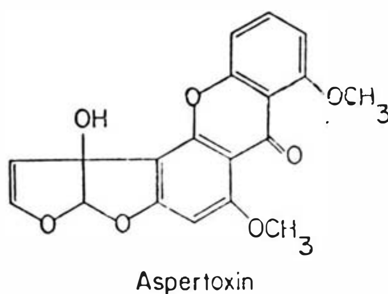

Figure 4. Structure of Aspertoxin

From: Goldblatt, L.A. Aflatoxin (New York: Academic Press, 1969) p. 8.

the liver. These investigations appear to indicate that the gerbil is relatively resistant to chronically administered aflatoxin B_1.

Since the majority of metabolic studies have been conducted with white rats, Rattus rattus, the results of one of the more pertinent investigations with this animal will be presented. In this study by Shank and Wogan (1966) white rats were sacrificed 24 hours after receiving an intraperitoneal injection of either methoxy or ring-labeled aflatoxin B_1. The urine, feces, and CO_2 excreted were counted by liquid scintillation. The total excretion amounted to 70 to 80 percent of administered dose over a 24-hour period. There was a significant difference in the feces ^{14}C content between the differently labeled isotopes. Approximately 60 percent of the administered radioactivity from the ring-labeled compound appeared in the feces. The difference is largely accounted for by the ^{14}C content of the animals dosed with the methoxy-labeled compound. Only about 1/2 percent of the ring-labeled ^{14}C appeared in CO_2. From this it can be concluded that aflatoxin B_1 or its metabolites are rapidly excreted by the rat after a single dose. Also, data on the methoxy-labeled compound indicated that a significant amount of administered radioactivity appeared in CO_2. Since this radioactivity could have arisen only from the methoxy carbon of aflatoxin B_1, it is thus apparent that O-demethylation must be a major pathway in the degradation of the compound. It is not known quantitatively what proportion of the demethylated fragment is fully oxidized to CO_2. The fate of the portion of

the group not fully oxidized remains unknown. Therefore, the proportion of the administered dose that undergoes demethylation cannot be estimated.

Since only small amounts of ^{14}C derived from the ring-labeled compound appeared in CO_2, it is apparent that very little ring cleavage of aflatoxin B_1 occurs or alternatively, if ring cleavage does occur, the fragments thus generated are not completely metabolized through oxidative pathways.

The distribution of unexcreted radioactivity from ring-labeled aflatoxin B_1 in the liver, kidneys, and spleen of the rat were determined by Falk _et al._ (1965) at intervals up to a 24-hour period following a single intraperitoneal dose. The aflatoxin B_1-^{14}C content of these organs is summarized in Figure 5. The high initial values reflect the presence of radioactivity in entrapped blood or urine in the tissues. At all times after administration the specific activity of liver exceeded the value for other tissues and at 24 hours the liver contained radioactivity at a level 5 to 15 times greater than any other tissue. These results clearly demonstrate that the liver accumulates aflatoxin B_1 or its metabolites to a much greater extent than other tissues.

Implications of Aflatoxins to Human Health

Extensive evidence has been collected concerning the action of aflatoxins on various animals. However, there is a lack of direct evidence regarding the degree of susceptibility of man to aflatoxins. Evidence presented by Tydskrif (1965)

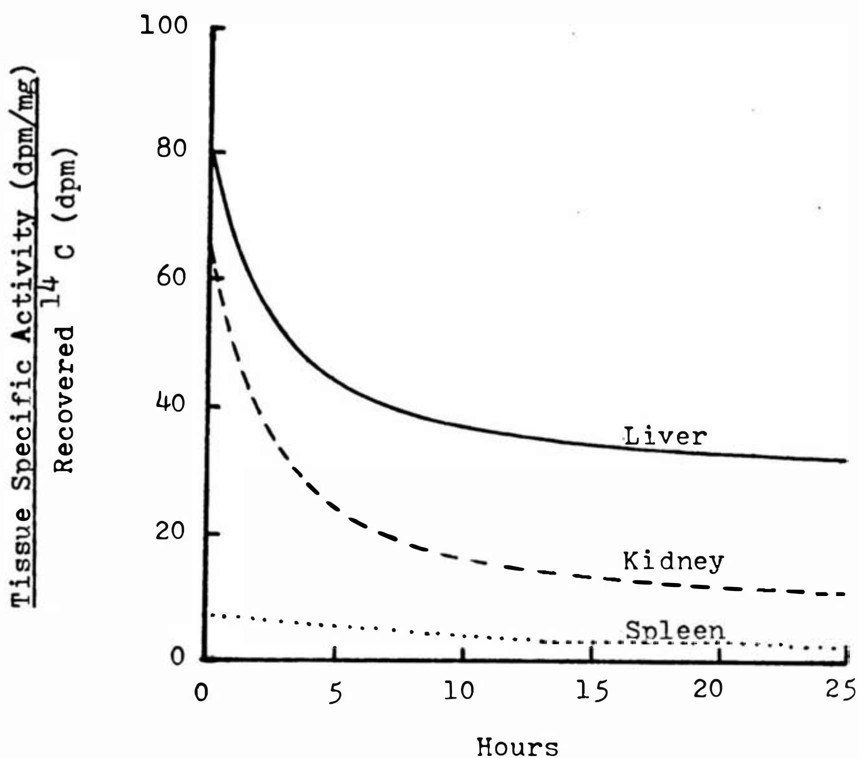

Figure 5. Relative Tissue Distribution of ^{14}C in Rat Tissue after Intraperitoneal Injection of Ring-labeled Aflatoxin B_1

From: Falk, H. L., Thompson, S. J., and Kotin, P., "Metabolism of Aflatoxin B_1 in the Rat," Proc. Am. Assoc. Cancer Res., 6, 18 (1965).

indicates that the epidemiological pattern of hepatomas in Africa is best explained by the spoilage of food by molds such as _A. flavus_. The suggestive evidence concerning the induction of hepatomas in animals and the pattern of hepatomas in Africa provides supportive evidence for a cause and effect relationship between the toxin and the induction of hepatomas in man.

Iron Metabolism Literature

The general concepts of iron metabolism in mammalian systems will be developed first. Then information concerning the validity of the technique used in this iron metabolism investigation will be presented.

Iron Absorption

The mechanism of iron absorption in mammalian systems through the gut mucosa was originally proposed by Granick using guinea pigs (White _et al._, 1964). The mechanism of iron absorption as modified from his original work is presented in Figure 6.

When iron in the diet is consumed and passes through the gastrointestinal tract it is reduced to the ferrous state, if it is not already in that state by gastric acidity or other reducing agents in the food and secretions. The ferrous iron is then absorbed into the mucosal cells of the duodenum and jejunum (Orten and Neuhaus, 1970). Ferrous iron entering the mucosal cells is rapidly oxidized to form ferric hydroxide-phosphate which combines with apoferritin. Within the mucosal

