1979

Distribution, production, analysis and effects of aflatoxin in animal tissues and effects of scirpene toxins on chicken embryos

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Distribution, production, analysis and effects of aflatoxin

in animal tissues and effects of scirpene

toxins on chicken embryos

by

Iheanyichukwu Wilfred Obioha

A Dissertation Submitted to the

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INTRODUCTION

Mold growth on food and feeds is a common occurrence; the occasional contamination of human food with biologically active compounds of mold origin (mycotoxin) is a situation that has been with man for many years. Molds are used in the manufacture of a variety of foods in many parts of the world. These include fermented foods, cheeses and certain cured and aged meats.

Mycotoxins are secondary fungal metabolites which cause a variety of acute or chronic toxicological manifestations in man and his domestic animals. Humans can be exposed to mycotoxin by direct consumption of mycotoxin contaminated food or by consumption of residues from milk or meat produced from animals which have ingested mycotoxin contaminated feed.

When one considers the ways in which molds are associated with human foods, it seems odd that the public health aspects involving the growth of these microorganisms on food have not been extensively investigated. However, there has been little direct evidence of aflatoxin involvement in human diseases. Nevertheless when the numerous biological systems affected by mycotoxins are considered, it is highly unlikely that these compounds would not also affect man.

Towards the end of last century, scientists found that certain products of microorganisms were responsible for various diseases in both man and animals. Subsequent research has increased the knowledge in the field of microbial toxins enormously, particularly for bacterial toxins. However,

food poisoning induced by molds is much less extensive and research concerned with the causes and prevention of mold intoxication of foods and feeds still has far to go (Chipley, 1977).

The world first became aware of the aflatoxin problem in 1960 when 100,000 turkey poults died in England when fed moldy peanut meal. The problem is a very old one. Research has shown that the toxins are primarily metabolites of Aspergillus flavus, a saprophytic fungus. This mold occurs world wide and grows on almost any organic matter, including foods and feeds. According to O'Brien (1976), the fungus is most active in tropical and subtropical regions; it is especially active across the southern United States; but it occurs throughout the corn belt. Under favorable conditions of high temperature and high humidity and adequate time, Aspergillus flavus produces a number of related toxins; some are the most hepatotoxic and hepatocarcinogenic agents known (Anonymous, 1966; Smith, 1969). Chemically these compounds can be readily separated by chromatography and observed by their fluorescence.

The present work has several objectives: to determine distribution of aflatoxin in animals fed aflatoxin by using chickens as test animals; to determine influence of nitrite on mold growth and toxin production in sausage (as an example of cured meat) and to evaluate effects of different scirpene toxins on chick embryos.

LITERATURE REVIEW

Aflatoxin

The term aflatoxin is derived from three source words, "a" from Aspergillus, "fla" from flavus, with toxin added. These toxins may be lethal if ingested in quantity, but when ingested in moderate amounts physiological functions are depressed (Kraybill and Shapiro, 1969). They suppress cell division, depress protein synthesis by binding of the toxin with DNA, a vital part of cell nuclei, inhibit DNA synthesis and depress fertility and reproductive function (Kraybill and Shapiro, 1969). In addition, these toxins may induce bleeding from the gut and urinary membranes, alter nervous function, induce generalized stress, depress the immune response and cause necrosis of the kidney and numerous histological changes in the liver (Wogan, 1969). Various mold toxins lower the white cell count, which partially explains the depression of disease resistance and lowered reproductive performance (Whittaker, 1975).

There are four commonly known types of aflatoxins. When exposed to long wave ultraviolet light, the most prevalent B1 and B2 give blue fluorescence whereas G1 and G2 fluoresce green. In addition to the four toxins specified, aflatoxins M1 and M2 are two other aflatoxins that are metabolically converted forms of aflatoxin B1. The "M" toxins were so named because they were first found in milk produced by lactating animals fed diets containing aflatoxin B1 (Masri et al., 1969). According

to Whittaker (1975), for every part of aflatoxin appearing in milk approximately 100 parts must have been ingested by the lactating animal. The "M" aflatoxins are also found in urine and body fluids (Nabney et al., 1967). Aflatoxin B1 is the most toxic and the most abundant. In order of toxicity, B1 is followed by G1, B2, and G2 (Ciegler and Lillehoj, 1968; Wilson et al., 1968). Different structural formulas of aflatoxins are shown in Figures 1 and 2.

Aflatoxins are toxic to many species of animals and the toxicity varies with the species. The duckling is the most susceptible to the affects of aflatoxin and the LD$_{50}$ value for the toxin when administered orally (single dose) to duckling ranges from 0.34 to 0.56 mg of aflatoxin B1 per kilogram of body weight. LD$_{50}$ values of aflatoxin B1 when administered to rats and hamsters are 7.2 and 10.2 mg of B1 per kilogram of body weight respectively (Wogan, 1966).

Aflatoxin B1 does not affect all animal species equally but in high dosages it is lethal to all and it is a slow acting poison (Ciegler and Lillehoj, 1968). The order of susceptibility at chronic or lower levels is hatchery raised trout; ducklings and turkey poults are most affected. Large animals in order of susceptibility are young pigs, sows, calves, mature cattle, horses and sheep (Allcraft, 1965; Borker et al., 1966).

Figure 1. Different structural formulas of aflatoxins B and their metabolites.
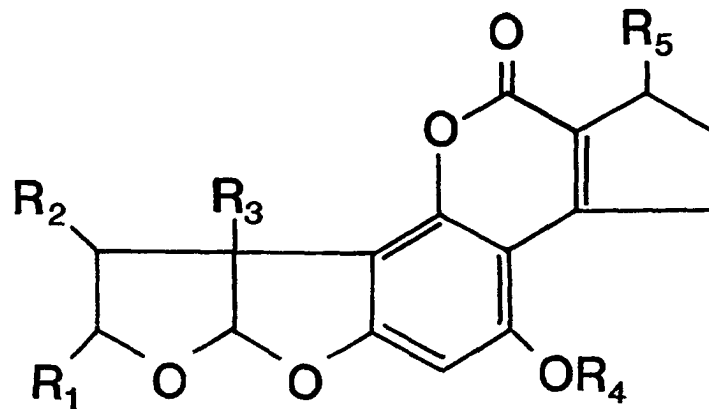
Aflatoxin	R_1	R_2	R_3	R_4	R_5
B_1	H	H	H	CH_3	$=O$
B_2	H_2	H_2	H	CH_3	$=O$
B_{2a}	OH,H	H_2	H	CH_3	$=O$
Aflatoxicol(Ro)	H	H	H	CH_3	$-OH$
M_1	H	H	OH	CH_3	$=O$
M_2	H_2	H_2	OH	CH_3	$=O$
P_1	H	H	H	OH	$-CH_3$
Q_1	H	H	H	CH_3	$-OH$

Figure 2. Different structural formulas of aflatoxins G.
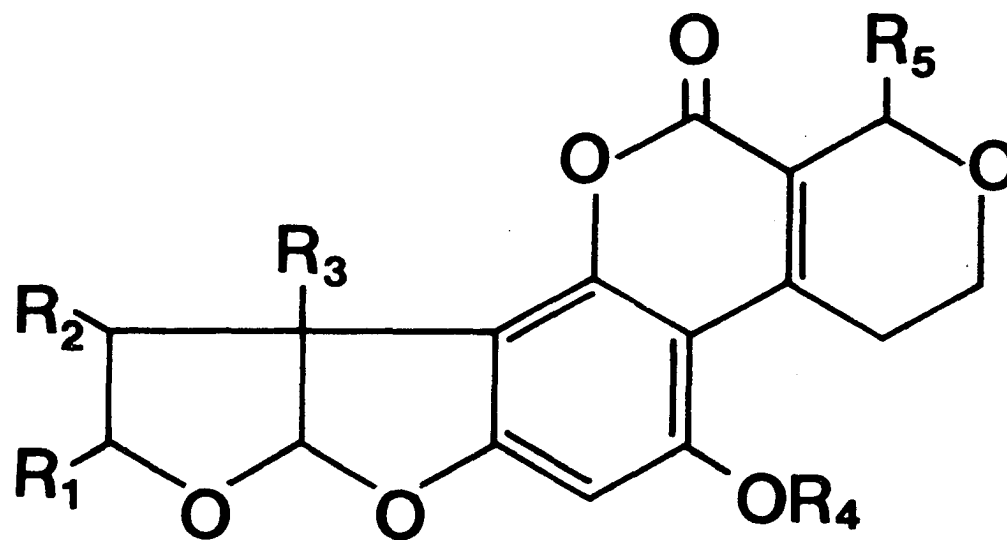
Aflatoxin	R_1	R_2	R_3	R_4	R_5
G_1	H	H	H	CH_3	O
G_2	H_2	H_2	H	CH_3	O
G_{2a}	OH,H	H_2	H	CH_3	O
GM_1	H	H	OH	CH_3	O

Occurrence and Conditions Necessary for Growth
of Aflatoxin Producing Fungi in Commodities

The occurrence of mold growth in stored and processed commodities

increased largely because of modern methods of harvesting, transportation

and storage, and failure to observe safety precautions of quick drying and

temperature control. Long storage of two to five years contributes to fungal

growth and toxin production, but only if favorable conditions for mold growth

occur. Aflatoxin was first believed to be produced by numerous genera and

species of fungi but new and sophisticated tests have shown that only a few

strains of Aspergillus flavus and Aspergillus parasiticus produce aflatoxin

(Cysewki et al., 1968; Wilson et al., 1968). In most cases these molds

are generally post harvest invaders of both cotton fiber and seed

(Ashworth et al., 1968; Smith, 1969).

The aflatoxin producing fungi have been found in a large variety of

commodities including peanuts (most frequently affected) cotton seed

(second in frequency of contamination), corn, sorghum, rice, wheat,

oats, millet, soybeans, coconut, egg solids, skim milk powder, fresh and

cured meat products, prepared feeds, ensilage, protein supplements, pecans

and numerous others (Borker et al., 1966; Ciegler and Lillehoj, 1968).

Conditions favoring fungal growth and toxin production are 1) high

moisture of the commodity (over 10% for peanuts; 12.5% and higher for

soybeans and over 13% for cereal grains), 2) high temperature (optimal

75 to 95° F), 3) time (average 7 to 15 days), 4) insect damage and 5) the

physical condition of the grain, weathering, mechanical handling and presence

of cracked grains (Christensen and Kaufman, 1965; Anonymous, 1967). The

presence of molds causes deterioration of quality but never a sure sign of

aflatoxin. According to Christensen and Kaufman (1965), only about 5% of the

molds are harmful. Most molds including some strains of Aspergillus flavus never

produce aflatoxin.

It is widely known that the most important factor in mold growth and

aflatoxin production is the moisture or relative humidity surrounding a natural

commodity such as peanuts. Safe storage (no mold growth) of seeds and other

natural substrates generally has been established at substrate moisture in

equilibrium with 70% relative humidity, at which few fungi grow. Diener

(1960) made many determinations of fungi inhabiting stock peanuts that had

been stored from 8 months in 26 farm size bins of several types of construction.

He found that the peanuts differed in initial moisture, damage to sound mature

kernels, type of bins for storage and length of time in storage. High mold

counts were correlated more often with high initial moisture of peanut

kernels than with any other factor. Mold counts were consistently high in

peanuts placed in storage with a kernel moisture content of 12.5%. After

3 to 4 weeks of storage, kernel moisture content of peanuts in all bins was about 7% and remained there for 8 to 56 months.

Research has indicated that certain conditions may result in either preharvest or postharvest infection and aflatoxin formation by Aspergillus flavus in peanuts. Most experimental data (Ashworth et al., 1965, Bampton, 1963; Barnes, 1971; McDonald, 1970) indicated that there was little invasion of intact and undamaged kernels, and immature and mature pods in the ground by Aspergillus flavus, and little or no aflatoxin present in kernels before lifting at normal harvest time. Peanuts are usually pulled up from the soil and left for 3 to 4 days before harvesting and this process is called lifting. According to Diener et al. (1965), McDonald and Harkness (1964) invasion of peanut pods and kernels by Aspergillus flavus and other fungi usually occurred during curing when the variety was dug near maturity. When drying of peanuts lowers the kernel moisture content rapidly and steadily downward within 4 or 5 days to safe storage moisture, little opportunity for fungal invasion occurs. Under tropical conditions, peanuts that were free of toxin at digging contained detectable toxin in 48 hours (Bampton, 1963). Also, kernels with testae damaged during shelling showed spontaneous growth of Aspergillus flavus in 4 days. Other field studies (McDonald and Harkness, 1964) demonstrated that contamination of kernels with aflatoxin occurred at least five to six days after lifting. According to Jackson (1967) in Georgia, high levels of

aflatoxin as well as high percentages of kernel invasion by <u>Aspergillus flavus</u> occurred experimentally in slowly dried pods as compared to rapidly dried pods.

Data demonstrating that invasion by <u>Aspergillus flavus</u> and aflatoxin formation in peanuts frequently occurs in the field before harvest has been steadily accumulating. As early as 1956, <u>Aspergillus flavus</u> was reported to be the dominant fungus in unblemished Spanish peanuts, having been isolated from 16% of the kernels and shells over a six-week period before and after harvest in Texas (Norton et al., 1956). Fungi grew from 22.2% of the nuts examined, with <u>Aspergillus flavus</u> occurring 79.3% of the time. In 1963 and 1964, peanut crops in northern Nigeria harvested at normal harvest time or earlier were free from aflatoxin whereas late harvesting usually resulted in some toxic peanuts (McDonald and Harkness, 1967).

Dickens et al. (1973), McDonald (1969), and Pettit et al. (1971) reported that drought stress has been closely associated with aflatoxin occurrence in peanuts before digging. Drought stress probably increases susceptibility to fungal invasion since it decreases the moisture content of the pod and kernels and greatly lowers the physiological activity of the peanut. Bampton (1963) reported that in Nigeria, aflatoxin was detected in late planted crop in sandy soils in semi-arid region, although the plants had been in the ground for only the normal length of time. Metabolic

activity of these peanuts probably had been reduced by a nine week drought.
McDonald and Harkness (1964 and 1965) and McDonald et al. (1964) reported
that it appeared that when kernel moisture content was above 30% or below
10%, Aspergillus flavus activity was restricted. It was also reported (Dickens
and Patee 1966) that kernels became more susceptible to Aspergillus flavus
invasion when the soil moisture in the pod zone approached levels at which
moisture moved from the pod into the soil and the kernel moisture content
dropped below 31%.