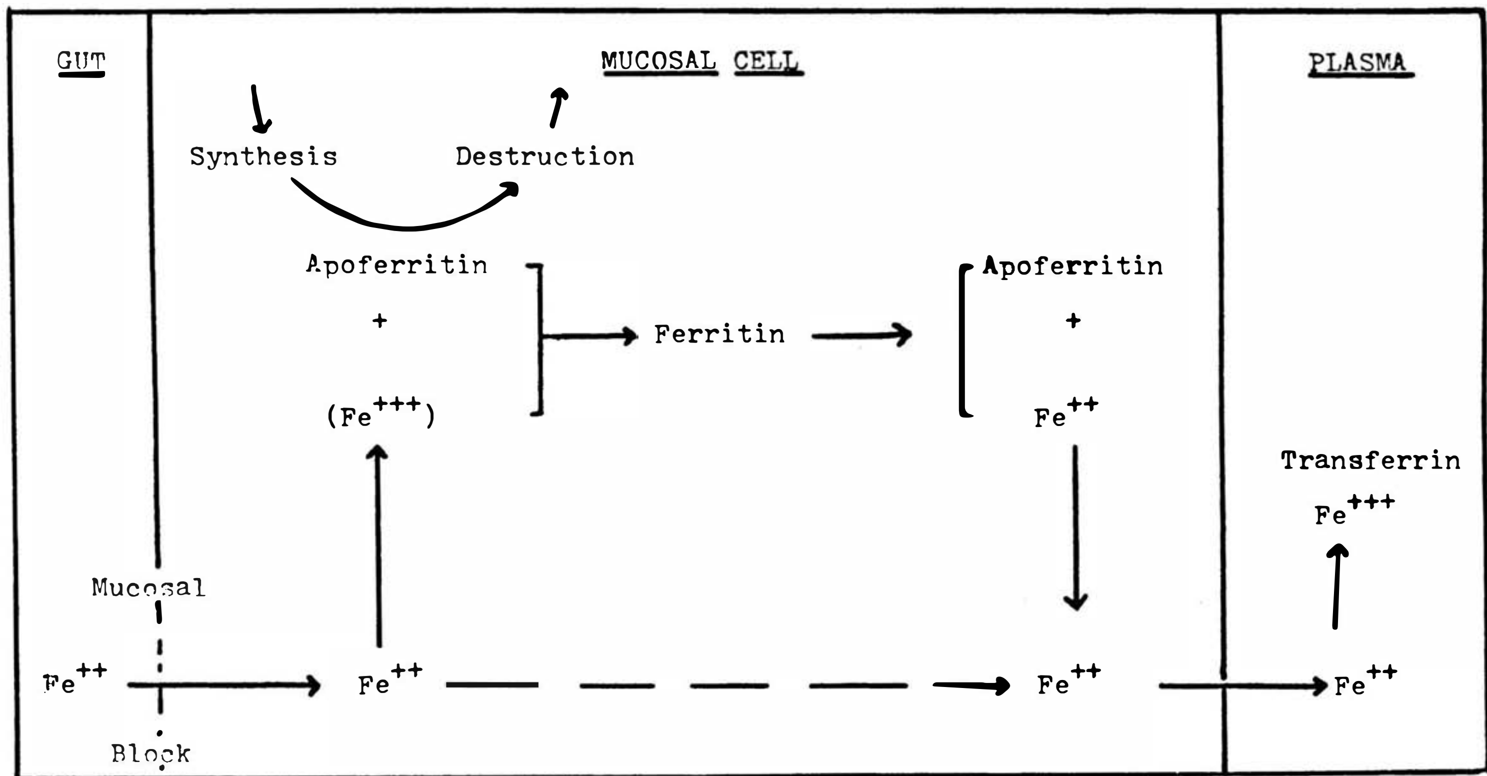

Figure 6. Iron Absorption Regulation in Mammalian Systems by the Mucosal Cell of the Intestinal Tract

From: White, A., Handler, P., Smith, E. G., Principles of Biochemistry (New York: McGraw Hill, 1964) p. 803.

cell, because of unknown circumstances which favor reduction of ferric to ferrous iron, breakdown of ferritin occurs, allowing absorption of additional iron from the intestine. It has been suggested that iron absorption is limited by the binding capacity of the apoferritin for iron which is indirectly related to ferritin concentration (White et al., 1964).

Iron Transport and Storage

Only the ferrous iron can pass into the blood from the mucosal cell. The ferrous iron is then auto-oxidized and becomes attached to one of the B_1-globulins called transferrin (White et al., 1964). This complex facilitates iron transport throughout the body and is in equilibrium with the ferrous iron and ferritin in the liver, spleen, and bone marrow. A diagram demonstrating the transport and storage of iron is presented in Figure 7.

Iron is mainly stored in the liver in the form of ferritin and hemosiderin. Hemosiderin is composed of large aggregates of ferritin molecules with a higher iron content. This iron is relatively inert (Bothwell and Finch, 1962).

Erythropoiesis

Iron is transferred to the individual red blood cell precursor by transferrin. The protein of transferrin probably becomes attached to the cell membrane since it has been shown that immature red blood cells are coated with a protein immunologically identical with transferrin (Bothwell and

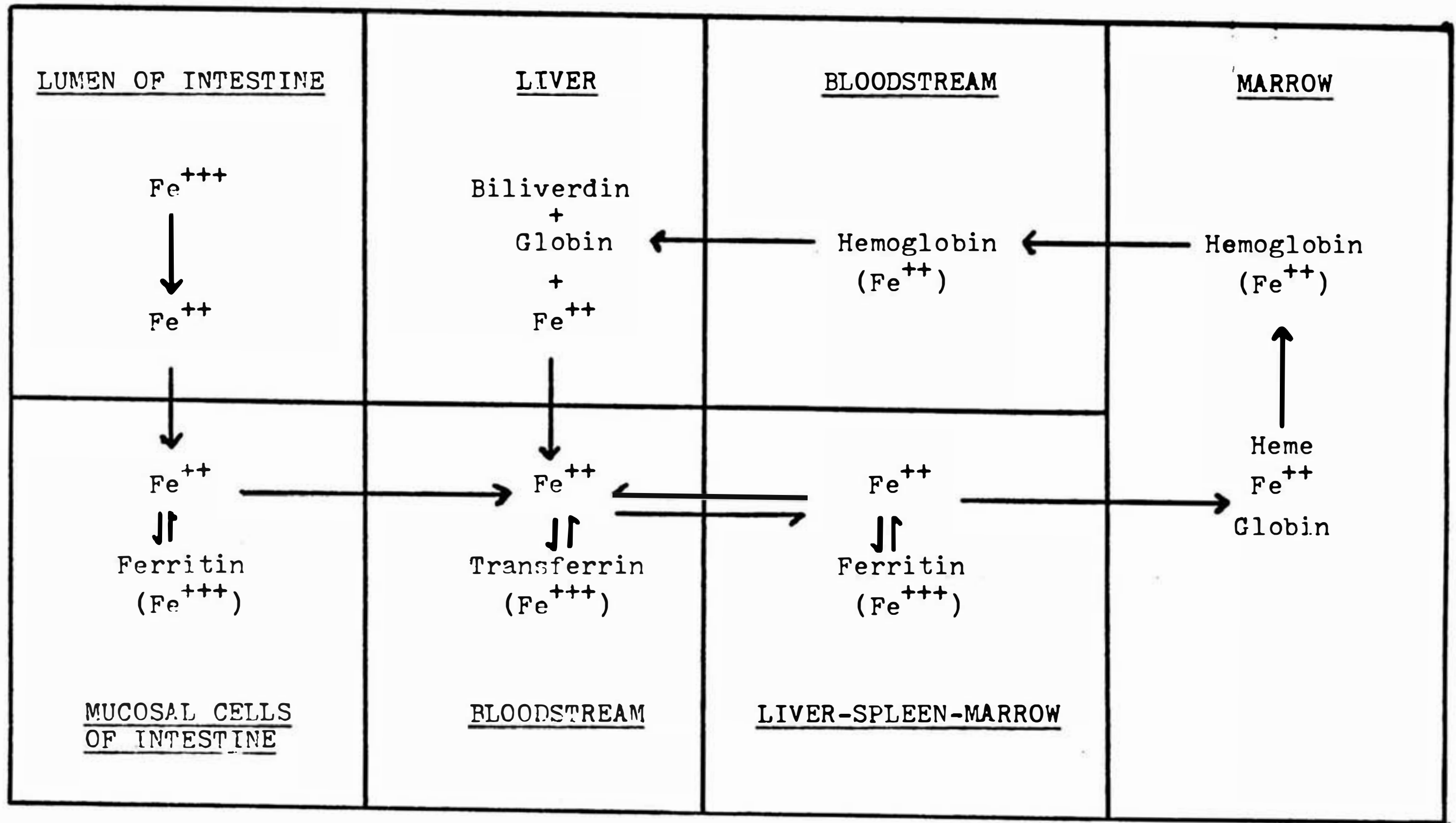

Figure 7. Scheme Showing the Transport and Storage of Iron in Mammalian Systems

From: Orten, J., and Neuhaus, O., Biochemistry (St. Louis: C. V. Mosby Company, 1970) p. 422.

Finch, 1962). Once within the red blood cell one of two alternate pathways is followed: hemoglobin formation or ferritin formation. Ferritin acts as an iron store within the cell that may contribute to hemoglobin synthesis or be excreted when the cell matures (Orten and Neuhaus, 1970).

Iron Excretion

There is no excretory pathway for excess iron, thus the adjustment of iron absorption to need is essential to the organism. No other nutrient is known to be regulated in this manner. The failure of the kidney to excrete iron results from the fact that all the plasma iron is bound to the transferrin, which is not filterable by the kidney (Bothwell and Finch, 1962).

Validity of Technique

Iron absorption and distribution were studied in this investigation. Since $^{59}Fe^{++}$ emits strong gamma radiation it is ideally suited for external counting and represents the best method for estimating iron absorption. This technique has been used extensively in human and animal investigations and its validity has been substantiated by numerous researchers. The method was validated in one of the earlier investigations by Krantz *et al*. (1959) by establishing a linear relationship between various levels of oral $^{59}Fe^{++}$ doses given to mice and the counts observed on a whole body counter. Once the iron had been absorbed by the animal the distribution was monitored

easily by sacrificing the animal and counting various tissue and blood samples.

EXPERIMENTAL

Introduction

The general objective of this investigation was to evaluate the difference in the absorption and distribution of $^{59}Fe^{++}$ between gerbils consuming a diet containing 10 ppm aflatoxin B_1 as compared to gerbils consuming a control diet. A brief summary of the investigation will be developed below and followed by a more detailed explanation of the various procedures used.

An experimental group consisting of 12 male gerbils fed a diet containing 10 ppm aflatoxin B_1 and a control group consisting of 6 male gerbils fed an identical diet which did not contain aflatoxin B_1 were established. Water was provided *ad libitum* from a drinking bottle. At this time the aflatoxin and control group animals had mean weights of 31.0 g with a standard deviation of 1.1 g and 30.9 g with a standard deviation of 1.7 g, respectively. The gerbils of both groups were fed their respective diet for a 75-day period. During this period weight and urinalysis were determined biweekly. The physical activity of three aflatoxin and two control gerbils was also monitored during this period. Due to the expense of the physical activity monitoring equipment only five units could be constructed.

On the sixty-eighth day of the investigation all the gerbils were anesthetized with anesthetic grade ethyl ether and intubated with 0.10 cc of $^{59}Fe^{++}$ in the form of a ferrous citrate solution. An initial whole body count was conducted two hours after intubation. Whole body counts were also conducted at intervals of 70, 90, 110, 130, and 160 hours after the initial whole body count.

On the sixty-eighth day, 160 hours after intubation, the final whole body counts were made. Percent absorption was calculated, and the animals were sacrificed. Liver, kidney, spleen, and muscle tissue samples were counted and blood samples were also counted. Iron concentrations were determined in the blood and tissue samples.

General And Aflatoxin Related Safety Precautions

All equipment and materials on the laboratory worktable were considered potentially contaminated; therefore, disposable examination gloves and a laboratory coat were worn while working. All laboratory records were kept on a pad beside the worktable with a designated pen. After laboratory work was completed, gloves and laboratory coat were removed and hands were thoroughly washed outside the laboratory.

The laboratory door had a biohazard sign posted and was kept locked to reduce the number of unauthorized people entering. Smaller biohazard stickers were also placed on the individual cages and on equipment potentially contaminated with aflatoxin B_1.

At the termination of the investigation all equipment was decontaminated with Clorox which is a five percent sodium hypochlorite solution. Aflatoxin B_1 is oxidized by this solution.

Diet Cake Preparation

Preparation of Aflatoxin B_1 Dietary Stock

Aflatoxin B_1 dietary stock having a concentration of 250 ppm was prepared under a ventilation hood. The first step taken after obtaining five vials containing 10 mg of pure crystalline aflatoxin B_1, grade B, dried in situ was to dissolve the contents of each vial by injecting 5 ml of certified spectranalyzed chloroform through the porous rubber tops of the vials. The chloroform was injected with a ground glass hypodermic syringe and needle and the solution was shaken vigorously for five minutes to insure complete solubilization. It is important to use a glass syringe because chloroform may react and dissolve a plastic syringe. The top was removed from the vial and the 5 ml mixture of aflatoxin B_1 in chloroform was poured into a 400 ml beaker. A total of five 10 mg vials were extracted in this manner yielding a 25 ml solution of chloroform in a beaker with 50 mg of aflatoxin B_1 dissolved in it. The 25 ml solution was diluted to 125 ml with 100 ml of chloroform of the same grade and stirred to insure proper mixing. The 125 ml solution of chloroform containing 50 mg of aflatoxin B_1 was poured from the beaker and mixed thoroughly with 200 g of Purine Laboratory Chow for Mice, Rats and

Hamsters in meal form which had been previously weighed and placed in a 20x20x5 cm aluminum pan. This type of Purina meal was used in all the preparations of this investigation. The pan containing the mixture was left under the exhaust hood in complete darkness for a 48-hour period to allow complete evaporation of the chloroform. This procedure left the 50 mg of aflatoxin B_1 equally dispersed in the 200 g of Purina meal yielding the 250 ppm aflatoxin B_1 dietary stock. It has been determined from previous research that the chloroform used in the preparation of aflatoxin dietary stock is not detectable after the evaporation procedure.

The dietary stock was stored in an air tight jar which was contained in an enclosed white, light proof, cardboard cylinder. The cylinder prevented chemical degeneration of the aflatoxin B_1 by preventing its exposure to visible light and especially the ultraviolet spectrum. The jar and the cylinder were labeled with biohazard stickers and stored in a designated cabinet in the research laboratory.

Preparation of the 10 ppm Aflatoxin B_1 Diet Cupcakes

The 10 ppm aflatoxin B_1 diet was prepared in a Kenmore electric mixer unit using the proportions listed in Table 1. Figure 8 illustrates the materials and equipment used.

The concentration of aflatoxin B_1 was based on the weight of Purina meal excluding the agar and water constituents of the diet. The aflatoxin B_1 dietary stock had a concentration of 250 ppm aflatoxin B_1, which is equivalent to 250 ug aflatoxin B_1 per g of Purina meal. In order to yield a 10 ppm

Figure 8. Materials and Equipment Used in Diet Cupcake Preparation

Table 1. Ingredients and Proportions of the 10 ppm Aflatoxin B_1 Diet

Material	Weight (g)
Agar	40.000
Aflatoxin B_1	0.004
Purina meal	400.000
Water	950.000

Table 2. Ingredients and Proportions of the Control Diet

Material	Weight (g)
Agar	40.000
Purina meal	400.000
Water	950.000

aflatoxin B_1 concentration in the selected 400 g of Purina meal it was necessary to add 16 g of the aflatoxin B_1 dietary stock to 384 g of Purina meal. The 16 g of the aflatoxin B_1 dietary stock contained 4,000 ug of aflatoxin B_1 and approximately 16 g of Purina meal. This would yield a mixture with 4,000 ug of aflatoxin B_1 in 400 g of Purina meal, which is equivalent to a 10 ppm aflatoxin B_1 concentration.

The initial step of the preparation was to weigh 40 g of agar and place it in the mixer bowl. Then 950 g of boiling tap water was poured into the mixing bowl and agitated thoroughly with the electric mixer unit until all the agar was dissolved. Finally 16 g of the aflatoxin B_1 dietary stock followed by 384 g of Purina meal were added and mixed thoroughly for approximately five minutes to insure adequate dispersion. With the use of spatulas the diet was placed into 30 cc clear plastic disposable medication cups. The medication cups were then placed on plastic trays and the trays were set in the freezer which remained at a constant temperature of $-10^{o}C$ to harden into cupcakes. An "X" was marked on the top of each of these cupcakes prior to freezing for identification purposes.

Confirmatory tests of the aflatoxin B_1 levels in these cupcakes were conducted at the Virginia Department of Agriculture and Commerce Mycotoxin Laboratory, Richmond, Virginia, on April 15, 1973. A concentration of 10.15 ppm was established verifying the accuracy of the cupcake concentration. A technique developed by Reynolds (1970) was used. The procedure

utilized several thin layer chromatographic systems and a visual dilution technique which was sensitive to less than 2 ppb.

Preparation of Control Diet Cupcakes

The control diet cupcakes for the control gerbils were prepared using the same methods and equipment utilized in the preparation of the aflatoxin B_1 diet cupcakes. However, the aflatoxin B_1 dietary stock was omitted and 400 g of Purina meal was used. The control diet proportions are listed in Table 2.

The tops of these cupcakes were not marked prior to freezing at $-10^{\circ}C$ in order to distinguish them from the 10 ppm aflatoxin B_1 cupcakes. The control diet was also tested using the same method as the aflatoxin B_1 diet at the Virginia Department of Agriculture and Commerce Mycotoxin Laboratory to determine if aflatoxin B_1 was detectable. The test was negative.

Feeding Procedure

The original proposal was to allot all the gerbils the same quantity of diet. The system implemented for this procedure consisted of providing each gerbil a new dietary cupcake at the beginning of the investigation and continuing to provide each gerbil a cake approximately every two days. Most of the gerbils would eat their cakes on schedule and none of the gerbils who consumed their diet cake earlier than the 2-day feeding period ever had to go without diet for more

than 12 hours. However, some of the gerbils failed to eat all of their dietary cake in the 2-day feeding period making it necessary to discard portions of their diet periodically. Careful measurement of the discarded diet of those gerbils was recorded so that the mean consumption could be calculated. It was determined that food wastage by the animals did not affect the quantity of diet consumed.

Gerbils

Environment

The gerbils were purchased from Tumblebrook Farm and were housed individually in disposable 20x50 cm polypropylene cages covered by matching indented galvanized lids which had space for the dietary cake and a water bottle. Disposable "Bed-o'cobs" and "Pel-i-cel" bedding were used in the cages. All cages were changed at least once a week during the investigation.