Overmaturity, drought stress, kernel moisture content and the level of
physiological activity in the peanut are interrelated and moisture-related,
although other factors may be involved. The high level of pod invasion by
Aspergillus flavus in the soil has been associated with overmaturity and with
low kernel moisture content due to lack of rain. Decreased vigor of the plant
and reduced physiological activity in seed coincided with the drop in the
kernel moisture content and overmaturity resulting in increased susceptibility
of pods and kernels to Aspergillus flavus invasion and aflatoxin formation
(McDonald and Harkness, 1964, 1967). McDonald (1969) also reported that
pods collected at Kano, Nigeria contained toxic kernels of 5 to 14% kernel
moisture content whereas living plants at harvest had no toxic kernels and
24 to 32% kernel moisture content. McDonald (1969) concluded that delayed

lifting could have undesirable results. Apparently, decreased physiological activity associated with maturity or resulting from low moisture in the soil environment favors invasion of kernels of such peanuts.

Production of Aflatoxin by Fungi

Aflatoxin production on substrates has been reviewed by several workers (Diener and Davis, 1969; Hesseltine et al., 1966). Aflatoxin has been produced by Aspergillus flavus when grown on every major cereal grain and oil seed crop. Aspergillus flavus produced aflatoxin experimentally on over 25 fruits and vegetable juices and other foods (Wildman et al., 1967). Aflatoxin production on any given substrate will vary quantitatively with the strain of the fungus, temperature, moisture in the substrate and/or surrounding relative humidity, aeration, length of incubation period and the method of aflatoxin analysis.

During the past decade, a large amount of literature has accumulated on aflatoxins. Studies have been carried out in order to elucidate the various factors that regulate the synthesis of aflatoxin (Diener and Davis, 1969) and as indicated previously, environment is known to play a very important role in the production of aflatoxin by the mold (Schroeder and Ashworth, 1966). Some workers (Mateles and Adye, 1965; Lee et al., 1966) have shown that zinc is very essential for the production of aflatoxin by Aspergillus parasiticus. Gupta and Venkitasubramanian (1975) carried out a detailed investigation in

order to establish the exact role of zinc on the metabolism of Aspergillus parasiticus. The fact that the growth of Aspergillus parasiticus is suppressed in a medium fortified with soybeans led to the suggestion that high concentration of phytic acid and low concentration of zinc in the soybeans might be responsible for inhibition of the growth of the fungus and aflatoxin formation.

Stutz and Krumperman (1976) observed that when conditions are favorable, walnut and filbert nutmeats will support heavy growth of Aspergillus parasiticus and production of aflatoxins. They also observed that extensive testing of culled nutmeats for aflatoxins over several seasons did not reveal their presence in the nut crops grown in Oregon and Washington. Several environmental factors might be responsible for this anomaly, but data on the natural occurrence of aflatoxins in agricultural commodities indicate that temperature is likely to be the most important.

Schindler et al. (1967) demonstrated that production of aflatoxins from Aspergillus flavus was limited by temperatures of less than 7.5°C and greater than 40°C. Diener and Davis (1966b, 1967) reported a minimal temperature for production in peanuts of $13 \pm 1°C$ after 21 days at a relative humidity of 97 to 99% although damaged kernels developed aflatoxin at $13 \pm 1°C$. They also claimed a maximum temperature for aflatoxin production of $41.5 \pm 1.5°C$. This is in contrast to the studies of

Schindler et al. (1967) demonstrating increasing production at 13°C at

3, 6 and 12 weeks after inoculation. Sorenson et al. (1967) studied

production of aflatoxin in the temperature range of 8 to 37°C and

reported that no aflatoxin were produced at 8°C. Walbeek et al. (1969)

studied five strains of Aspergillus flavus at 7.5 and 10°C and reported

aflatoxin production at both temperatures. Four isolates were investigated

by Schroeder and Hein (1967) for aflatoxin production at temperatures from

10 to 40°C and for a ten day period. They reported that only small amounts

of aflatoxins were produced at the two temperature extremes. Northolt

et al. (1976) reported that aflatoxin B1 was produced by Aspergillus

parasiticus (NRRL 2999) at temperatures as low as 13°C and as high as

32°C. Diener and Davis (1966b) showed Aspergillus parasiticus strain

(NRRL 2999) to produce the highest level of toxin between 25 and 30°C

with a significant decrease occurring at 35°C and only slight to trace amounts

at 40°C. With the exception of work done by Schroeder and Hein (1968)

studies on the relationship of aflatoxin production and temperature have

been conducted at constant temperature. Whereas these studies have

provided considerable and significant information, aflatoxin generation

in a natural environment is likely to occur under conditions of varying

temperature. Schroeder and Hein simulated conditions that might be

found in a natural environment by cycling temperature once over a

24 hour period. The programmed temperature provided an average temperature of 25°C per 24 hours. Since the experiment was designed to provide an average temperature of 25°C, the exposure time at lower temperature extreme was of short duration. In the high temperature range, growth and aflatoxin production declined rapidly, whereas cycling temperature at the lower range had little effect upon either mycelial growth or toxin production. Stutz and Krumperman (1976), in their study of the effect of temperature cycling on the production of aflatoxin by Aspergillus parasiticus, suggested that under conditions of diurnal and nocturnal time-temperature sequencing, the time the culture is exposed to a given temperature is an important factor for aflatoxin production. They continued that the relationship between the two can better be expressed in terms of total heat input during the period of the cycle. They suggested that thermal input is more definitive and provides a finite number which can be related to observable changes in the culture such as sporulation and toxin biosynthesis. Their studies were designed to characterize the effect of heat input upon the functions of Aspergillus parasiticus (NRRL 2999) and to determine the minimal thermal input required for aflatoxin production. No growth was detected at a thermal input less than 208 degree hours per day. Between 208 and 270 degree hours per day, mycelial growth was observed as well as biogenesis of copious

amounts of a yellow pigment; yellow in color in the early stages of growth
and turning deep orange as the culture aged. Above 270 degree hours per
day, cultures consistently sporulated and produced aflatoxin.

Northolt et al. (1976) reported that optimum water activity for
aflatoxin B1 production was 0.99 and Diener and Davis (1967) found
optimum water activity values of 0.95 and 0.99 depending on the substrate.
In the study of aflatoxins in cotton seed, McMeans et al. (1977) concluded
that moisture was the main factor in the invasion of cotton seed by Aspergillus
flavus and the production of aflatoxin. Aflatoxin production appears to be
self-limiting; that is, as mycelial growth reaches the end point, autolysis
occurs followed by a decline in the amount of toxin produced. The fungus
appears to use the aflatoxin in its metabolic processes or to alter the toxin
to the extent that it cannot be detected by standard assay procedures
(Schroeder, 1966).

Competition by Fungi

Growth of other microorganisms may change available nutrients or
produce volatile and/or nonvolatile end products which may stimulate,
inhibit, detoxify or have no influence on growth of fungi or on mycotoxin
production (Barr, 1976; Denizel et al., 1976; Moore-Landecker and Stozky,
1972, 1973). Weckbach and Marth (1977) studied the effect of several
microorganisms of importance on growth and aflatoxin production by

Aspergillus parasiticus. They incubated Yeast Extract Sucrose broth (YES) with Rizopus nigricans; Saccharomyces cerevisiae; Acetobacter aceti; or Brevibacterium linens incubated for three days at 28°C then inoculated with Aspergillus parasiticus spores and incubated at 28°C for seven days. They found that when Aspergillus parasiticus was grown in association with R. nigricans, both molds grew but the amount of aflatoxin produced was much less in the competitive situation than when Aspergillus parasiticus grew alone. Weckbach and Marth (1977) suggested that the reduction in the amount of aflatoxin present when both molds grew together as compared to when A. parasiticus grew alone may have been caused by R. nigricans degrading or detoxifying the aflatoxin as it was formed. Certain strains of Rhizopus have been found that can metabolize preformed aflatoxin B1 and G1 (Cole and Kirksey, 1971; Jarvis, 1971). Another possibility is that the growth of A. parasiticus was not sufficient to result in appreciable amount of aflatoxin being produced. Weckbach and Marth (1977) further showed in their studies that Rizopus nigricans is an effective competitor to A. parasiticus by adding spores of A. parasiticus to an established culture of Rhizopus nigricans; slight growth of A. parasiticus was evident but no aflatoxin was detected. Adding A. parasiticus to a poor culture of Rhizopus nigricans stimulated growth of Rhizopus nigricans.

When activity of A. parasiticus was compared with S. cerevisiae growth and aflatoxin production by A. parasiticus was inhibited. When A. aceti was

the competitor, A. parasiticus grew and produced more aflatoxin than

when the latter grew alone. Culturing A. parasiticus in the presence of

B. linens resulted in less growth and aflatoxin production than when the

former was grown alone.

Aspergillus flavus-oryzeae group

The Aspergillus flavus group of species is a normal constituent of the

microflora in the air and soil and is also found in or on living or dead plants

and animals throughout the world (Raper and Fennell, 1965). It has been found

associated with peanut soil and peanuts wherever they are grown (Diener,

1973). Aspergillus flavus is an important storage fungus associated with

the deterioration of wheat, corn, rice, barley, bran, flour, soybeans and

other seeds (Christensen, 1957; Christensen and Kaufman, 1969). It has

also been reported as a pathogen of man, animals and plants (Clinton, 1960).

The Aspergillus flavus-oryzeae group includes A. flavus and A. parasiticus,

which produce aflatoxin, as well as A. oryzeae and A. tamarii, which do not

(Hesseltine et al., 1970). Investigators in England, Holland, India, Israel,

South Africa and United States indicated that 60% of about 1,400 isolates of

Aspergillus flavus group were aflatoxin producers (Diener, 1973). Isolates of

Aspergillus flavus and Aspergillus parasiticus vary widely in the amount of

aflatoxin produced on peanuts (Diener and Davis, 1966a).

Prevention of Aflatoxin Contamination

Contamination of food grains by storage fungi such as Aspergillus

flavus and Aspergillus parasiticus is known to occur very widely. During

their growth, these fungi produce a group of toxic metabolites collectively

known as aflatoxins which are potent hepatotoxins and carcinogens.

Consumption of such contaminated foodstuffs have been shown to be

hazardous to a variety of animals including monkeys (Goplan et al.,

1972; Tilak, 1975) and more recently to man (Krishnamachari et al.,

1975a,b).

Considerable effort was directed towards preventing aflatoxin

contamination of food grains. Among the new approaches to the problem

have been an attempt to identify and develop varieties which are resistant

to aflatoxin production. Nagarajan and Ramesh (1972, 1973) have shown

that varietal differences do occur among genotypes of maize and groundnuts

with respect to their capacity to support production of aflatoxins by

Aspergillus flavus and Aspergillus parasiticus.

Peanuts and corn are good substrates for the growth of the Aspergillus

flavus group and are prone to aflatoxin contamination especially under high

moisture conditions (Wilson et al., 1977). Landers, Davis and Diener (1967)

and Sanders, Davis and Diener (1968) used modified atmospheres in pilot studies

to reduce aflatoxin contamination of peanuts. Wilson and Jay (1975, 1976)

extended these studies to include corn and different levels of carbon monoxide

with peanuts. Wilson et al. (1975) found that Aspergillus flavus and

Fusarium moniliforme survived in high moisture corn and Aspergillus flavus

survived in uninoculated peanuts.

In the studies of Wilson et al. (1977) the exposure of two 70 kilogram

containers of corn to a modified atmosphere for 36 and 109 days demonstrated

that larger quantities of corn behave similar to smaller quantities in aflatoxin

content and fungal survival. The atmosphere inside the containers was

modified by purging the containers and their contents with 14.3% CO_2,

0.5% O_2 and 85.2% N_2, and then the containers were sealed and stored

for the above number of days at about 25°C. They also stated that

visual observations demonstrated almost complete control of insect activity.

They further used the exposed corn to demonstrate feasibility of feeding the

corn to pigs. The studies summarized that many insects, growth of

Aspergillus flavus and aflatoxin contamination can be controlled with an

atmospheric oxygen content less than 1%, but with an oxygen level above 1%,

aflatoxins probably will be formed.

Among the currently approved feedstuff preservatives, calcium and

sodium propionate, propionic acid and copper sulfate are the most popular.

Despite the widespread use of antifungal compounds in grain and feed,

mycotoxin related problems continue to exist leading to the conclusion that

the compounds used as preservatives either are being added to feed in

insufficient concentration or they do not possess adequate antifungal activity to preserve the feeds for the desired length of time (Stewart et al., 1977).

Chen and Day (1974) compared 19 antifungal agents for activity on five different mold species by agar plate assay and found gentian violet to have the highest overall activity. Sauer and Burroughs (1974) determined that propionic and acetic acid were superior to either calcium or sodium propionate, sorbic acid and potassium sorbate in preserving high moisture corn in a mold free state. Vandergraft et al. (1975) determined that propionic acid prevented aflatoxin and ochratoxin formation in high moisture corn. The studies of Stewart et al. (1977) demonstrated that propionic acid was superior to other chemicals in regard to mold retarding capabilities and inhibition of aflatoxin production. An in vitro concentration of 4 microgram per ml resulted in total inhibition of mold growth and aflatoxin formation. Crystal violet at the level of 4 microgram per ml resulted in slight inhibition of mold growth accompanied by an 11.9% inhibition of aflatoxin formation. Chen and Day (1974) reported gentian violet to have one of the highest antifungal activities of 19 fungal inhibitors tested on five mold species including four different genera. However, the minimal growth inhibitory concentration determined by Chen and Day (1974) exceeds that reported in the studies of Stewart et al. (1977). Graded levels of crystal violet resulted in dose related retardation in growth of Aspergillus parasiticus. This strain of Aspergillus parasiticus becomes resistant to the effect

of crystal violet with continued exposure to the compound. During the course

of their study, Stewart et al. (1977) noted that after 10 days of incubation,

control cultures (no crystal violet added) would sporulate, resulting in

greenish coloration of the mycelial mat. However, those cultures with

crystal violet added had only a slight greenish coloration and presumably

less spores. Hence, sporulation was monitored and compared with growth

of mold in response to graded levels of crystal violet. A level of $2.0 \mu g/ml$

of crystal violet was required to retard growth, however, a level of $1.0 \mu g/ml$

significantly retarded sporulation. Sporulation, therefore, appears to be

more sensitive to crystal violet than does growth. These results were

consistent with those of Doupnik and Bell (1971) who noted a marked reduction

in sporulation of Aspergillus parasiticus NRRL 2999 when visually compared

to control cultures.

Sporulation of molds is a means of reproduction. The above works indicate

that crystal violet will impair sporulation and therefore reduce subsequent

mold growth resulting from spore germination. If this activity occurs in

finished feeds this could justify continuous or long term use of crystal violet.

An in vitro concentration of $8 \mu g/ml$ of crystal violet was required to retard

the growth of Aspergillus parasiticus after 10 days of incubation. Total

aflatoxin produced per culture flask (mycelium plus medium) was reduced by a

level of $1 \mu g$ and greater percent. In comparison to growth and sporulation,

total aflatoxin production is the most sensitive parameter to crystal violet.

The prevention of aflatoxin contamination is essentially a problem of the prevention of the growth of the ubiquitous aspergilli during storage of the substrates. This entails harvesting without damage to the shell and kernel and rapid drying to levels of moisture that will prevent fungal colonization. Insect or rodent damage and uptake of moisture should be avoided during storage. Fumigation protects crops against insects and rodent damage but only drying protects crops against fungal growth. Some fungicidal agents such as mecuric chloride and thiourea cannot be used on food grains for toxicological reasons (Srinivasan and Majumder, 1961). However, some antimicrobial agents such as methyl bromide, ethylene oxide and chloropicrin could be useful on substrates under hermetic storage. But the irritant nature of these agents and the need for special apparatus such as fumigation and storage chambers may limit the usefulness of these techniques in rural small scale farm practice especially in poor countries. Golumbic and Kulic (1969) stated, "The practical use of fungicides to control fungal infestation in grains must overcome the same hurdle that exists for insect treatments, namely the problem of harmful chemical residues. Thus far there appears to be no fungicidal treatment that has been successful for large scale application despite the considerable effort that has been exerted in this area." Even adequately dried substrates may subsequently increase in moisture content through improper

storage especially in rainy weather and damp store houses and may then be susceptible to fungal spoilage. In poor countries where the groundnut is an important crop it is cultivated by rural farmers ignorant of the implications of fungal spoilage and aflatoxin accumulation. Hence effective and cheap crop protection against fungal contamination are urgently needed for economic and toxicological reasons.

Mycotoxin in Tissues

Mycotoxins are known to affect a variety of tissues and to lower some vitamin and amino acid concentrations in chicken. Aflatoxins are known to affect liver and kidney functions (Tung et al., 1973). Schmidt et al. (1976) speculated that there is the possibility of a peculiar retinal requirement in the chicken as there is in the cat. It was thought that perhaps lowering the concentration of some vitamins and amino acids in chickens might affect the photochemistry of the retina to make electroretinogram recordings diagnostic of early mycotoxicoses. If mycotoxicoses could be detected in broiler chickens before retarded growth and other overt clinical signs became evident, some of the losses associated with mycotoxicoses could be prevented. Tung et al. (1971) found that relatively small amounts of aflatoxin over a relatively short period of time are known to cause insidious changes in muscle tissue prior to an effect on growth rate. Neural disturbances such as loss of righting reflex have been reported to occur at dosages of T-2 toxin

which retarded growth (Wyatt et al.,1973). Again, if other neural tissues are susceptible to mycotoxins, it was reasoned that checking retinal functions by electroretinogram despite the lack of any apparent visual problems in affected chickens could be a sensitive method of monitoring the effects of mycotoxins. Coulter et al. (1977) in their study of electroretinograms from broilers fed aflatoxin and T-2 toxin concluded that photochemistry of the retina was not sufficiently altered to permit early detection of mycotoxicoses by electroretinograms.

A mycotoxin is said to have a potential for accumulating "toxic residues" in body tissues when its metabolism in vivo is known to be slow or when a significant pathway of metabolism results in the formation of one or more metabolites with no appreciable loss in toxicity and when such a product is slowly eliminated from the body. This potential problem is greatest when the animal in question is destined for use as human food. According to Patterson (1973) aflatoxin B1 may be detoxified in the liver by its transformation into three hydroxylated metabolites (aflatoxicol, aflatoxin Q1, and aflatoxin P1) that are theoretically capable of conjugation to polar compounds suitable for rapid elimination from the body. However, none of the toxins are detoxified so well that animals are entirely unaffected by toxin ingestion. In certain animal species, these detoxifying pathways appear to be better developed than others. Aflatoxin is metabolized more

rapidly by some animal species than others (Patterson, 1973) and consequently, in "slow metabolizing" animals (Patterson, 1973; Krieger et al., 1975) aflatoxin B1 can itself constitute a residue problem particularly in tissues of farm animals. Biotransformation of aflatoxin B1 into M1 by microsomal enzymes is to some extent detoxification since conjugates of this metabolite have been identified in bile (Bassir and Osiyemi, 1967) and urine (Harland and Cardeilhac, 1975). However, because unconjugated M1 is almost as toxic as B1, it must be regarded as a potential toxic residue. As is well known, a considerable health problem exists in the excretion of aflatoxin M1 in the milk of dairy cows consuming rations contaminated with aflatoxin.

Radioactive Studies with Mycotoxin

Studies have been done to determine the fate of aflatoxin in some animal species. Radioactive aflatoxin B1 frequently has been used in such work for quantitative measurements (Dalezios and Wogan, 1971; Mabee and Chipley, 1973a; 1973b; Wogan et al., 1967). Lijinsky et al. (1970) used ^{3}H-labelled toxins to study the interaction between aflatoxin B1 and G1 in rat tissues. ^{14}C aflatoxin was used by Wogan et al. (1967), Dalezios and Wogan (1971), and Mabee and Chipley (1973a, 1973b) to study the effect of aflatoxin in the rat, monkey and chicken respectively. Chou and Marth (1976) studied the distribution of aflatoxin in the tissues of mink.

They injected a single dose of 100 μg aflatoxin B1 (^{14}C-labelled and

unlabelled) intraperitoneally in seven female minks, which were sacrificed

1, 2, 4 and 24 hours after dosing. Liver, intestines, stomach, lungs, kidney,

brain, pancreas, spleen, urinary bladder, uterus and bile were removed and

examined for retained radioactivity. One hour after dosing, intestines and

their contents retained the largest amount of ^{14}C-radioactivity (18.9% of

the amount that was administered) which was followed by the liver (13.2%)

and bile (10.8%). All this time all other tissues retained less than 1% of the

administered radioactivity. Generally, the amount of radioactivity retained

in all tissues declined with time. Only 1.2% and 0.6% of the administered

radioactivity was found in the intestine and bile respectively 24 hours after

dosing; however, the liver still contained 6.6% of the initial radioactivity.

The early appearance of large-amounts of radioactivity in bile, liver and

intestines suggested that bilary secretion plays an important role in aflatoxin

excretion by mink. Chou and Marth also believed that after intraperitoneal

injection, a large portion of aflatoxin B1 and or its derivatives were

absorbed and transported to the liver through the portal system and then

secreted into the intestines from the gall bladder. The considerable decrease

in radioactivity in intestines in mink at 2, 4 and 24 hours after dosing

probably resulted because radioactivity was excreted with feces.

Distribution of ^{14}C derived from ^{14}C aflatoxin B1 in tissues of layer and broiler chickens was studied by Mabee and Chipley (1973a, 1973b). They dosed the chickens daily for 14 days by crop intubation with 0.1 ppm of the body weight. The aflatoxin was in propylene glycol. Differences were found between the two types of chickens. The percent distribution of radioactivity detected in the blood, liver, and heart, gizzard breast muscle and leg muscle was 11.04, 9.83, 4.30, 12.52, 31.66 and 30.66 respectively, of the total amount retained by the broilers. On the other hand, 19.5, 16.1, 3.9, 7.22, 26.4 and 26.9 percent of total retained radioactivity was found in blood, liver, heart, gizzard, breast muscle and leg muscle respectively of layers. The broiler chickens excreted 90.64% of the ^{14}C administered. The daily administration of 0.1 mg of aflatoxin B1 $-^{14}C$ per kilogram of body weight to layer chickens for 14 consecutive days did not significantly affect the weight, feed consumption and egg production. Sims et al. (1970) observed weight loss in laying hens resulting from daily intubation of 8.0 ppm of crude aflatoxin to each hen. There are other reports that describe the effects of various levels of aflatoxins on chicken (Platnow, 1965; Cottier et al., 1969; Muller et al., 1970); however, these workers administered aflatoxin by incorporating it into feed at various levels. Their results, therefore, are based on the amount of the test feed the bird consumed and this could introduce variability.

Effects of Consumption of Mycotoxin

There is evidence that dietary mycotoxins affect the intestinal tracts

of animals. In trout, dietary T-2 toxin cause sloughing of intestinal mucosa

(Marasas et al., 1969). This effect of T-2 on the intestinal mucosa is

apparently related to the tissue irritating and inflammatory properties of the

tricothecene mycotoxins. Aflatoxin, perhaps the most widespread mycotoxin

in nature, also appears to exert an effect on the intestinal tract. Wilson

et al., 1975) observed that some of the adverse effects of aflatoxin in

quail could be alleviated by supplying vitamins and antibiotics in drinking

water. Witlock et al. (1977) initiated a study to better understanding the

action of the dietary aflatoxin and to determine the effect of this mycotoxin

on the gross morphology of the avian intestinal tract. Specimens of the

mid-intestine from birds fed 5 μg aflatoxin per gram displayed no

morphological differences from the control birds. However, at 10 μg per

gram level, slight changes included varied morphology of villi from an

acute triangular appearance to a parallel-sided apically rounded configuration.

It is somewhat surprising that aflatoxin did not have any severe effect on the

birds because aflatoxicoses have been reported to inhibit lipid transport

(Tung et al., 1972), decrease plasma carotenoid concentration (Tung and

Hamilton, 1973), increase capillary fragility (Tung et al., 1971) and impair

kidney functions (Tung et al., 1973).

Identification of aflatoxin as a potent hepatotoxic and carcinogenic agent

has emphasized the dangers of mold contamination of human and animal foodstuff.

While the data on toxicity to animals from field and experimental studies are

extensive, cogent circumstantial evidence of the toxicity of these mold

metabolites to humans has only recently been published. Krishnamachari et al.

(1975a) described an epidemic of aflatoxicoses in an Indian village, caused by the

consumption of moldy maize in which 106 patients died and 291 showed signs

of hepatic dysfunction. There are marked differences in susceptibility between

different animal species. Single doses of aflatoxin produced cancers in rats

a year after ingestion (Carnaghan, 1967), but continued feeding for six years

was required to produce tumors in monkeys (Adamson et al., 1973). The

aflatoxins were soon recognized as health hazards in those countries where

liver cancer was reported as being particularly frequent. In those countries

the dietary staples which might be contaminated are major sources of food.

In 1968 it was considered essential by a number of scientists to demonstrate

whether the aflatoxins were in fact ingested by man, whether an association

existed with the liver cancer incidence and finally if there was a dose response

relationship. In these studies relating liver cancer to aflatoxin ingestion, it

has been assumed that it was legitimate to compare current exposure to aflatoxin

to current cancer rates. The areas where these studies are taking place are in

Africa and Asia and it was felt that the diet, storage and cooking habits have

not changed over the recent past. Most of the food is grown in small individual farms using traditional methods of subsistence farming. The study by Alpert et al. (1971) in Uganda examined the major dietary staples and the result showed an association between the levels of contamination of beans, maize and sorghum, the staples most frequently involved and a tribal distribution of liver cancer was demonstrated.

Prior to the discovery of aflatoxin, Aspergillus flavus was reported to cause the hemorrhagic anemia syndrome of chickens (Forgacs and Carll, 1962). The hemorrhagic anemia syndrome which results from eating moldy feed was characterized by spontaneous hemorrhages into the musculature and internal organs and by aplastic anemia. Whether aflatoxin is involved in the economically important hemorrhagic anemia syndrome has not been investigated directly, but Tung et al. (1970, 1971) found that dietary aflatoxin causes increased capillary fragility in young chickens and increased susceptibility to bruising but not spontaneous hemorrhaging. Brown and Abrams (1965) observed a slight anemia in ducklings and New Hampshire chicks fed 0.5 ppm of aflatoxin for six weeks but noted that too few animals were used to permit conclusions as to significance. On the other hand, Juskiewicz et al. (1967) did not find any significant changes in erythrocytes and leukocyte counts or in haemoglobin determinations in ducklings fed 0.9 ppm aflatoxin for two weeks. Gagne et al. (1968) observed only minimal

deviation of haemoglobin and blood cells in pigs fed aflatoxin for four

months. Harding et al. (1963) observed an increase in both leucocytes and

erythrocytes in pigs fed aflatoxin for nine weeks. No significant changes

were found in hemoglobin or packed cells volume from pigs given daily

oral doses of aflatoxin for three weeks (Sisk et al., 1968). The status of

the hematopoietic systems during aflatoxicoses in chickens was investigated

by Tung et al. (1975) and they found that aflatoxin administration caused

an anemia at a dose of 1.25 μg per gram and that a dose of 2.5 μg and above

of dietary aflatoxin decreased packed cell volume. Also, a dose of 0.625

micrograms per gram decreased the number of circulating erythrocytes. The

hepatotoxic effect of aflatoxin B1 and the isolation of aflatoxin B1 at necropsy

from tissues of 22 out of 23 cases of Reye's syndrome in Northern Thailand

have stimulated interest in the possible role of aflatoxin B1 in the etiology

of the syndrome (Hogan et al., 1978). Aflatoxin has been associated

at necropsy with Reye's syndrome in 2 cases in New Zealand, 2 cases in

Czechoslovakia, 1 case in the United States and reported two cases in which

aflatoxin B1 was found in the blood of patients with Reye's syndrome during

the acute phase of the disease (Hogan et al., 1978). They said that both

cases were preceded by viral illness and the patient showed vomiting,

hyperventilation, hepatomegaly, decerebrate posturing, seizure and coma.