After the gerbils had arrived from the supplier and been placed in their respective cages they were allowed to acclimate for a period of seven days. The gerbils were maintained at a room temperature of 27 ± 2°C during the investigation.

Weight and Urinalysis

The initial weight of the gerbils was determined at the beginning of the investigation and the gerbils were weighed two times a week during the investigation. Frequently the gerbils would urinate in the balance cage while being weighed. This enabled a urinalysis to be conducted at that time using a

Bili-Labstix Number 2814 test strip. The urinalysis yielded values for six parameters. These included determination of the pH and the protein level and also if glucose, ketones, bilirubin or blood were detectable in the urine. The urinalysis measurements were conducted to evaluate the general health of the animals.

Physical Activity Monitoring

The physical activity based on movement in the cage of five of the gerbils, three aflatoxin and two control gerbils, was monitored daily to determine if a difference in physical activity between animals influences weight gain. The system employed a Calrad 95-866 photoelectric relay system and an IT&T Number CE40BN52 digital counter.

The system was constructed by bolting the exciter lamp and the receiver unit of the photoelectric relay system to opposite ends of two pieces of 18-inch angle iron. Next the counter was attached and wired to the receiver unit. The complete system was then placed on top and in the center of the gerbil cage. A wiring schematic of the system as positioned on the cage is illustrated in Figure 9.

Every time the gerbil would cross the center of the cage and break the infrared light beam of the photoelectric relay system, a count would be registered. If the gerbil remained in the path of the beam the counter would register only 1 count.

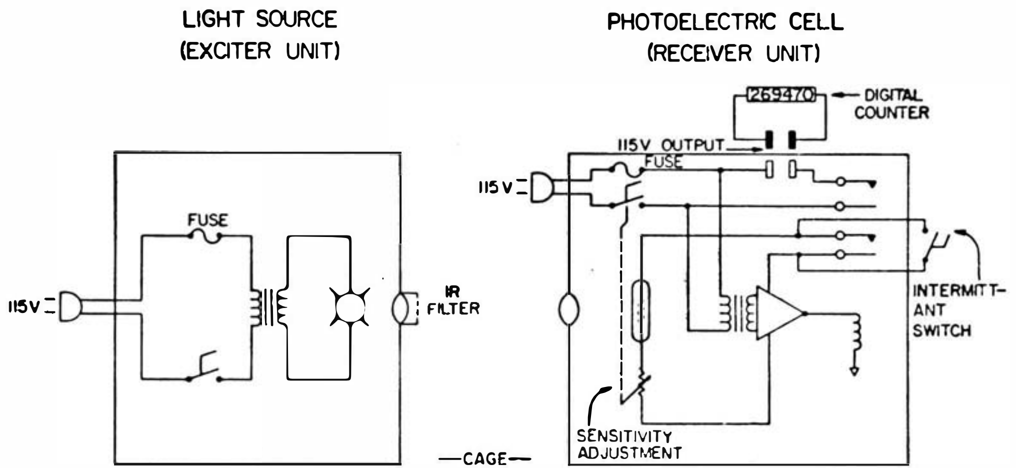

Figure 9. Wiring Schematic of the Physical Activity Monitoring System on the Cage Showing the Calrad 95-866 Photoelectric Relay System and the IT&T Number CE40BN52 Digital Counter

Radioisotope Procedures

Radioisotope Safety Precautions

Gloves and laboratory coat were worn when intubating and conducting counting procedures. A radiation area warning sign was posted on the laboratory door and radiation stickers were placed on each cage and on all equipment contaminated with the radioisotope.

The gerbils were intubated on a tray and absorbent paper towels were placed under them to absorb a radioactive spill or drip if it occurred. When the gerbils were sacrificed, work was also done on absorbent paper towels on a tray. After all these procedures using the radioisotope had been completed, the towels were disposed of in the solid radioactive waste container. The paper towels, syringes, disposable bedding and the disposable animal cages were collected in the solid waste container and disposed of via the Office of Radiological Health located at the Health Sciences Division of Virginia Commonwealth University.

A cylindrical lead shield surrounded the gerbils during whole body counting. The lead shield counting well of the Nuclear Chicago 412 Detector surrounded tissue and blood samples during planchet counting to shield the detector crystal from background radiation. These lead shields would also act to decrease human exposure from the samples. A square 20x20x1 cm lead shield was also placed in front of samples being counted and in front of the animals during intubation to

decrease human exposure.

Radioisotope Specifications

The radioisotope, $^{59}Fe^{++}$, in the chemical form of ferrous citrate was supplied by the Isotope and Nuclear Division of International Chemical and Nuclear. The isotope met the specifications listed in Table 3.

$^{59}Fe^{++}$ Intubation Technique

On the sixty-eighth day of the investigation, all 18 gerbils of the investigation and 2 additional controls of the same approximate weight were intubated with 0.10 cc of $^{59}Fe^{++}$. It was necessary to use two extra control animals, numbers 19 and 20, because a portion of the intubation dose went into the lungs of animals number 2 and number 4. The intubation dose contained 21.15 uCi. The two extra control animals were used exclusively for the iron determinations of the investigation while using gerbil two and gerbil four for all other parameters. The 12 experimental gerbils were also intubated with 0.10 cc of $^{59}Fe^{++}$. A Tomac 1 cc disposable plastic syringe with a Popper & Sons mouse intubation needle was used for all intubations.

Immediately before the gerbils were intubated, they were anesthetized in an ether chamber located on a bench in front of our ventilation fan. Anesthetization was done by dropping anesthetic grade ethyl ether with a dropper onto absorbent towels in the bottom of the chamber. The ether was directed

Table 3. $^{59}Fe^{++}$ Specifications as Established by the Supplier, International Chemical & Nuclear Corporation

Chemical form	Ferrous citrate in 0.85% NaCl
Concentration	225 uCi/ml
Specific activity	25.2 Ci/g
Radionuclidic purity	99%
Half-life	45 days
Emission	Beta and gamma radiation

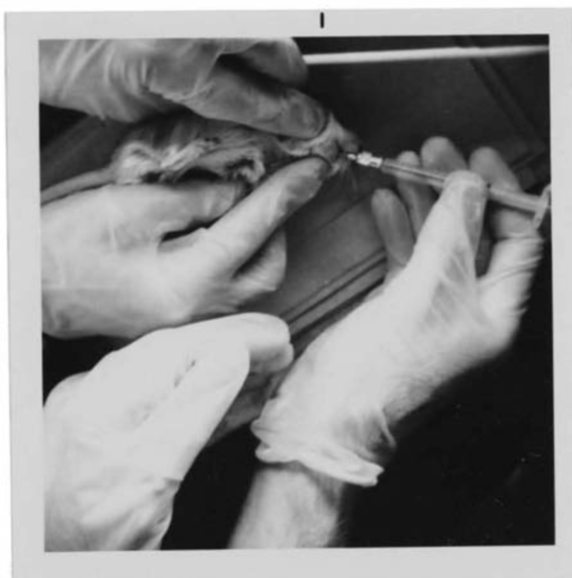

Figure 10. Intubation Technique Consisting of Pushing the Intubation Needle into the Esophagus of the Animal and Injecting the 0.10 cc of the Ferrous Citrate Solution

through a 1/2 inch hardware cloth platform which had been placed in the chamber. The gerbil was placed on top of the wire platform until he became recumbent. He was then taken out of the chamber and placed on a tray for intubation.

The intubation needle on the syringe was pushed through the esophagus of the gerbil and into his stomach where the 0.10 cc of the isotope was injected. This procedure is illustrated in Figure 10. After the intubations had been completed, the gerbil was placed in the bottom of a polypropylene cage which was covered with soft paper towels to prevent self-injury and allowed to recover. When the gerbil had recovered from the anesthesia he was placed in his proper cage. Two hours were allowed for the gerbil to recover from anesthesia following the last intubation before conducting the initial whole body count. This time delay was necessary because of the relative sensitivity of gerbils to ethyl ether.