A commonly reported symptom of mycotoxicoses is poor feed conversion efficiency. One possible explanation according to Osborne et al. (1976) is that the process of digestion and absorption is impaired. Osborne et al. (1976) evaluated the effects of aflatoxin, ochratoxin and T-2 toxin on digestion in broiler chickens and determined fecal lipid content, bile concentration and the activities of pancreatic lipase, amylase trypsin, RNAse and DNAse utilizing the concept of the dose response curve. Aflatoxin decreased the level of all digestive enzymes and bile and increased lipid fecal excretion even at concentrations too small to inhibit growth. Ochratoxin had no effect on any of the parameters measured except for a slight increase in bile volume. T-2 toxin affected all parameters but growth inhibition was a more sensitive indicator of T-2 toxicoses. These studies suggested that the three mycotoxicoses can be differentiated by their effects on digestion and that the economically important factor of feed utilization can be intimately affected by mycotoxins.

On the assumption that in commercial laying hen operations, aflatoxin would be consumed intermittently rather than continuously Garlich et al. (1976) carried out an eleven week experiment to determine the effects of intermittent consumption of aflatoxin by laying hens. For the first five weeks, feed consumption, egg production and egg weight were the same for the control hens and hens intermittently fed 10 ppm aflatoxin. After five

weeks there was a decline in egg weight and production. Despite the decline

in egg production, serum alkaline phosphotase and liver lipid content were not

elevated. The conclusion was made that aflatoxin is a cumulative toxin for

laying hens. After prolonged intermittent consumption, the first sign of toxicity

is a decline in egg production. Lötzsch and Leistner (1977) studied the

transmission of aflatoxin into eggs and egg products. They found that

aflatoxin fed to laying hens of quail as well as hens of the laying chicken

were transmitted into eggs in measurable amounts in albumen and yolk but

more in the latter. In their study, they found that quail was the most

sensitive poultry for the transmission effect with a critical aflatoxin B1

concentration of 100 ppb in the feed leading to the detectable residue. As

compared to the quail, an increase of about 30 to 50 fold quantity of

aflatoxin B1 was necessary in the feed to induce measurable residues in

the eggs of white and brown laying hens respectively. These results

contrast with the findings of Jacobson and Wiseman (1974) who observed

aflatoxin B1 residues in eggs of Arbour Acre strains fed with the low aflatoxin

B1 concentration of 100 ppb in the diet. High concentrations of aflatoxin

in the feed caused excessively toxic amounts in the eggs of test poultry.

Lötzsch and Leistner (1977) also concluded that if hens were fed

continuously with diets containing more than about 100,000 ppb of

aflatoxin B1, egg production ceased after a few days thus excluding

hazardous aflatoxin residue in eggs. This agrees with the work of Garlich et al. (1976). For that reason, the egg production is an important natural limiting factor for the transmission of aflatoxins into eggs.

The transmission of aflatoxin into foods of animal origin was reviewed by Purchase (1972). Krough et al. (1973) fed diets containing 300 or 500 μg of aflatoxin B1 + B2 per kilogram feed to pigs for 120-231 days. During the growth period when weight increased from 20-90 kilograms, they reported that pigs on the aflatoxin contaminated diet had impaired weight gain and feed conversion. The pigs were slaughtered four hours after the last feeding and tissue samples were analyzed. Various amounts of aflatoxin B1, B2 and M were found in liver and kidney and trace amounts were found in the heart muscle and adipose tissue of the pigs on the contaminated diet. Murthy et al. (1975) reported that the response of swine to aflatoxin depends on whether the aflatoxin contaminated protein source is fed separately or incorporated into the total diet ration. In their study, pigs fed the aflatoxin source separately developed toxic symptoms and aflatoxin B1, B2 and M1 were found in their tissues. The pigs fed the mixed diet did not develop toxic symptoms, and no aflatoxin residues were found in the tissues of one pig examined.

Jacobson et al. (1978) conducted a study to determine if aflatoxin residues could be measured in tissues of swine receiving aflatoxin in the diet

at levels approximating the range of field contamination. Appreciable amount of B1 were found in samples from pigs fed the B1 contaminated diet and all except two muscle samples contained measurable amounts of M1. These data agree with those reported by Krough et al. (1973) and Murthy et al. (1975) for the transmission of B1 into liver and kidney tissues.

Fusarium Toxins

The ability of Fusarium, one of the main field fungi, to elaborate toxic secondary metabolites on cereal grains throughout the world is well documented, accompanied by specific pharmacological effects which occur in farm animals on consumption of Fusarium infected grains. Toxins that are found in field grains naturally contaminated by Fusarium and that have been associated with a specific farm animal syndrome are zearalenone, a potent estrogen, vomitoxin, an emetic refusal principle (Vesonder et al., 1973, 1976); and T-2 toxin, a vesicant and hemorrhagic (Hsu et al., 1972). Structures of scirpene compounds are shown in Figure 3.

Growth of Fusarium on grains requires a high moisture content (20 to 22%). Temperature may also be critical and may determine which toxic trichothecene will be produced. For example, Fusarium poae elaborates T-2 toxin at low temperature (8° C) rather than a high or room temperature (25°C). In contrast, trichothecenes of the type which vary in structural

Figure 3. Structures of scirpene compounds. Deoxynivalenol (vomitoxin) in structure 1 has the following substitutions: $R_1 = H$, $R_2 = R_3 = OH$, $R_4 = 0$.

Structure I
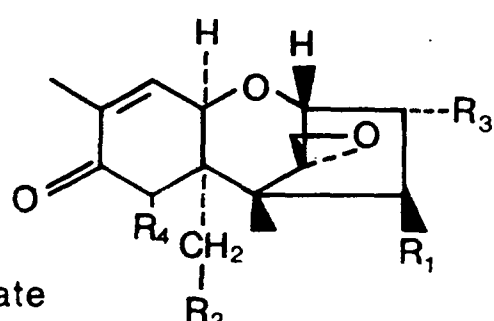
Compound Name	Substituents (R) in Formula				
	R_1	R_2	R_3	R_4	R_5
Calonectrin	H	OAC	OAC	H	H
Diacetylcalonectrin	H	H	OAC	H	H
Trichodermol	OH	H	H	H	H
Pentahydroxyscirpene	OH	OH	OH	OH	OH
Trichodermin	OAC	H	H	H	H
Diacetylverrucarol	OAC	OAC	H	H	H
Verrucarol	OH	OH	H	H	H
Scirpentriol	OH	OH	OH	H	H
Diacetoxyscirpenol	OAC	OAC	OH	H	H
T-2 Tetraol	OH	OH	OH	OH	H
T-2 Toxin	OAC	OAC	OH	OIV	H
HT-2 Toxin	OH	OAC	OH	OIV	H
Neosolaniol	OH	OAC	OAC	OH	H

OAC = Acetate   OIV = Isovaleryl

Structure II

Compound	Substituents			
	R_1	R_2	R_3	R_4
Trichothecolone	OH	H	H	H
Trichothecin	O-iso-crotonyl	H	H	H
Nivalenol	OH	OH	OH	OH
Nivalenol Diacetate	OAC	OAC	OH	OH
Fusarenone	OAC	OH	OH	OH

Structure III
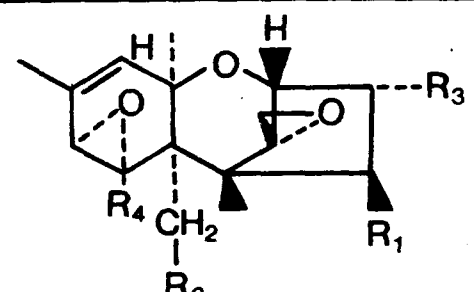
Compound	Substituents			
	R_1	R_2	R_3	R_4
Crotocol	OH	H	H	—
Crotocin	O-iso-crotonyl	H	H	—

Figure 3.   Continued.

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constituents at the C8 position from T-2 such as Fusarium -X require a

moderate temperature (25 to 27°C) for production by Fusarium nivale

(Smalley and Strong, 1974). Vomitoxin which is a toxic metabolite of

Fusarium graminearium NRRL 5883, is also produced at moderately high

temperature (28°C). The trichothecene toxins consists of about 34 derivatives,

most are produced by species of Fusarium, Tricoderma and Trichothecene.

Hsu et al. (1972) reported T-2 toxin, diacetyoxyscirpenol, present in

moldy corn associated with a lethal toxicosis in dairy cattle. Mirocha

et al. (1976) reported the presence of diacetyoxyscirpenol, T-2, vomitoxin

and other Fusarium toxins in feeds implicated in mycotoxicosis of swine and

cattle.

Reports in the literature on the incidence of Fusarium toxins, especially

the trichothecenes, in animal feeds have been lacking because of the lack

of suitable methods of analysis. As these methods are developed, it may

become apparent that toxins from Fusarium may be more prevalent in feed

and foodstuff than the aflatoxins. Analyses are difficult because the

analyst is often not certain which trichothecene to analyze for since

(there are about 50 known derivatives) and each has different separation

characteristics making extraction and resolution difficult.

Stored corn may be the source of a number of mycotoxins when

moisture content and other conditions permit fungal growth and toxin

production. Pier et al. (1975) fed calves four mycotoxins that had been
associated with corn at the following dose levels (mg/kg/day): Aflatoxin
(B1 equivalence) 0.1, 0.2 and 0.5; ochratoxin (as A) 0.1, 0.5, 1.0 and
2.0; rubratoxin (as B) 8.0, 12.0 and 16.0; and T-2 toxin 0.08, 0.16,
0.32 and 0.64. Combined doses of rubratoxin and aflatoxin were given
in the following combinations (Afla/Rubra): 0.1/8.0, 0.2/12.0, 0.2/
16.0. The calves were examined clinically each day and experimental
observations continued for 30 days. The calves became clinically ill and
died during the experimental period as follows: those fed 0.5 mg
aflatoxin died in 14 days, for 16.0 mg rubratoxin they died in 7 days,
for 0.6 mg T-2 toxin they died in 20 days, for 0.2 mg aflatoxin plus
10.0 mg rubratoxin, they died in 7 days. Aflatoxin caused moderate
reduction in liver function (0.1 and 0.2 mg), moderately depressed weight
gains at 0.2 mg, and severely at 0.5 mg. Pathologic changes with
aflatoxin include yellow liver, portal fibrosis, bile duct hyperplasia and
hepatic necrosis. Ochratoxin (0.1 to 2.0 mg) caused frequent urination
and reduced urine specific gravity; liver functions were not impaired.
Weight gains were moderately depressed at 1.0 mg and severely at 2.0 mg.
There was mild degeneration of urinary tubules. Rubratoxin caused dramatic
depression of liver function (8.0 mg) and caused moderate depression in rate
of gain at 12 mg. The liver was mottled, yellow and degenerative changes

include hepatocyte necrosis (12.0 mg) and disorganization of hepatic cords

(16.0 mg). T-2 did not affect liver functions but body weight gain was

depressed moderately at 0.32 mg and severely at 0.64 mg. Pathological

changes were limited to ulceration of the rumen and abomasum and edema

of the small intestine. The combination of aflatoxin and rubratoxin caused

marked reduction in liver function and severe reduction in rate of body weight

gain, suggesting that there is synergism between these toxins in cattle as

well as in other species (Richard et al., 1974). As predicted by Stahr in

1970 (Buck, 1970), if a toxicant was present in animal feed, it might be

possible for metabolites of other mycotoxins to also be present.

Moldy corn toxicosis in the north central United States has been reported

to be associated predominantly with infection of Fusarium roseum var.

graminearium and Fusarium tricinctum (Hsu et al., 1972; Mirocha et al.,

1967). Fusarium tricinctum has been reported as one of the most prominent

fungi associated with moldy corn toxicosis in the northern temperate region

(Gilgan et al., 1966; Smalley et al., 1970). Among several mycotoxins

produced by this species, the major toxic metabolite was identified as

4,15-diacetoxy-8-(3-methylbutyrloxy)-scir-9en-3-ol and was designated

as T-2 toxin (Bamburg et al., 1968). The identified toxic metabolites

produced by isolates of Fusarium tricinctum include a butenolid (Yates

et al., 1968), zearalenone an estrogenic compound (Mirocha et al., 1968)

and members of 12,13-epoxytrichothecenes (Bamburg and Strong, 1971). In the last group, T-2 toxin, diaceloxyscirpenol and HT-2 toxin are produced in major quantities, depending on the toxic strain of the fungus and culturing conditions. In addition, alimentary toxic aleukia, moldy bean hull intoxication, stachybotryotoxicosis and fusariotoxicosis have been considered primarily trichothecene intoxications (Smalley and Strong, 1974).

Mycotoxicosis in farm animals is complex since a species of fungus produces not only different kinds, but also different amounts of toxins. Mold toxins are metabolized into even more complex molecules when ingested by animals. Ellison and Kotsonis (1974) demonstrated an in vitro conversion of T-2 toxin into HT-2 toxin by incubation of T-2 toxin with 9,000 X g supernatant fluid of both human and bovine liver homogenates.

Many of the naturally occurring tricothecenes have been reported as being very toxic to rats and mice, having median lethal doses (LD_{50}'s) below 10 mg/kg of body weight when administered orally or intravenously. However, comparative studies using a uniform application method and a single animal species for the acute toxicity of the 12,13-epoxytricothecenes are lacking (Chi et al., 1977). Chi et al. (1977), in their study of acute toxicity of 12,13-epoxytricothecenes in one day old broiler chicks, reported that no deaths occurred in the control groups for each toxin tested. Most deaths in the toxin treated birds occurred during the period of 8 to 60 hours

after dosing. Birds administered with T-2 toxin, diacetoxyscirpenol, died more rapidly after dosing than those intubated with HT-2 toxin or T-2 tetraol. They reported that birds treated with each tricothecene derivative developed similar clinical signs asthenia, inappetence and diarrhea within 4 to 10 hours after dosing. Birds treated with high doses of each toxin were in a coma before death. The weight gain and feed conversion of the surviving birds treated with each toxin decreased proportionally with the amount of each toxin administered.

Methods of Analysis

Studies of aflatoxin metabolism have shown that small portions of any aflatoxin ingested by an animal may be found either as a metabolite or as original aflatoxin, deposited as a residue in some edible tissue (Armbrecht, 1971). Methods of analysis, therefore, involve both aflatoxins per se and their metabolites.

In the past, many methods have been used for the extraction and determination of aflatoxins from products of plant origin. There is a strong possibility that aflatoxin can be found in food products of animal origin just as they are found in cereals, especially when processing and storage practices result in a suitable environment for mold growth. The elaboration of a method for extraction and determination of aflatoxins in meat products, therefore,

seemed purposeful. In the study of Strzelecki (1973), samples of meat were cut, blended and then defatted by a solvent mixture of petroleum ether, hexane and benzene. Other solvents, according to the investigator did not improve the defatting of the meat samples. The extraction of aflatoxins was performed by the use of a methanol water solvent during the blending of the sample and then chloroform extraction from the methanol water layer in a separating funnel to which sodium chloride was added before shaking. Purification of the aflatoxin was achieved by the use of column chromatography. Aluminum oxide covered with anhydrous sodium sulfate was used as the column packing. With this method, he reported an average recovery of 64.4% for the total amount of aflatoxins in raw ham.

There are numerous methods available for the determination of aflatoxins in a number of food products; however, most of these were developed for use on peanut products, seed meals or cereals. The methods employ a variety of solvents and extraction procedures. Aflatoxins can be produced on a number of substrates and the substrate often determines the type of extraction procedure to be used. Since the substrate affects the purity of the extract, not all methods are suitable for all substrates.

Lee (1965) found that direct chloroform extraction of defatted peanut meals in the presence of small amounts of water gave results comparable to methanol extraction. Eppley (1966) verified this finding and improved on

Lee's method by incorporation of a silica gel column purification step. Brown and Abrams (1965) used a method which consisted of a methanol extraction, purification of the extract by transfer from aqueous methanol to chloroform, silica gel column chromatography, partition chromatography according to Pons on a cellulose aqueous methanol column and quantitation by thin layer chromatography, fluorodensitometry for determination of aflatoxin in animal tissues.

Several workers have shown that ^{14}C acetate could be incorporated into aflatoxins (Ayres et al., 1971; Bassir and Osiyemi, 1967; Basappa et al., 1970; Hsieh and Mateles, 1971; Mabee and Chipley, 1973a,b). Several methods have been used for the preparation of ^{14}C-labelled aflatoxin. These involve addition of precursor to rice culture, or to mold culture after mycelial growth or to mold mycelia in nitrogen-free resting cultures. The above methods all have certain disadvantages. One, which produces good yields and good specific activity, involves the difficulty of extracting from a natural commodity, such as rice. Other methods result in B1 either with a low specific activity or with a low percentage of precursor incorporation. The main disadvantage of most of the methods is that they are designed to produce less than 1 mg of labelled B1 aflatoxin.

Jackson and Ciegler (1976) developed a unique technique to prepare ^{14}C-labelled aflatoxin B1 from chlamydospores of Aspergillus parasiticus

NRRL 2999. The chlamydospores result from, according to workers, a lack

of manganese in the synthetic medium. This they pointed out has two advantages.

First, aflatoxin is extracted from a liquid medium instead of a natural commodity.

Secondly, chlamydospores act as resting cell cultures by converting added

nutrients into secondary metabolites rather than into more cell mass. Hence,

the percentage incorporation of labelled acetate is better by utilizing the

chlamydospores of Aspergillus parasiticus NRRL 2999 rather than its hyphal form.

Gupta et al. (1974) studied the production of aflatoxin and acetate

1-^{14}C incorporation with Aspergillus parasiticus NRRL 3240 with two media

1) yeast extract sucrose medium (YES medium) (Davis et al., 1966) and

2) synthetic medium (SL medium) (Reddy et al., 1971). The flasks were

incubated at $26 \pm 1°C$ as stationary cultures. After growing for the requisite

number of days, mycelium and medium were separated and the mycelium was

washed with double distilled water. The mycelium was resuspended in sterile

resuspension medium and incubated for required period.

For incorporation studies, acetate 1-^{14}C was added directly to the

culture or to the resuspension medium. At the end of the experiment the

mycelium and the medium were separated. The mycelium was blotted and its

wet weight determined. The aflatoxins from the medium and mycelium were

extracted with chloroform and were separated by thin layer chromatography

(Gupta et al., 1974).

Jacobson et al. (1970) worked on determination of aflatoxins B1 and M1 in milk. They claimed that the method that was used gave good recoveries within the limits of visual comparison on samples containing 0.5 ppb aflatoxin. In this method, milk was blended with methanol. Deproteinized milk (80% methanolic filtrates) was adjusted to 50% by addition of 4% salt solution and defatted by extraction with hexane. Aflatoxins were extracted from the alcoholic phase with chloroform which was removed in vacuo. The residue was dissolved in benzene hexane (1:1) and chromatographed on a partition column of celite coated with an aqueous solution of calcium chloride to separate aflatoxins B1 and M1. The eluted B1 was passed through another partition column of celite coated with methanol water (1:1) for additional clean up. Aflatoxin eluates were evaporated to dryness and residues were dissolved in chloroform and spotted on thin layer chromatographic plates for fluorescence comparisons.

Specifically, aflatoxin B1 has been found in both the yolk and white of eggs produced by hens receiving aflatoxin B1 experimentally in their rations (Jacobson and Wiseman, 1974; Sawhney et al., 1973). Trucksess et al. (1977) pointed out that the analytical methods used in these latter studies were impractical for routine and survey purposes. They devised a method for the routine determination of aflatoxin M1 in dairy products. The original method specified extraction with acetone-water, precipitation

with a soluble lead salt, defatting with hexane, partition into chloroform,
reverse phase chromatography on a cellulose column and quantitation after
separation on a silica thin layer chromatographic plate. They modified
this method by adding acetic acid to the extraction solvent, using a low
boiling petroleum ether (30-60°C) for defatting, adding a silica adsorption
column for clean up and substituting two dimensional thin layer chromatography
for the reverse phase cellulose column. They stated this modified method
proved practical for use in survey analysis and sufficiently sensitive to
detect aflatoxin B1 at levels less than 1 nanogram per gram in eggs from
hens on experimentally contaminated feed and in "spiked" eggs. They also
pointed out that with this method, recovery was greater than 75% when the
aflatoxin was extracted immediately after spiking and usually less than 30%
when extracted after more than 24 hours.

Amount of lipid affects methods used for different products on cured
and aged meats often are very high in lipid content (Bullerman et al., 1969c),
and this is the determining factor in the selection of suitable extraction and
assay methods. The official AOAC method for peanut products is suitable for
both high lipid peanut butters and defatted peanut meals. With this method,
however, all samples must be subjected to partition column chromatography.

Other methods likewise involve many steps or the use of solvents unsuitable

for extraction of aflatoxin from tissues.

MATERIALS AND METHODS

Aspergillus parasiticus NRRL 2999 was the aflatoxigenic organism used

for the production of aflatoxin B1 in this study. The stock subculture was

maintained by regular subculturing on modified Sabouraud glucose agar

(Abou-Gabal, 1970). The subculture was stored at room temperature for about

two weeks for sporulation. The synthetic liquid medium (Jackson and Ciegler,

1976) was used for ^{14}C-labelling of aflatoxin from Aspergillus parasiticus.

A spore suspension of the A. parasiticus strain was prepared by growing

the fungus at 26°C for a week on a slant of the modified Sabouraud glucose

agar and then harvesting the surface growth in 10 ml sterile saline solution.

One liter of the synthetic liquid medium in a Fernbach flask was inoculated

with the saline spore suspension. The inoculated liquid medium was incubated

at 28°C in a water bath on a rotary shaker (Gyrotory shaker, New Brunswick

Scientific Company, New Jersey) at slow speed. After 48 hours of incubation,

100 microcuries of sodium acetate-2-^{14}C (New England Nuclear Corp.,

Boston, Massachusetts) was added to the Fernbach flask and another 100

microcuries of the same sodium acetate-2-C^{14} was added after 60 hours of

incubation. Incubation was continued for additional 24 hours. The culture

formed chlamydospores within 40 hours of incubation (Plate 1). An electron

micrograph of a cross section is shown in Plate 2.

The culture was labelled in two stages as indicated because aflatoxin

production was shown to lag behind growth, (Jackson and Ciegler, 1976), and was not

initiated until about 40 hours but was essentially completed by 72 hours. Also

coinciding with the beginning of aflatoxin production, a yellow color appeared

in the culture broth and it increased in intensity with time. According to

Jackson and Ciegler (1976) precursor addition is best made on first appearance

of yellow pigment (about 48 hours after inoculation).

Extraction

After incubation, the chlamydospores were filtered out and the medium was

extracted twice with equal volumes of chloroform. The chloroform extract was

dried with anhydrous sodium sulfate and then taken to dryness in the flash

evaporator. Residual solids were dissolved in 5 mls of chloroform for thin layer

chromatography.

Thin Layer Chromatography

Thin layer chromatography was used for separation of the aflatoxins. Thin

layer chromatography was done twice. The chloroform solution of the extract

was spotted on a thin layer chromatographic plate of 2 mm thick layer of silica

gel G-60 and developed in an unlined unequilibrated tank of acetone-chloroform

(12 + 88). A long wave (360 nm) ultraviolet light (UVL 56, Ultraviolet Product,

Inc., San Gabriel, California) was used to visualize the aflatoxins. Standards

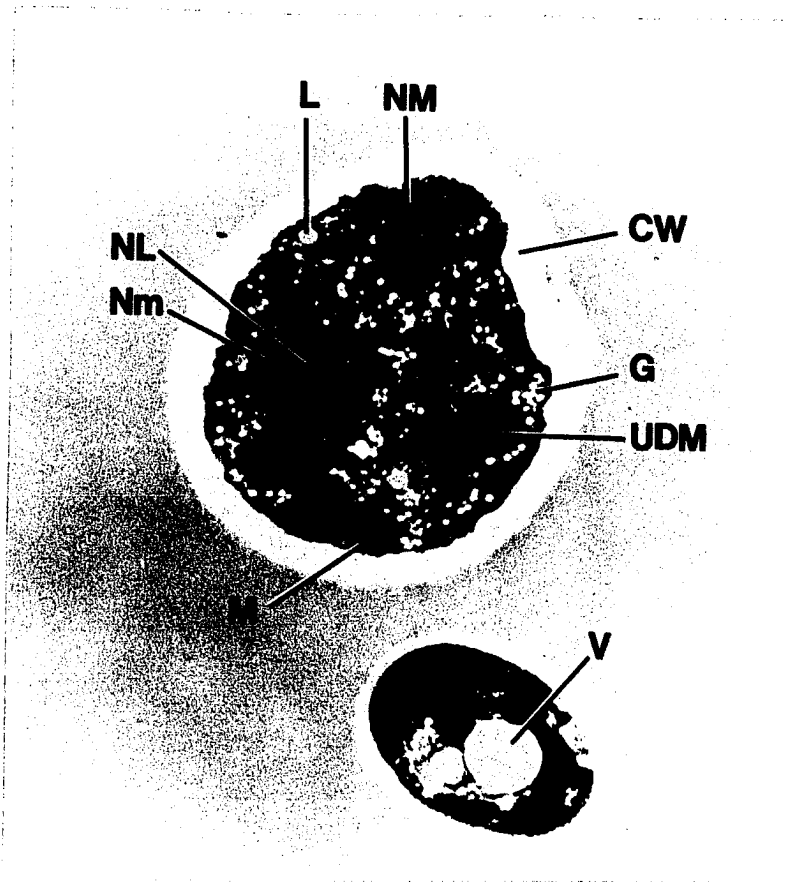Plate 1. Chlamydospores of *Aspergillus parasiticus* (NRRL 2999) from synthetic liquid medium.
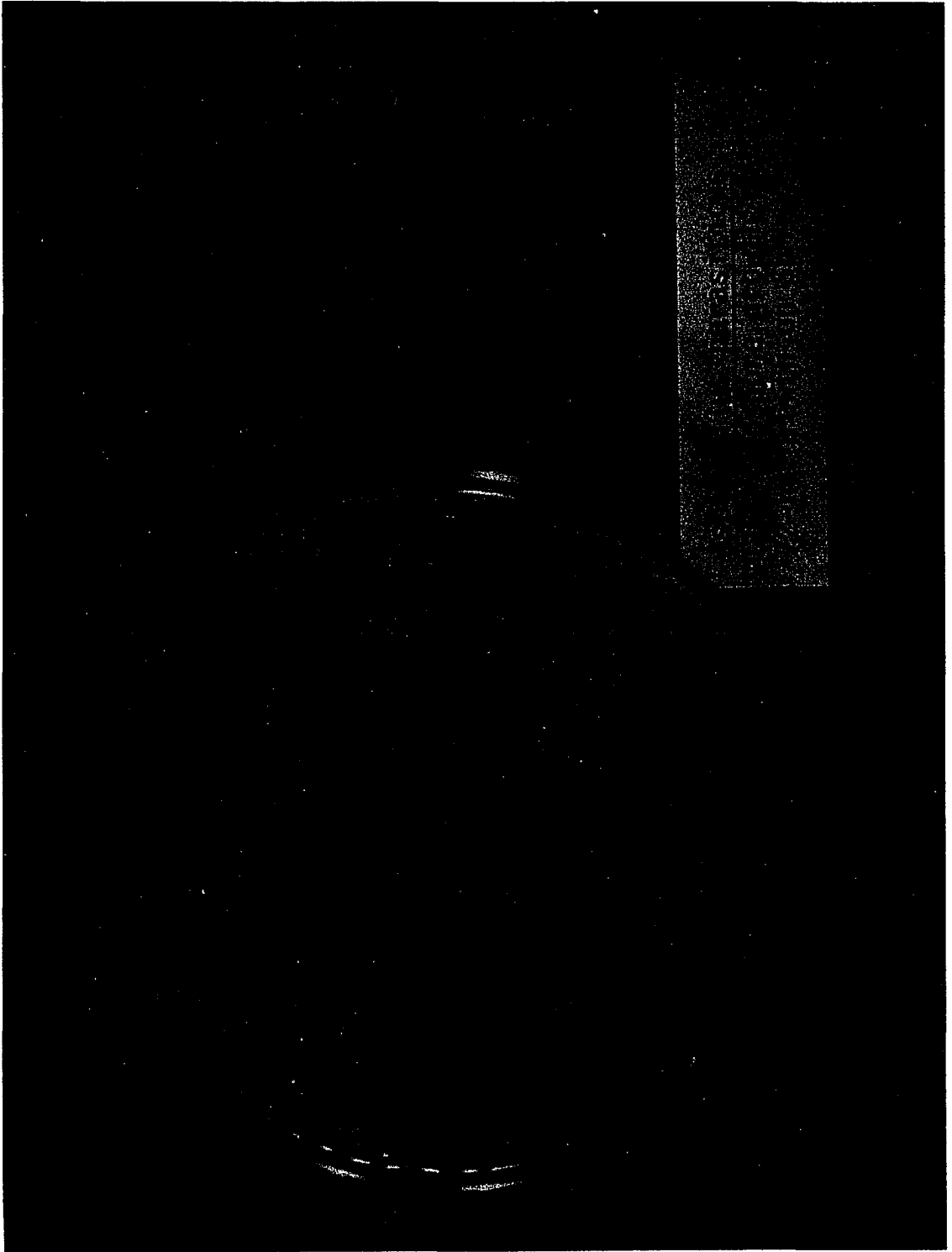
Plate 2. Electron micrograph of the chlamydospore growth phase
of A. parasiticus on synthetic liquid medium. CW -
cell wall; NM - nuclear material; L-lipid; NL-nucleus;
Nm-nuclear membrane; M-mitochondria; V-vacuole;
UDM-unidentified deposited material. (19,000X)