Counter and Detector

A Nuclear Chicago 151A Counter and a Nuclear Chicago 412 Scintillation Detector with a sodium iodide (thallium activated) scintillation crystal which was 1-inch in diameter by 1-inch thick and counting well were used for detecting the isotope. The proper operating voltage of the counter was determined by placing a planchet sample containing $^{59}Fe^{++}$ into the well and taking counts between 0 and 1,800 volts. The counts for these voltages were plotted and a plateau was established. The proper operating voltage of 1200 volts was determined from the

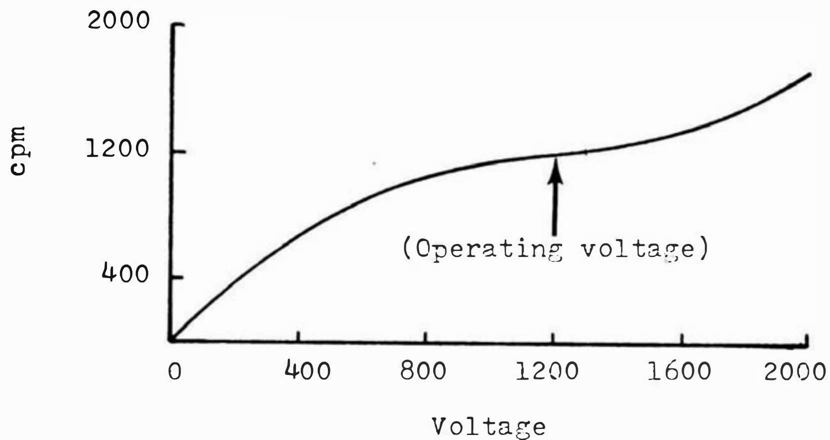

Figure 11. Plateau and Operating Voltage Determination Graph for the Nuclear Chicago Counter (151A)

plateau of the graph illustrated in Figure 11. After the proper operating voltage of 1200 volts had been determined the efficiency of the instrument was calculated by placing a sample containing 0.4572 uCi of $^{59}Fe^{++}$ in a planchet, counting, and utilizing the following formula:

$$\text{Percent efficiency} = \frac{\text{cpm}}{\text{dpm}} \times 100$$

An efficiency of 19.6 percent was established.

Whole Body Counting

A counting chamber for conducting whole body counts was devised. It consisted of a large lead cylinder having an interior diameter of 10 cm and an exterior diameter of 11 cm and 1 cm thick with a metal plate placed on top. The Nuclear Chicago 412 Scintillation Detector was placed on top of the plate with its scintillation crystal extending through a hole in the metal plate. Each gerbil to be counted was placed inside a perforated wax paper cup and placed inside the lead cylinder.

The first whole body counts were conducted for five minutes on each gerbil approximately two hours after intubation and these values were used to establish a baseline for iron absorption calculations. The cpm for each animal was considered as 100 percent of the original amount intubated. Each animal was counted for 5 minutes in the chamber at this time and also at 70, 90, 110, 130, and 160 hours after the intubation. The percent absorption of each animal was

calculated by comparing the corrected original cpm with the corrected cpm at 160 hours. The cpm were corrected for background radiation and radioactive decay in both cases.

Blood Counting Technique

After the gerbils' whole body count had been completed on the seventy-fifth day of the investigation, they were all sacrificed with chloroform. The same chamber which had been used to anesthetize them was used for this procedure. A mid-line incision was made on the ventral side of the gerbil's body. A Tomac 1 cc disposable syringe with a 27 gauge needle was pushed into his posterior vena cava while exposing the vessel with a blunt probe and approximately 0.30 cc of blood was drawn up into the syringe. The blood was injected into a planchet and weighed on an analytical balance. The planchet sample was then placed in the counting well and counted for a five-minute period. The observed cpm was corrected for background, decay, and the efficiency of the instrument. After corrections had been made for these parameters the data was calculated as ng $^{59}Fe^{++}$/g of blood for each gerbil and tabulated. Mean values for the control and the aflatoxin groups were calculated at this time.

Tissue Counting Technique

The first step was to remove the whole liver from the animal. A portion of the whole liver was placed in a planchet, weighed, and counted. The liver tissue sample and all the

other tissue planchet samples were counted in exactly the same manner as the blood samples had been counted. The two kidneys were then removed and one was placed in the center of a planchet, weighed and counted. The spleen, lungs, and the gastrocnemius muscle from the right leg were also removed, weighed, and counted. The leg muscle tissue sample was prepared by removing the gastrocnemius muscle from the right leg and placing it in the center of a planchet. The remainder of the liver was placed in a jar containing formalin solution and taken to the Virginia Department of Agriculture and Commerce Mycotoxin Laboratory for histopathological evaluation. The observed cpm of all the tissue samples were corrected for background, decay and the efficiency of the instrument. After corrections had been made for these parameters the data was calculated as ng $^{59}Fe^{++}$/g of tissue for each gerbil and tabulated. Mean values and standard deviations for the control and experimental groups were calculated at this time. A table showing the percent distribution of the $^{59}Fe^{++}$ in the blood, liver, kidney, and spleen of all animals was also calculated at this time. The mean of the two groups and the standard deviation of these means were included.

RESULTS AND DISCUSSION

Statistical Evaluation of Data

The t-test was used to determine if the difference in the means of all the various parameters evaluated between the control group and the experimental group fed aflatoxin B_1 diet were significant. A five percent level of significance was selected to determine if the differences evaluated were significant. The t_c values for the t-test were obtained from Spence *et al.* (1961). Standard error of the control group mean, S_1; standard error of the aflatoxin group mean, S_2; standard error of the difference of the means, S; and the standard deviation, SD were calculated in the statistical evaluation of the data. The formulas used for calculating these values were obtained from Steel and Torrie (1960).

The probable percent error of the total counts of the radioisotope in the various counting procedures in the investigation were determined from Table 4.5 from Chase and Rabanowitz (1970). All the total counts measured in this investigation had a probable percent error below ten percent at the two-sigma level of confidence. This was considered an acceptable level of error.

Evaluation of Weight Gain

The mean weight gain of the control and aflatoxin animal groups were determined twice a week, biweekly. The mean weight gain values are given in Table 4 and graphed in Figure 12. A difference in the mean control group and mean aflatoxin group weight gain was evident on the first biweekly weighing. The difference between the mean biweekly weight gain of control and aflatoxin animal groups became progressively larger as the investigation proceeded. On the seventy-fifth day, when the investigation was terminated, the mean weight gain of the control group animals was 36.9 g with a SD of 6.13 g as compared to 29.8 g with a SD of 2.97 g for the aflatoxin group. The mean weight gain of the aflatoxin group was 7.1 g less than the control group which is a difference of 19.24 percent. A reduction in weight gain in juvenile gerbils when fed a diet containing 10 ppm aflatoxin B_1 is suggested since the t-test at the five percent level confirmed a significant difference between these two means. The statistical analysis of this data is presented in Table 5.

A reduction in weight gain has been observed in numerous investigations where aflatoxin B_1 was fed chronically to juvenile animals. Syrian hamsters chronically fed aflatoxin B_1 at a concentration of 1 ppm (Llewellyn _et al._, 1970) gained less weight than control animals over a 250-day feeding period. In an unpublished investigation by Llewellyn _et al._ (1973) a reduction in weight gain was observed when juvenile Syrian hamsters consumed a diet containing 10 ppm aflatoxin B_1 over

Table 4. Mean Biweekly Weight Gains for the Control Group Animals and the Aflatoxin Group Animals

Day	Control group weight gain	SD	Aflatoxin group weight gain	SD
0	0.0	0.69	0.0	1.06
3	4.6	1.14	3.9	1.23
8	7.2	2.18	5.2	1.85
11	11.1	2.25	9.3	1.83
15	10.6	3.25	10.4	2.64
18	17.7	3.50	15.5	2.26
22	17.7	4.12	17.9	1.97
26	21.7	3.41	19.9	2.35
29	23.2	3.75	20.9	2.62
32	25.9	2.26	22.6	2.91
36	27.2	4.76	24.7	3.27
39	27.5	3.55	24.3	3.53
43	26.1	3.47	24.5	3.72
47	30.5	4.84	27.4	3.41
51	31.0	5.47	28.0	3.71
54	34.1	5.32	29.6	4.23
58	34.2	5.87	30.5	3.90
61	36.4	6.30	32.2	3.54
65	37.9	7.67	33.4	3.79
68	36.6	6.61	32.0	3.02
72	37.7	6.50	32.0	3.08
75	36.9	6.13	29.8	2.97