were spotted on the same plate as the samples. The aflatoxin B1 and B2 band were scraped into a flask containing 100 mls of chloroform-methanol (70 + 30). The G1 and G2 toxins were also scraped into a similar solvent. Both flasks were agitated by hand and then were allowed to stand overnight. The silica gel was filtered off using glass fiber filter paper 934AH (Fisher Scientific Company) and the chloroform-methanol was then taken to dryness by blowing a gentle stream of nitrogen over it. Small amounts of chloroform (5 mls) were again added to the flask to redissolve the residue. The chloroform containing the residue rinsed from the flask was again spotted on a thin layer plate. Standards were also spotted on the same plate as the samples. Aflatoxin B1 and B2 were visualized with a long wave ultraviolet light. Both bands were separated by scraping each band into a different flask. The B1 was scraped into a flask containing 100 mls of chloroform-methanol (70 + 30) and the silica gel was filtered off as previously described. The solution was dried by passing a gentle stream of nitrogen over it. The flask was thoroughly rinsed with 10 mls of chloroform and the solution was measured in a UV spectrophotometer (Spectronic 600, Bausch and Lomb, Rochester, NY) to determine the amount of aflatoxin B1 present.

The radioactivity of aflatoxin B1 was determined by a liquid scintillation spectrophotometer system. A known quantity of the above chloroform containing

B1 toxin was transferred into a scintillation vial and a gentle current of air was passed over it to evaporate the chloroform. Then 15 mls of ^{14}C-cocktail was introduced into the vial and the vial was capped. The same amount of chloroform containing aflatoxin B1 was added to a combustion boat containing a small amount of mannitol. The contents of the boat were combusted in a biological material oxidizer (R. J. Harvey Instrument Corporation, Hillsdale, New Jersey). Oxygen gas was admitted at the rate of 300 mls per minute to facilitate complete combustion. The combustion products were passed through a series of catalysts at 700°C and the carbon-14 was trapped in the ^{14}C-cocktail. The cocktail was transferred into another vial and capped. Both vials were placed in a scintillation counter (Packard Model 2425 Tri-Carb) for measurement of the activity. The activity of the uncombusted material was used to calculate the percent incorporation of the radioactivity or acetate into aflatoxin B1. In this case the percent incorporation was less than 2%. Also, activity of the noncombusted material was compared to the activity of the combusted material to obtain the percent recovery of activity during combustion since the tissue analysis was to be done by combustion. The average yield of labelled B1 was 8.7 mg per 500 ml medium with specific activity of 68.2 mCi per mole.

Autoradiography

Sodium acetate-2-^{14}C-labelled aflatoxin was spotted on thin layer chromatographic plate and the plate was placed face down on an X-ray film
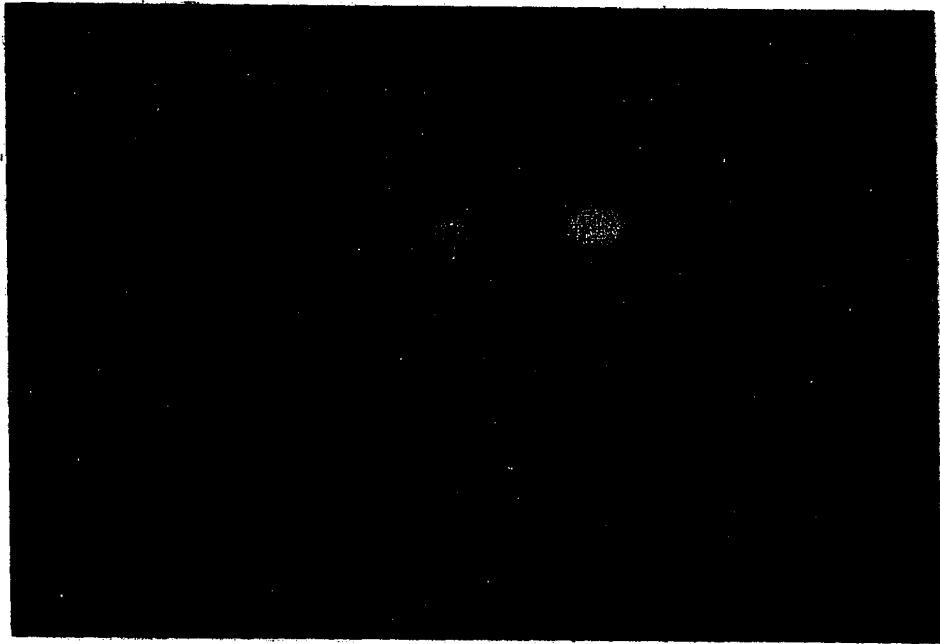
Plate 3. Thin layer chromatographic plate of labelled aflatoxin B1 extract from A. parasiticus (NRRL 2999).
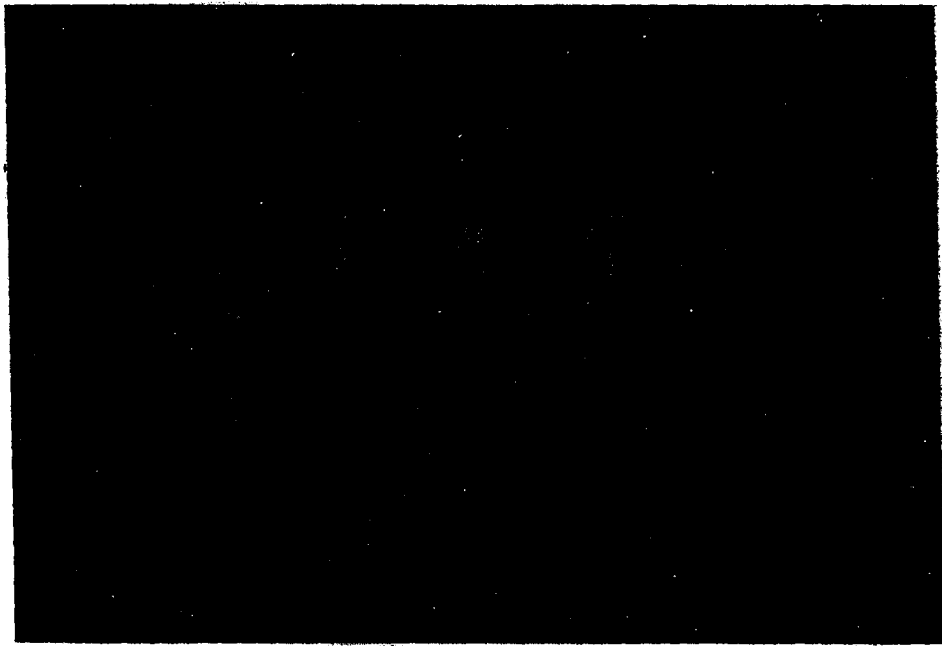
Plate 4. Thin layer chromatographic plate of labelled aflatoxin
B1 extract from A. parasiticus (NRRL 2999) with X-ray
film superimposed to show radioactive spot (dark spot).

in the dark and stored in darkness for 72 hours. The film was processed and dark spots on the processed film designated corresponding area of radioactivity on the thin layer chromatographic plate (TLC).

In this work, autoradiography was used to confirm the radioactivity of aflatoxin B1 that was fed to chickens. Aflatoxin B1 from the labelled culture was extracted and separated as described previously and visualized under UV light (Plate 3). The plate was placed on X-ray film as described above. After the film was processed, the intensity of radioactive spots varied with amount spotted on the plate when measured in a densitometer. The spot with 100 μl of the extract was most visible. The film was superimposed on the TLC plate under UV light to show the radioactivity spot (Plate 4).

Biological Material Oxidizer

The biological material oxidizer combusts any biological and most organic materials (wet or dry) in a stream of oxygen gas at 900°C, passes the combustion products through a series of catalysts at 700°C, then traps the ^{14}C-dioxide. The furnace compartment of the biological material oxidizer is divided into two portions which are maintained at separate temperatures. The combustion side (the side in which the sample is introduced) is maintained at 900°C, and the catalyst bed side (the side to which the traps are attached) is maintained at 700°C. The combustion side initiates the oxidation of organic materials and converts the sample to the gaseous state. The gaseous combustion products are swept through the catalyst by the oxygen gas stream. The catalyst bed converts any compound

fragments, which have not been completely oxidized, to carbon dioxide and

water. The catalyst bed is maintained at 700°C within narrow limits, since

this temperature allows the bed to operate at optimum efficiency. Operation

above 700°C destroys the catalyst physically and operation below 700°C

reduces its efficiency to the point where incomplete oxidation may result.

The gaseous forms of other compounds are removed at this time by the

catalyst bed; only oxygen, nitrogen and nitrogen oxides are expelled from

the combustion tube exit into a trap. These gases have little effect on the

counting efficiency of the trapping-counting solutions.

Feeding Studies

For the feeding studies, the labelled and unlabelled (Calbiochem)

aflatoxin B1 were mixed and a concentration of 1 mg/ml of aflatoxin B1

was made by dilution with chloroform and by measuring with the UV

spectrophotometer. Since chloroform is toxic to chickens if used as

a solvent for toxin, gelatin capsules were utilized instead. With

a chloroform solution of aflatoxin B1 of 1 mg/ml, 100 μl pipettes were

used to introduce the solution into empty #5 gelatin capsules (Eli Lilly and

Company, Indianapolis, Indiana). The capsules were left open in the dark

inside the hood to allow the chloroform to evaporate and to leave the aflatoxin

as a residue inside the capsule. This was done 10 times to get 1 ml of the solution into the capsule because 1 ml was calculated to contain 1 mg of aflatoxin. The capsules were then covered. Two capsules were then analyzed individually for aflatoxin content and each was found to contain approximately 1 mg/ml of aflatoxin B1. Also, two capsules were combusted individually to get an idea of radioactivity content of each capsule to be fed to chickens.

The chickens used in this experiment were obtained from the Iowa State University poultry farm. They were three week old chicks. The chickens were fasted for 12 hours before feeding the aflatoxin capsules. The chickens were divided into four groups of eight; one group was fed capsules containing ^{14}C-aflatoxin; another group received capsules with solvent but with no aflatoxin added. This group served as a control for the solvent in which the aflatoxin was dissolved. The third group received empty gelatin capsules to serve as a control for the gelatin capsules. The last group remained as the chicken control with no capsules given.

The capsules were fed to chickens by using a pair of tweezers to place the capsule behind the tongue after the beak was opened. The beak was then held shut for a moment, forcing the chicken to swallow. The chickens

were then given only drinking water for the next two hours before any feed was provided. Two chickens from each group were necropsied after decapitation with a pair of scissors at 12, 24, 48 and 72 hours. The following organs were collected for analysis: liver, kidneys, heart, lungs, gizzard, gastrointestinal tract, also breast muscle, and blood. The feces were collected from the cage each time chickens were necropsied. These samples were individually weighed and homogenized in a Sorvall mixer. A known amount of each sample was taken out from the homogenate for determination of ^{14}C activity and the rest was analyzed for aflatoxins. For the radioactivity, the samples were combusted in order of collection.

Sausage

The same A. parasiticus strain NRRL 2999 was used as inoculum for the sausage study. The fungus was grown as described previously. The spore suspension was prepared two hours before inoculation in a sterile saline solution. Three loopsful of spores and or mycelial fragments were inoculated from the agar slant cultures into a sterile saline solution for the sausage inoculum.

The sausage was made from pork with a ratio of lean to fat of 80 to 20. The meat was obtained from the Iowa State University meat laboratory. The sausage was made in two five pound batches. Each batch had 2.5% NaCl and one batch was made to have 156 ppm $NaNO_2$ and the other 200 ppm $NaNO_2$. The sausage was stuffed into a collagen casing and made into links

about the size of weiners (hot dogs). After the sausage was made, it was

stored at 5°C in a walk-in cooler for approximately 24 hours before inoculation.

Each sausage was inoculated with 0.2 ml of the <u>Aspergillus parasiticus</u> spore

suspension by injecting the spores with a 1.0 ml sterile syringe into the sausage at

different locations. After inoculation, the sausages were divided into three

groups. Each group consisted of sausages containing 0 ppm (control),

156 ppm and 200 ppm nitrite. The three groups were incubated at three

different temperatures: 5°, 26°, and 37°C. Sausages from which samples

were taken to analyze for residual nitrite and water activity were incubated

at 26°C. The sausages were analyzed for aflatoxins at 0, 3, 7, 14, 21, and

28 days. Residual nitrite and water activity were determined at each sampling

time.