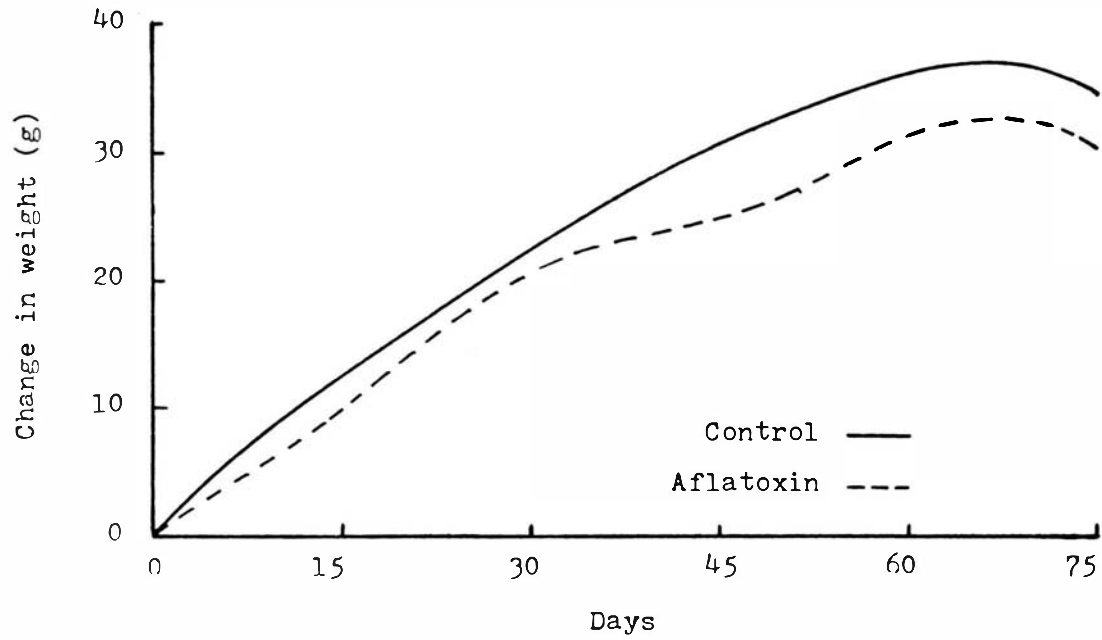

Figure 12. Mean Biweekly Weight Change for the Control and Aflatoxin Groups

Table 5. Analysis for Difference Between Weight Gain in the Control Group and the Aflatoxin B_1 Group at the Termination of the investigation

	Change in weight of control group		Change in weight of aflatoxin B_1 group
	30.1		30.0
	46.6		30.5
	39.4		31.3
	36.6		28.4
	30.7		26.3
	38.6		29.2
			27.8
			29.3
			34.6
			36.2
			27.6
			27.2
Sum	222.0		358.4
n	6		12
Mean	36.9		29.8
S		2.701	
t		2.970	
t_c		2.571	

The aflatoxin B_1 group weight gain was significantly different from the control group at the 5 percent leval.

a 50-day feeding period. In an investigation conducted by Asplin and Carnaghan (1961) where chickens were fed a diet containing aflatoxin B_1, the chickens failed to gain as much weight as control group chickens. When pigs, *Sus scrofa*, were fed a diet containing 0.81 ppm aflatoxin B_1 a reduction in weight gain occurred (Keyl *et al.*, 1968). In another investigation with pigs (Armbrecht, 1971) a reduction in weight gain as a result of consumption of a diet containing between 1 and 4 ppm aflatoxin B_1 and G_1 over a 16-week period occurred. Numerous other investigations have also reported depressed weight gain in pigs as a result of consumption of aflatoxin concentrations between 0.28 and 0.80 ppm (Armbrecht, 1971). Keyl *et al.* (1968) also observed a significant decrease in weight gain in cattle, *Bos taurus*, fed aflatoxin B_1 at 0.7 and 1 ppm. In an unpublished experiment conducted by Llewellyn and Novak (1969) where adult male gerbils were fed 1 ppm aflatoxin B_1 for 300 days no weight changes took place.

Physical Activity Monitoring

The physical activity counts registered on the digital counter of two of the control gerbils and three of the aflatoxin animals and the statistical analysis of this data are presented in Table 6. The mean count of the two control animals was determined to be 44,301 and the mean count of the three animals of the aflatoxin group was determined to be 40,818. A difference of 7.8 percent was observed between the means of the two control gerbils and the three aflatoxin

Table 6. An Analysis for Difference Between Physical Activity Monitoring Results from the Control and Aflatoxin B_1 Groups

	Control animal counts		Aflatoxin B_1 animal counts
	34,446		55,378
	54,157		27,659
			39,414
Sum	91,603		122,451
n	2		3
Mean	44,301		40,818
S		12,714	
t		0.273	
t_c		12.706	

The aflatoxin B_1 group was not found to be significantly different from the control group at the 5 percent level.

gerbils, but the difference was not significant at the five percent level. This data indicated that no inference based on the difference in the physical activity of these five gerbils could be made concerning the mean weight gain difference between the control and aflatoxin groups.

Diet Consumption

Sixty-six percent of the control and 58 percent of the aflatoxin animals consumed exactly 1063 g of diet. Two of the 6 control animals and 5 of the 12 aflatoxin animals consumed less than this quantity. The quantity of diet consumed by each of the control animals is depicted in Table 7 and the quantity consumed by each of the aflatoxin animals is depicted in Table 8. A mean total diet consumption of 1048 g was determined for the control group and 1044 g for the aflatoxin group during the 75-day investigation. The slight difference indicates that the total quantity of diet consumed apparently did not influence the difference in weight gain between the control group and the aflatoxin group.

Feed Efficiency

The mean feed efficiency, mean weight gain (g)/feed weight consumed (g), was calculated for the control group and the aflatoxin group. A value of 0.035 was calculated for the control group and 0.028 for the aflatoxin group. This is a difference of 20 percent and was found to be significant. A significant reduction in feed efficiency was observed by Keyl

Table 7. Diet Consumption of the Six Control Animals

Animal number	Diet consumption (g)
1	1016
2	1024
3	1063
4	1063
5	1063
6	1063

Mean diet consumption = 1048 g
SD = 22 g

Table 8. Diet and Aflatoxin B_1 Consumption of the 12 Animals Fed Diet Containing Aflatoxin B_1 at 10 ppm

Animal number	Diet consumption (g)	Aflatoxin B_1 consumption (mg)
7	1008	10.08
8	1032	10.32
9	985	9.85
10	1063	10.63
11	1063	10.24
12	1024	10.44
13	1044	10.63
14	1063	10.63
15	1063	10.63
16	1063	10.63
17	1063	10.63
18	1063	10.63

Mean diet consumption = 1049 g SD = 24 g
Mean aflatoxin B_1 consumption = 10.49 mg SD = 0.24

et al. (1968) when cattle were fed a diet containing aflatoxin B_1 at concentrations of 0.7 and 1 ppm. In the swine investigation by Armbrecht *et al.* (1971) feed efficiency was also reduced with the consumption of aflatoxin at concentrations ranging from 0.2 to 2.0 ppm.

Aflatoxin B_1 Consumption

The quantity of aflatoxin B_1 consumed by each gerbil in the aflatoxin group was calculated from the aflatoxin diet consumption values and is depicted in Table 8. The total mean aflatoxin B_1 consumption was determined to be 10.49 mg with a standard deviation of 0.24 mg. A mean daily consumption value and a value indicating the mean mg of aflatoxin B_1 consumed/kg of body weight were calculated. The values were determined to be 139.86 ug/day/animal and 0.170 mg aflatoxin B_1/kg of body weight. A 50 g wet weight fecal sample was collected from the aflatoxin group animals and tested at the Virginia Department of Agriculture and Commerce Mycotoxin Laboratory, Richmond, Virginia, on May 1, 1973. A concentration of 0.164 ppm aflatoxin B_1 in the fecal sample was determined indicating that over 99 percent of the aflatoxin B_1 consumed by these animals was either absorbed or decomposed as it passed through the gastrointestinal tract. The method of Reynolds (1970) was used in this determination.

Pathological Evaluation

The liver tissue of all the animals was evaluated for pathological injury on May 15, 1973, at the Virginia Department of Agriculture and Commerce Mycotoxin Laboratory by F.W. Rea (D.V.M.). Liver tissue samples of five of the six control animals appeared normal with only slight hemorrhage present in two of them. However, one of the controls, number five had a small foci containing necrotic cells.

The livers of two-thirds of the 12 aflatoxin animals appeared normal; one-third of these animals had pathological injury present. Gerbil number 18 had foci present containing necrotic cells. Gerbil number nine also had foci present containing necrotic cells with cellular infiltration of the interlobular vein. Gerbils number 13 and 16 developed toxic hepatitis which is the expected toxic response from aflatoxin B_1 consumption. Their livers also had widespread areas of hemorrhage and large numbers of liver cells were replaced by red blood cells.

Iron Absorption

The whole body cpm of the control and aflatoxin group animals conducted at 0, 70, 90, 110, 130, and 160 hours after intubation are presented in Tables 9 and 10 and graphed in Figure 13. The mean whole body counts of both control and aflatoxin animals progressively decreased after the initial count and had completely leveled off after 130 hours. It was determined after 160 hours that all the unabsorbed radioactive

Table 9. The Initial cpm and the cpm at 70, 90, 110, 130, and 160 Hours of the 6 Animals of the Control Group

Animal Number	Initial cpm	cpm at 70 hours	cpm at 90 hours	cpm at 110 hours	cpm at 130 hours	cpm at 160 hours
1	10,403	760	442	508	408	406
3	13,381	398	240	210	232	141
5	11,183	2049	1723	1574	2514	1912
6	9,604	194	130	116	141	84
19	12,032	2036	2328	1993	1542	1023
20	10,909	410	309	301	275	253
Mean	11,252	974	862	783	852	636
SD	1,319	847	926	796	965	710

Table 10. The Initial cpm and the cpm at 70, 90, 110, 130, and 160 Hours for the 12 Animals Fed a Diet Containing 10 ppm Aflatoxin B_1

Animal Number	Initial cpm	cpm at 70 hours	cpm at 90 hours	cpm at 110 hours	cpm at 130 hours	cpm at 160 hours
7	6,134	185	168	143	185	142
8	11,874	704	415	413	431	331
9	12,100	391	171	251	190	151
10	11,909	229	183	196	208	166
11	11,479	149	108	113	112	84
12	8,777	373	314	358	297	260
13	11,789	2679	2762	3252	3531	2861
14	9,522	1120	928	834	945	775
15	10,495	635	609	642	678	541
16	9,921	133	131	158	152	115
17	8,552	1527	1573	1891	2349	1442
18	10,023	1358	934	1074	964	709
Mean	10,214	790	691	777	836	631
SD	1,794	764	789	935	1056	805

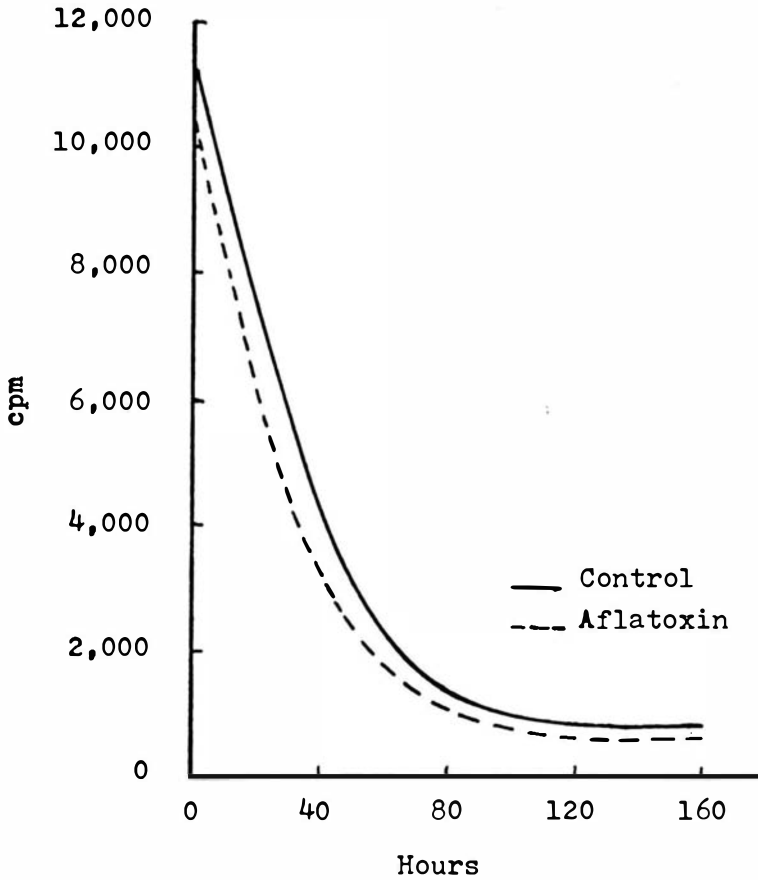

Figure 13. Mean Whole Body cpm from $^{59}Fe^{++}$ at 0, 70, 90, 110, 130, and 160 Hours after Intubation for the Control and Aflatoxin Groups

iron intubated must have passed through the gastrointestinal tract. Therefore, the radioactive iron present in the animal at this point was considered absorbed. This is in agreement with studies conducted using mice where a period of 144 hours was allowed after intubation to determine percent absorption by whole body counting (Krantz *et al.*, 1959).

The cpm at 160 hours were corrected for radioactive decay and background radiation. The corrected cpm at 160 hours of each animal were compared with its initial count to calculate percent absorption using the formula given below:

$$\text{Percent absorption} = \frac{\text{cpm at 160 hours}}{\text{Initial cpm}} \times 100$$

The percent iron absorption of the control and aflatoxin animals are presented in Tables 11 and 12. The mean control percent absorption was determined to be 6.25 percent with a SD of 6.98 and the mean aflatoxin group percent absorption was determined to be 6.89 percent with a SD of 8.11. This is in agreement with the 7.5 percent absorption value observed in mice (Krantz *et al.*, 1959).

If the number five control animal which had a small foci with necrotic cells present on its liver is excluded from the control group iron absorption analysis a mean control percent absorption of 3.70 can be calculated. This value would indicate increased absorption in the aflatoxin group which is in agreement with the general trend of increased iron absorption when liver injury is present. However, with the small number

Table 11. The Mean and Individual Percent Absorption Values for the Control Animals

Animal number	Corrected cpm at 160 hours	Percent absorption
1	451	4.34
3	157	1.17
5	2128	19.02
6	93	0.96
19	1139	9.46
20	282	2.58
Mean	710	6.25
SD	790	6.98

Table 12. The Mean and Individual Percent Absorption Values for the Aflatoxin Animals

Animal number	Corrected cpm at 160 hours	Percent absorption
7	158	2.57
8	368	3.09
9	168	1.38
10	185	1.55
11	93	0.81
12	289	3.29
13	3184	27.00
14	862	9.05
15	602	6.06
16	128	1.29
17	1605	18.76
18	789	7.87
Mean	702	6.89
SD	897	8.11

of samples involved in this investigation the elimination of one of the control animals was not considered justifiable.

Iron Distribution

The blood, liver, kidney, and spleen counts due to $^{59}Fe^{++}$ were corrected for background radiation, radioactive decay, and the efficiency of the instrument. Then the concentration of iron in ng $^{59}Fe^{++}$/g of each of these samples for every animal in the control group and aflatoxin group was calculated. The means and standard deviations of these concentrations for both groups were calculated and are presented along with the individual values in Tables 13 and 14.

The sum of the iron concentration in blood as extrapolated to the entire blood volume of the animals, in the whole liver, in both kidneys, and in the spleen of each animal was calculated. Then the percent which each of these constituents composed of the total iron was calculated and this data presented in Tables 15 and 16. These four constituents were chosen because the majority of iron in the body is present in them. In addition it was determined that a more accurate comparison between animals could be made with the percentage values.

There was a higher but not significantly different value of 89.83 percent of the $^{59}Fe^{++}$ present in the blood of the control group versus 88.80 percent in the blood of the aflatoxin group. The large proportion of iron present in the blood of both groups resulted from its incorporation into

Table 13. Mean and Individual Iron Concentration Values of the Blood, Liver, Kidneys, and Spleen for Control Animals

Animal Number	Total Blood Fe^{++} (ng)	ng Fe^{++}/Whole Liver	ng Fe^{++}/Whole Kidney	ng Fe^{++}/Whole Spleen
1	3.1655	0.2319	0.0188	0.0053
3	2.0571	0.2545	0.0161	0.0016
5	6.2053	0.5863	0.0578	0.0240
6	1.1506	0.1169	0.0072	0.0009
19	6.2193	0.5422	0.0772	0.0004
20	1.1953	0.1142	0.0079	0.0004
Mean	3.3321	0.3078	0.0308	0.0054
SD	2.3481	0.2073	0.0294	0.0092

Table 14. Mean and Individual Iron Concentration Values of the Blood, Liver, Kidneys, and Spleen for Animals Fed Aflatoxin B_1 at 10 ppm

Animal Number	Total Blood Fe^{++} (ng)	ng Fe^{++}/Whole Liver	ng Fe^{++}/Whole Kidney	ng Fe^{++}/Whole Spleen
7	1.7256	0.4291	0.0163	0.0044
8	2.2852	0.7823	0.0211	0.0053
9	3.4926	0.4761	0.0217	0.0030
10	5.0768	0.2796	0.0270	0.0054
11	1.5270	0.1828	0.0108	0.0000
12	2.0849	0.2103	0.0231	0.0021
13	12.9956	1.1694	0.0988	0.0456
14	10.5127	0.7107	0.0693	0.0126
15	8.9092	0.2766	0.0466	0.0122
16	3.6451	0.1425	0.0135	0.0000
17	6.2662	0.6631	0.0715	0.0095
18	8.0351	0.4839	0.0421	0.0074
Mean	5.5463	0.4005	0.0384	0.0089
SD	3.8090	0.2252	0.0279	0.0122