Analysis for Residual Nitrite

Residual nitrite in samples was determined spectrophotometrically as

described by methods of the Association of Official Analytical Chemists in

section 24.014 and 24.015 (AOAC, 1970). The reproducibility of nitrite

determinations was considered to be about 5%.

Modified Greiss Reagent was prepared in this manner: Five-tenths

of a gram of sulfanilic acid, (Fisher certified ACS) was dissolved in 150 mls

of 15% (v/v) glacial acetic acid (Fisher reagent). One-tenth gram of

α-naphthylamine (Baker chemical) was boiled in 20 ml deionized, distilled

water in a hood until dissolved and was mixed with sulfanilic acid solution while still hot. This mixture was tightly capped and stored in a brown glass bottle in a refrigerator.

The standard curve was prepared as follows: 0.1514 g of sodium nitrite (Baker analyzed reagent, 99.1% sodium nitrite) was dissolved in 100 ml of distilled deionized water in a volumetric flask. One ml of this solution was further diluted into a liter of distilled deionized water. Each ml of the solution was equal to 1 μg of sodium nitrite per ml of water or 1 ppm nitrite. Aliquots of 1, 3, 5, and 10 ml of the 1 ppm nitrite solution were pipetted into separate 50 ml volumetric flasks and then diluted with deionized distilled water to 50 mls. The nitrite concentration in each flask was 0.0, 0.06, 0.1 and 0.2 ppm respectively. Two ml of Greiss reagent was added to each flask and mixed well then allowed to stand at room temperature for approximately one hour for color development. About 5 mls of the solution was transferred to a cuvette and absorbance measured at a wavelength of 520 nm in the spectrophotometer.

Determination of Residual Nitrite in Sausage

A five-gram sample was removed from each sausage group and blended with 50 mls of 80°C deionized distilled water in a Waring blender for two minutes. The homogenized sample was transferred into a 500 ml volumetric flask and the blender jar was rinsed with 250 ml distilled water (80°C). The 500 ml flask

containing the blended sample with the rinse water was placed on the steam bath for two hours with occasional shaking. After steaming, 5 mls of saturated mecuric chloride solution (Analytical Reagent, Mallinkrodt) was added to the hot mixture and mixed vigorously by shaking. The mixture was allowed to cool at room temperature and the flask was filled to the mark with deionized distilled water. The mixture was filtered through Whatman #1 fiber glass filter paper. Different aliquots of the filtrate were transferred into 50 ml volumetric flasks and diluted to the mark with deionized distilled water and then 2 mls of Greiss reagent was added. Flasks were allowed to stand for one hour for color development. About five mls of the solution was transferred into a cuvette and absorbance of the solution was measured with the spectrophotometer at 520 nm. The residual nitrite concentration was determined from the standard curve.

Study of the Effects of Nitrite on the Growth and Sporulation of <u>A</u>. <u>parasiticus</u>

A weighed amount of sodium nitrite (Baker's reagent) was dissolved in a known volume of sterile distilled water. Modified Sabouraud glucose agar was autoclaved and cooled down to 45°C. The nitrite solution was mixed with the agar to give a concentration of 50, 156, 200, and 500 μg of nitrite per ml of medium. Four plates were poured for each concentration. Control plates containing no nitrite were included.

Two week surface growth of <u>A. parasiticus</u> was harvested in 10 ml of sterile saline as described previously. Two-tenths of a milliliter of the spore suspension was placed on the surface of the agar and was aseptically spread in a circular fashion with the aid of inoculating loop. All plates were incubated at 24°C and the rate of both growth and sporulation was checked every two days for a period of four weeks.

Water Activity in Sausage

The samples were equilibrated and the water activity was measured with a hygrometer (Model 15-3050, American Instrument Co., Silver Spring, MD). Measurements were made as follows: Twenty grams of sample from each nitrite level were placed in mason jars and capped with sensor-fitted screw caps. The hygrosensors (type Th-3) were made up of a strip of paper coated with a hygroscopic material (lithium chloride) and enclosed in an aluminum case and sealed at the factory. The closed system (mason jars) was allowed to equilibrate at room temperature for about four hours and equilibrium relative humidity (E.R.H.) readings were taken every hour. Known correction values were applied to each sensor's reading. The four hour readings were averaged and converted to water activity values.

Scintillation Counting

The combustion boats were boiled in concentrated nitric acid and then placed in a muffle furnace at 600°C overnight to completely burn off any organic material that was not removed by the nitric acid.

The biological material oxidizer operated overnight with a stream of oxygen going through it to condition the tube for combustion of samples. The following morning, the samples to be combusted were weighed into the combustion boats. Mannitol was weighed into three boats. The first mannitol portion was combusted to prime the tube and the total combustion cycle was four minutes. Fifteen mls of carbon 14 cocktail was transferred into the collection tube and then attached in position. The second mannitol boat was then combusted and the vapor bubbled into the cocktail. This served as a blank to correct for the background. A known amount of sodium acetate with known amount of activity was added to the third boat of mannitol and combusted and collected in another 15 mls of cocktail. Equal amount of sodium acetate as above with known amount of activity was added directly into 15 mls of the cocktail without combustion. From counts obtained from the non-combusted and the combusted acetate, relative efficiency of the system or percent recovery could be calculated after background adjustment with the blank. The samples were combusted in a similar way and collected in 15 mls of ^{14}C cocktail. The collection tube was rinsed thoroughly with methanol.

between samples. After combustion of two samples a mannitol blank was combusted and collected in 15 mls of the "cocktail" to check the rinsing of the tube between samples. After the last sample was combusted, another blank and another ^{14}C-acetate was tested to check reproducibility of the system and analysis throughout the combusting operation. The vials were then kept in a walk-in cooler for 24 hours to cool down. Before counting, the vials were allowed to warm up to room temperature and then set into the scintillation counter and allowed to stay for one hour to adjust to the conditions inside the counter, after which the counting was started. The counter was set to count for two cycles of 10 minutes and two counts on each vial.

Analysis of Sausage for Aflatoxin B1

Chemicals used in this work were analytical reagent grade. Solvents were re-distilled in glass except where otherwise indicated.

This study was designed to determine if known aflatoxin producing mold could produce aflatoxin in meat under different storage conditions. The extraction of aflatoxin from tissues is hampered by pigments, fats, that are soluble in chloroform. With pork, the primary interfering substance is fat.

Since a large number of samples were to be analyzed in this study, it was important that the method be simple and fast without sacrificing accuracy and precision. Direct chloroform extraction was used by Lee (1965) to extract

aflatoxin from defatted peanut products in the presence of a small amount of water and this provided quantitative recovery of aflatoxin. Eppley (1966) modified Lee's method by addition of a silica gel column purification step similar to that used by Pons et al. (1966). Both methods were further improved by Bullerman et al. (1969c) by extracting twice with chloroform and by shaking the mixture of homogenized tissue and chloroform for thirty minutes.

In this study, the sausage was weighed and then blended with 200 mls of acetonitrile:water (90:10) in a Waring blender. One hundred milliliters of the acetonitrile water extract was filtered through a filter paper into a measuring cylinder.

The extract was then transferred into a separatory funnel where it was defatted twice with 100 mls of petroleum ether. The petroleum ether layer was discarded. One hundred milliliters of water was then added to the separatory funnel with the defatted extract; 100 mls of chloroform was added and the funnel and its contents were vigorously mixed by shaking by hand. Chloroform was then allowed to separate from the water. The chloroform layer was drained into a beaker and 0.5 g of charcoal was added to remove some pigments that interfere with analysis or occur at about the same retention time as aflatoxin B1. The added charcoal was stirred and left in contact with the chloroform extract for about two minutes, and then, the cleaned extract was filtered into an Erlenmeyer flask through a glass fiber filter

paper. The chloroform was concentrated on a steam bath under nitrogen. One ml of chloroform:methanol (70:30) mixture was used to redissolve the sample and 0.1 ml was spotted on a thin layer chromatography plate. An aflatoxin standard of 20 nanograms was spotted on the same plate. The plate was developed in one direction with chloroform-acetone-2-propanol (85:10:5) in an unlined unequilibrated tank. When development was complete, the plate was removed from the tank and allowed to air dry in a hood.

The plate was examined in the dark under long wave ultraviolet light. From the aflatoxin B1 standard spotted on the plate in the reference channel any aflatoxin band with the same retention time as the standard was scraped from the plate into vials. Three mls of chloroform:methanol (70:30) were added to the vials which were vigorously shaken by hand. Vials were allowed to stand overnight for the silica gel to settle. The solution was filtered through filter paper (934AH; Fisher Scientific Company) into another clean vial.

The same procedure described above was also used to analyze chicken tissues, except for a few changes. In the extraction solvent methanol is substituted for acetonitrile. The tissue was extracted with 100 mls of the extraction solvent by blending in a Sorvall mixer and the entire 100 mls were filtered into the separatory funnel. Extraction was done as described previously. The chloroform extract was concentrated on a steam bath,

re-dissolved, and concentrated again. Chloroform was again added and 0.1 ml

or 50% of the sample was spotted on a thin layer chromatographic plate.

For both tissues, concentration of aflatoxin was determined by measuring

the absorbance of the filtered solution in the spectrophotometer at 362 nm. The

amount of aflatoxin present was calculated with the following formula:

$$A = e \; l \; c, \text{ where}$$

$$A = \text{absorbance reading of the sample}$$

$$e = \text{extinction coefficient of aflatoxin B1}$$
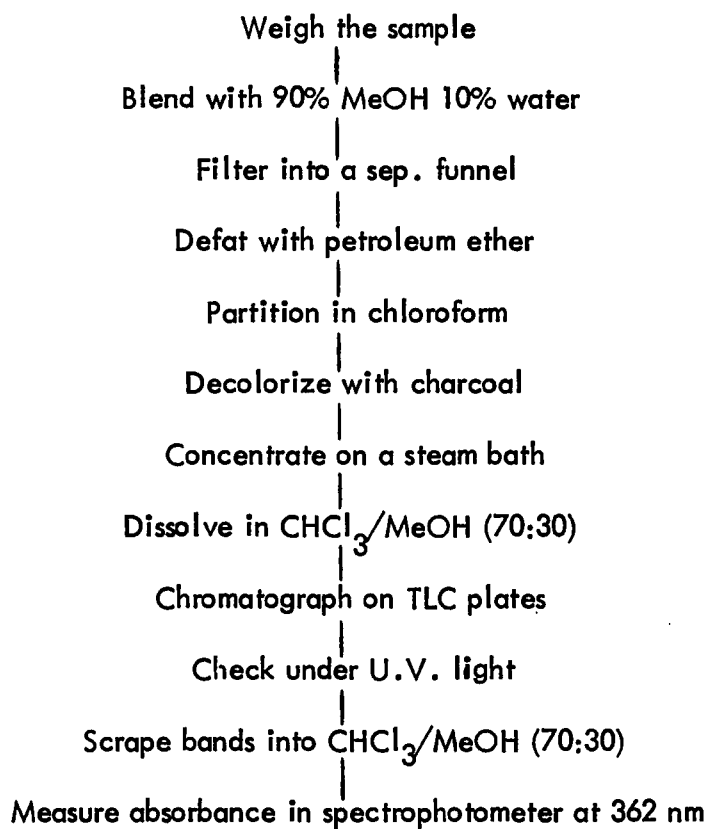
$$l = \text{length of UV cell}$$

$$c = \text{concentration}$$

A flow chart for aflatoxin analysis is presented in Figure 4.

Preparation of Thin Layer Chromatographic Plates

Glass plates of 20 X 20 cm were cleaned in an acid bath, rinsed in

milipore filtered water, and then oven dried. Forty grams of silica gel

G-60 for thin layer chromatography was weighed into a bottle with 90 mls

of 95% ethanol added and vigorously mixed by shaking. A thickness of 2 nm

of the silica gel-ethanol slurry was coated on the 20 X 20 glass plates lined

on a plexiglass mounting board, with the aid of stainless steel adjustable

applicator for coating (Brinkman Instruments, Inc., New York, NY).

The plates were dried for 2-4 hours in the hood to allow the ethanol to completely

evaporate. The plates were then activated by drying in the oven overnight at

Analysis of tissues for aflatoxin.

Weigh the sample
|
Blend with 90% MeOH 10% water
|
Filter into a sep. funnel
|
Defat with petroleum ether
|
Partition in chloroform
|
Decolorize with charcoal
|
Concentrate on a steam bath
|
Dissolve in $CHCl_3$/MeOH (70:30)
|
Chromatograph on TLC plates
|
Check under U.V. light
|
Scrape bands into $CHCl_3$/MeOH (70:30)
|
Measure absorbance in spectrophotometer at 362 nm

Figure 4. Flow chart for aflatoxin analysis.

105°C. Plates were stored in a desiccator to avoid absorption of moisture and thus deactivation.

Aflatoxin Standards

The aflatoxin standards used in this study were purchased from Calbiochem (San Diego, California). Ten milliliters of chloroform was added to the vial to dissolve the contents and dilutions were made to obtain a working standard of 1 nanogram of aflatoxin B1 per microliter of the solution. The concentration was confirmed by measuring the absorbance of the solution at 362 nm in a U. V. spectrophotometer. The sample was also spotted on a thin layer chromatographic plate and developed in unlined unequilibrated tank with chloroform-acetone-2-propanol (85:10:5) to confirm its purity by the presence of only one band when the TLC plate was examined in the dark under long wave UV light for fluorescence.

Scirpene Toxins

Another study was designed to determine the effect of scirpene toxins on fertile eggs as a biological test for the toxins. The toxins used were diacetoxyscirpenol (DAS), T-2 toxins, and Vomitoxin. DAS and T-2 toxins were obtained from Calbiochem (San Diego, California) and Vomitoxin from Dr. R. F. Vesonder (NRRL, Peoria, Illinois). The eggs were checked for live embryos by candling. A hole was drilled through the shell on top of the air sac.