Table 15. The Mean and Individual Iron Distribution of the Control Animals Based on Percentage

Animal number	Percent in blood	Percent in liver	Percent in kidneys	Percent in spleen
1	92.01	6.74	1.09	0.15
3	87.70	10.85	1.37	0.06
5	89.52	8.45	1.66	0.34
6	89.68	9.11	1.12	0.07
19	89.91	7.83	2.23	0.01
20	90.16	8.61	1.19	0.03
Mean	89.83	8.59	1.46	0.11
SD	1.38	1.37	0.42	0.12

Table 16. The Mean and Individual Iron Distribution of the Aflatoxin Group Animals Based on Percentage

Animal number	Percent in blood	Percent in liver	Percent in kidneys	Percent in spleen
7	78.73	19.57	1.48	0.20
8	73.36	25.11	1.35	0.17
9	86.90	11.85	1.08	0.07
10	93.74	5.16	0.99	0.09
11	88.19	10.55	1.24	0.00
12	88.96	8.97	1.97	0.08
13	90.19	8.11	1.37	0.31
14	92.42	6.24	1.21	0.11
15	95.88	2.97	1.00	0.13
16	95.55	3.73	0.70	0.00
17	88.48	9.36	2.01	0.13
18	93.31	5.61	0.97	0.08
Mean	88.80	9.76	1.28	0.11
SD	6.73	6.56	0.39	0.08

transferrin and hemoglobin. There was also a correspondingly higher mean of 9.76 percent of the $^{59}Fe^{++}$ present in the livers of the aflatoxin animals versus 8.59 percent of the iron in the livers of the control animals. The large proportion of iron present in the liver of both groups results from its incorporation into ferritin and hemosiderin in the liver. There was no significant difference between the control kidney and spleen values versus the corresponding aflatoxin group.

The aflatoxin group iron concentration of muscle tissues was not found to be significantly different from the muscle tissue in the control group. These values were calculated in ng $^{59}Fe^{++}$/g of tissue in exactly the same manner as the other samples tested. Mean values of 0.0048 ng $^{59}Fe^{++}$/g and 0.0051 ng $^{59}Fe^{++}$/g of muscle were observed in the control and aflatoxin groups, respectively.

The lung concentrations were only used to determine if some of the intubation dose had entered the lungs. As stated earlier, animals 2 and 4 were eliminated from iron calculations since a portion of the intubation had entered their lungs and extra controls, 19 and 20, were added.

SUMMARY AND CONCLUSION

The data indicated that a reduction in weight gain and feed efficiency occurs when juvenile male gerbils consume diet containing 10 ppm aflatoxin B_1. These observations are in agreement with investigations conducted using other animals where aflatoxin B_1 was also present in the diet. The reduction in weight gain was substantiated by the determination that neither the physical activity nor the diet consumption difference between the control group and the aflatoxin group significantly affected the reduction in weight gain of the aflatoxin group. Thus, a reduction in the growth rate of the gerbils consuming the 10 ppm aflatoxin B_1 diet occurred. A general positive relationship between the extent of growth rate inhibition and the level of aflatoxin B_1 in the diet has been established by Barber _et al_. (1968). In Barber's investigation numerous studies which were in agreement with this observation were cited.

Aflatoxin B_1 must have altered some biochemical process in the gerbils of the aflatoxin group inducing the reduction in growth rate. The decrease in feed efficiency substantiates this conclusion. It would appear that the biochemical change induced by aflatoxin B_1 was associated with the toxicity response to the compound. It had originally been speculated that a significant reduction in iron absorption or an alteration in

iron distribution associated with the toxicity response to aflatoxin B_1 causes the reduction in growth rate. However, the data indicated that a statistically significant difference between the mean control and mean aflatoxin group iron absorption or distribution was not present.

Table 17 depicts the relationship between the blood and liver iron concentrations and the percent iron absorption of the 12 gerbils in the aflatoxin group. The numbers corresponding to the aflatoxin group animals are presented in the order of the highest to the lowest for each of the three parameters evaluated. An asterisk was placed beside the animals having histopathological liver damage and a line was placed between the upper 50 percent and the lower 50 percent.

Three-fourths of the gerbils with liver damage had blood $^{59}Fe^{++}$ levels in the upper 50 percent of the aflatoxin group indicating a correlation between histopathological liver damage, symptomatic of toxic hepatitis, and highest $^{59}Fe^{++}$ levels in the blood. Animal number 13, the animal with the highest percent absorption, also had toxic hepatitis indicating a possible correlation between liver damage and iron absorption. However, contradictory evidence was also present with animal number 16 having toxic hepatitis and a very low percent iron absorption. Control animal number five which had a small foci containing necrotic cells had the second highest percent iron absorption indicating that there may be a correlation between increased iron absorption and liver damage which is not necessarily symptomatic of aflatoxin-induced toxic hepatitis.

Table 17. The Relationship of the Blood and Liver Concentrations and the Percent Absorption of the 12 Aflatoxin Group Gerbils

Blood	Liver	Percent Absorption
15	8	13*
16*	7	17
10	9*	14
18*	11	18*
14	12	15
13*	17	12
17	13*	8
12	14	7
11	18*	10
9*	10	9*
7	16*	16*
8	15	11

This would be in agreement with the general trend of increased iron absorption when liver injury is present.

Muscle tissue which is composed mainly of protein constitutes a large portion of the weight of an animal. If a reduction in muscle tissue formation occurs then reduction in the growth rate of the animal would be expected. This would be consistent with the ability of aflatoxin B_1 to inhibit protein synthesis (Clifford and Rees, 1967).

The relatively low concentrations of iron present in the muscle tissues of the control and aflatoxin groups probably resulted from the slow incorporation of iron into myoglobin (Theorell et al., 1951). Myoglobin which is an iron containing intracellular protein of muscle cells functions in the storage of oxygen in these cells (Orten and Neuhaus, 1970). Although no significant difference was observed between the control and aflatoxin iron muscle concentrations this could possibly be a factor to consider in future research concerning reduced growth rate.

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REFERENCES

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APPENDIX

LIST OF MATERIALS AND EQUIPMENT

Materials and Equipment	Source
Bed - o'cobs Bedding	Anderson Cob Mills, Inc. Maumee, Ohio
Bili Labstix Number 2814	Ames Company Elkhart, Indiana
Clear Plastic Disposable Medication Cups	Richmond Surgical Supply 1400 North Boulevard Richmond, Va.
Digital Counter Number CE40BN52	International Telephone & Telegraph Company New York, N. Y.
Ground Glass Hypodermic Syringe	Industrial & Scientific Instrument Company Tokyo, Japan
Intubation Needle	Popper & Sons, Inc. New York, N. Y. 10010
Kenmore 10 Speed Electric Mixer	Sears Roebuck & Co. Chicago, Illinois
Laboratory Grade Agar	Fisher Scientific Co. Fairlawn, N. J.
Lead Cylinder 1x12x12 cm	Welch Scientific Co. Skokie, Illinois
Lead Shield 1 cm thick with diameters of 10 and 11 cm	Welch Scientific Co. Skokie, Illinois
Juvenile Male Gerbils	Tumblebrook Farm West Brookfield, Mass.
Nuclear Chicago 151 A Counter with Scintillation Detector 412, with Model 414 Base	Nuclear Chicago Corp. 333 East Howard Ave. Des Plaines, Illinois

Parke-Davis Disposable Examination Gloves	Parke Davis & Company Detroit, Michigan 48232
Pel-i-cel Bedding	Vivarium Research, Inc. White House Station, N. J.
Photo-Electric Relay System Number 95866	Calrad Corporation Tokyo, Japan
Polypropylene Cages with 20x50 cm Galvanized Lids	Maryland Plastics Inc. Scientific Division New York, N. Y.
Purina Laboratory Rat Chow	Ralston Purina Co. Checkerboard Square St. Louis, Missouri
Sweetheart R-9 Cold Drink Cups with Plastic Lids, SL R-9	Sweetheart Cup Division Maryland Cup Corp. Baltimore, Md.
Tomac 1 cc Disposable Tuberculin Syringe	American Hospital Supply Evanston, Illinois 60201

LIST OF REAGENTS

Reagents	Grade	Manufacturer
Chloroform		Fisher Scientific Co. Fairlawn, N. J.
Crystalline Aflatoxin B_1	B	Calbiochem Co. 10933 N. Torrey Pines Road La Jolla, Calif. 92037
Ethyl Ether	Anesthetic	Mallinckrodt Chemical Works St. Louis Mo. 63160
Ferrous Citrate (^{59}Fe)	Radionuclidic Purity 99%	International Chemical & Nuclear Corporation Isotope & Nuclear Division 2727 Campus Drive Irvine, Calif. 92664

VITAE

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