The eggs with live embryos were divided into four groups of thirty eggs. One group received 0.1 microgram of ethanol into the air sac introduced with a micro syringe; groups 2, 3, and 4 received 0.1 microgram of DAS, T-2 toxin and vomitoxin respectively. After inoculation the drilled hole was sealed with Duco cement (DuPont). The eggs were then stored in an incubator and were checked for live embryos by candling after three weeks. A second batch of eggs of four groups with thirty eggs per group were again inoculated with the same toxin but with one-tenth the concentration of the above and were incubated for hatchability for 21 days.

RESULTS AND DISCUSSION

Effect of Temperature on Aflatoxin Production in
Sausage Containing Nitrite

Among the factors considered in this study in relation to aflatoxin

production in pork sausage, temperature had the most pronounced effect

on both toxin production and growth of the organism. With both 200 and

156 ppm added nitrite the highest level of aflatoxin B1 was obtained at 26°C

(1.7 μg per gram) with storage for 28 days. The lowest amount detected

at 26°C was 0.01 μg per gram after three days of storage. AT 37°C the

greatest level was 0.80 μg per gram, this was found with 200 ppm added

nitrite after 7 days. The mold grew profusely all over the sausage with

greenish yellow aerial growth. Sausages became sticky and the casing

dissolved at the point of contact with another sausage. Sausage at 37°C

became oily due to fat exuding because of the temperature of the

environment. Control samples were covered with fluffy white growth

unlike the treated samples, but were also sticky and slimy.

Sausages at 26°C were similar to samples incubated at 37°C except

that they were less oily and the casing was not dissolved at the point of

contact with other sausages of the same group.

No aflatoxin was detected in samples stored at 5°C for the same

time periods as sausage stored at other temperature. There was no

mold growth and sausages appeared acceptable. Storage of sausage at

5°C, therefore, provided a means of retarding mold growth and aflatoxin formation.

Several workers have produced more aflatoxin B1 both in liquid media and natural substrates than was observed in the present study. In those cases, the work was done with sterile substrates whereas the meat used in this study was not sterile. While an attempt was made to keep other microbial competition as low as possible by thoroughly cleaning the sausage making equipment, with the long incubation time required for the growth of A. parasiticus, it is very likely that competitive bacteria and yeasts were present in the natural flora of the meat. Also, with mixed cultures, the possibility of microbial detoxification of any small amounts of aflatoxin that might have been produced cannot be ruled out. From visual observation, it appeared that pork sausage stored at 26 and 37° C would become unacceptable due to the growth of bacteria and yeasts before the sausage would become toxic as a result of A. parasiticus growth.

Different temperatures have been reported as optimum for growth of A. parasiticus. These temperatures range from 18 to 30°C, although different temperatures were used with different substrates. Diener and Davis (1966b) reported maximum yield after 15 days at 20°C or 11 days at 30°C. In work reported here yield at 26°C was observed to increase with time up to a maximum at 28 days or at 7 days at 37°C (Figures 5 and 6). The longer the storage time, the higher the accumulation of aflatoxin at 26°C. However, there did not seem to be any significantly greater growth of the mold with increase in storage time after 14 days. At 37°C, profuse growth of the mold

Figure 5. Recovery of aflatoxin in sausage with 200 ppm
            added sodium nitrite and stored at 26° and 37° C.
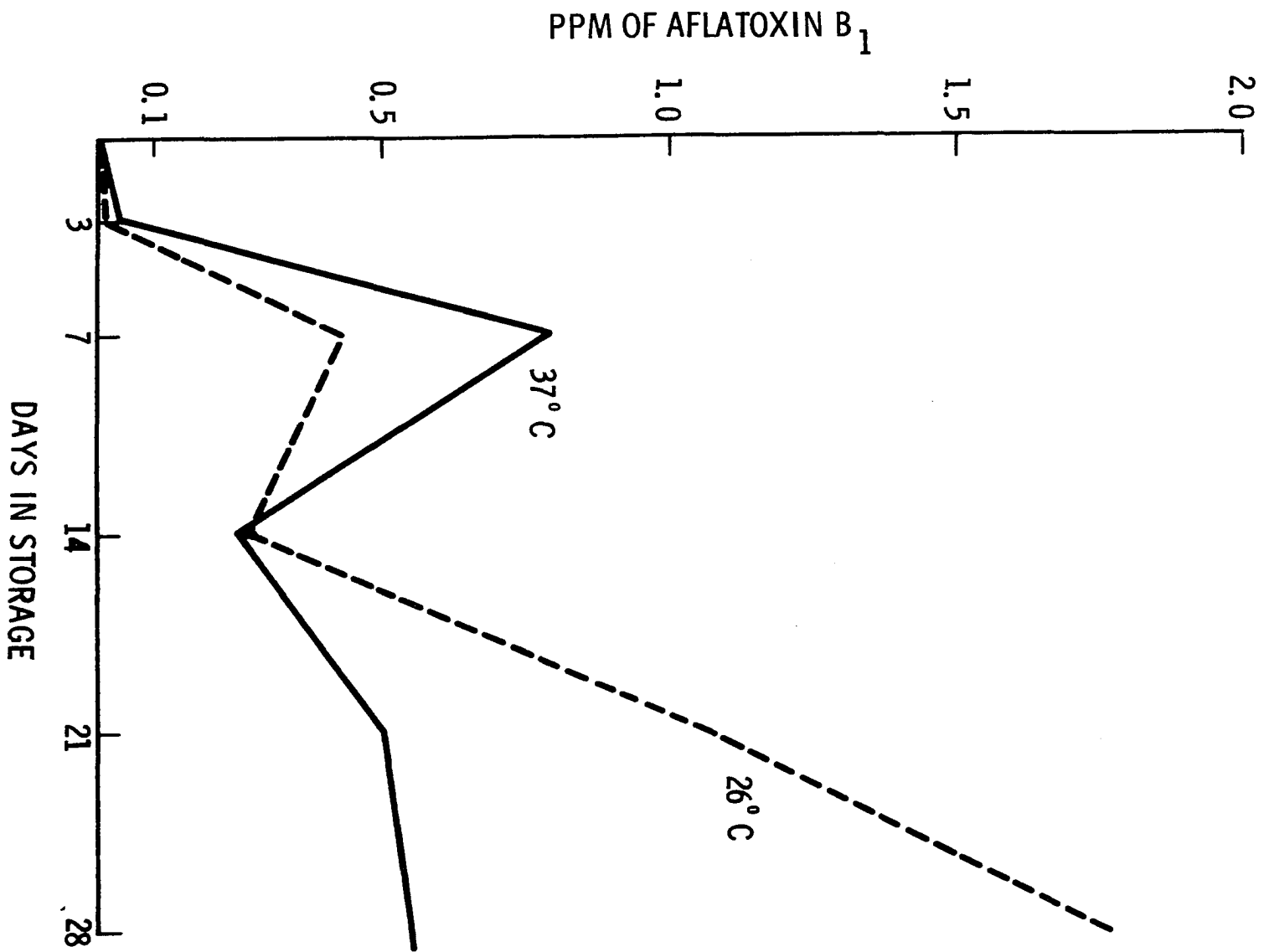
Figure 6. Recovery of aflatoxin in sausage with 156 ppm added sodium nitrite stored at 26° and 37° C.
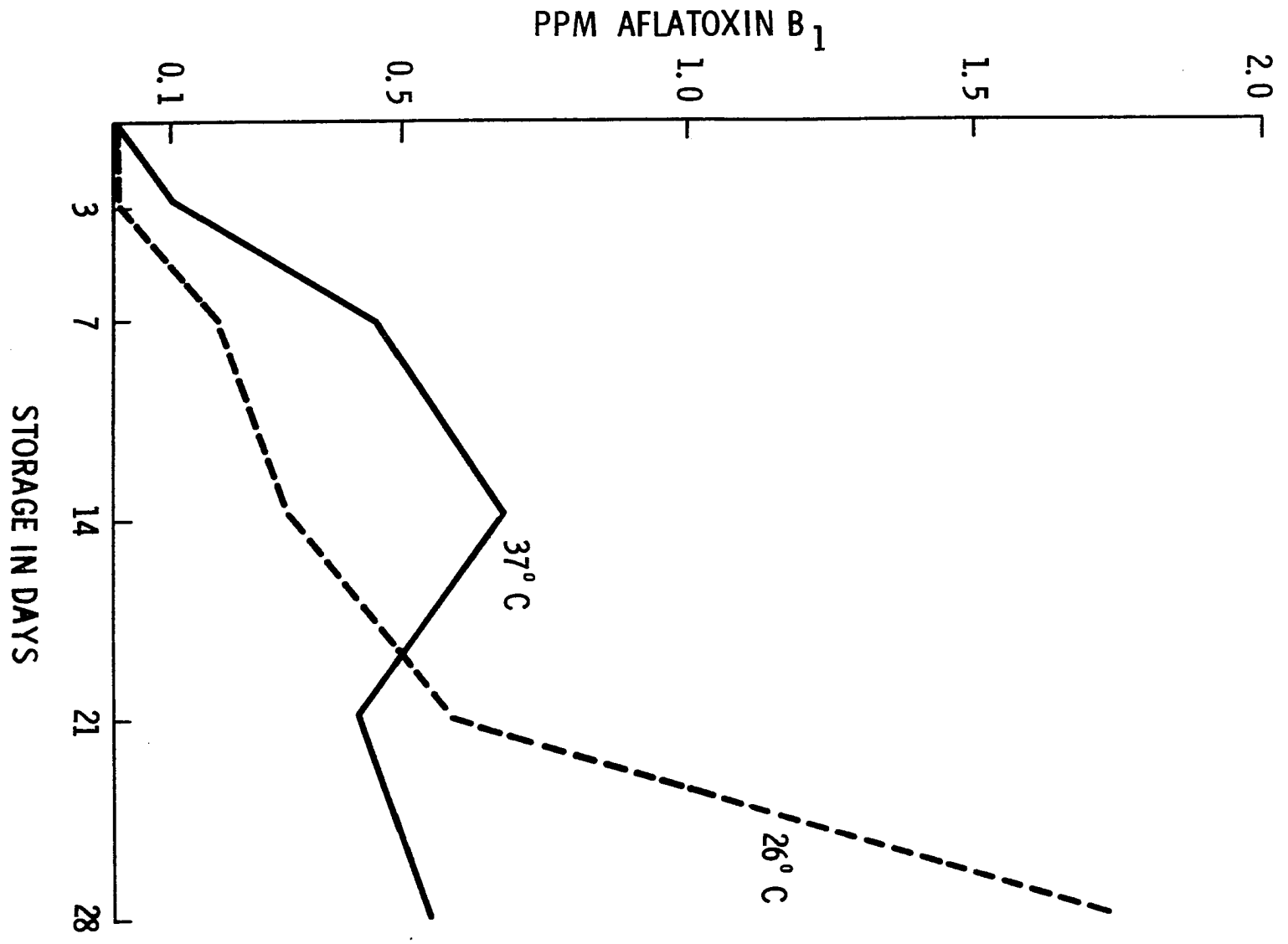
occurred in the sausage samples earlier than it did at 26°C. A difference

was noted in the rate of aflatoxin production at the two temperatures. At

37°C, for sausage samples with 200 ppm $NaNO_2$, the highest aflatoxin

production occurred after 7 days of storage (Figure 5) and declined as

sharply as it had increased. Similar samples incubated at 26°C

showed a peak at 7 days of storage and aflatoxin accumulation was not

as high compared to storage at 37°C after 7 days. However, at 26°C,

aflatoxin accumulation had declined by the 14th day of storage, and then

reversed the trend to continually increase after 14 days up to 28 days of

storage. Aflatoxin production at 37°C appeared to be reaching a plateau

by 28 days. Samples with 156 ppm incubated at both 26 and 37°C

(Figure 6) showed some differences from sausage containing 200 ppm

sodium nitrite (Figure 5). The peak of aflatoxin production for 156 ppm

$NaNO_2$ samples incubated at 37°C occurred on the 14th day (Figure 6)

as opposed to 7 days for 200 ppm $NaNO_2$ (Figure 5); while samples incubated

at 26°C showed a distinct trend toward continuous accumulation of aflatoxin

up to 28 days of storage.

Changes in aflatoxin B1 concentration are emphasized by the graphs

in Figures 5 and 6. It is generally accepted that even in pure culture,

aflatoxin concentration falls after reaching a peak and this study indicated

that the time when the peak was reached was a function of temperature.

Other investigators observed that certain molds, including A. niger and

A. flavus caused a decrease in the total amount of aflatoxin B1 which was

originally present when the molds were grown in an aflatoxin containing

liquid substrate or in toxin containing peanuts (Ashworth et al., 1965).

About a year later, Ciegler et al. (1966) observed the same phenomenon

when aflatoxigenic strains of A. parasiticus and A. flavus were studied.

However, no one has yet fully determined the conditions which are optimal

for biodegradation of aflatoxin or the mechanism which controls it (Doyle

and Marth, 1978b).

In this study, the decline in aflatoxin B1 concentration could be due to

one or two things: a) since the sausage samples were not sterile, there exists

the possibility of bacterial detoxification of aflatoxin B1, or b) mycelial

degradation of aflatoxin B1 could have occurred. Graphically, it may be

seen that the rate of aflatoxin B1 accumulation is proportional to the rate

of decline at both temperatures. At higher temperature, A. parasiticus produced

more mycelia and less aflatoxin than it did at the lower temperature this

agreed with the work done by Shih and Marth (1972). It is then possible

that the presence of large numbers of mycelia and large numbers of yeasts and

bacteria may be the cause of the sharp decline in the concentration of

aflatoxin B1 after it had reached a peak. Several theories to explain this

observation have been propounded. Ciegler et al. (1966) showed that

nondegraders of aflatoxin B1 could be induced to degrade aflatoxin by

lysis of the mycelium and they suggested a nonspecific chemical mechanism

of degradation. It is possible that at higher temperatures most of the

available nutrients were utilized for more mycelial and spore formation and

not enough remained for conversion into secondary metabolites after

degradation of aflatoxin. Hence a high initial accumulation occurred with

less accumulation with time. At $37°C$, aflatoxin B1 was degraded to a

point and then it started to accumulate again but not to as great a level

as its previous high concentration. Conversely, at $26°C$, after an early

decline in the amount of aflatoxin B1 present (Figure 5), the amount that

accumulated with time increased sharply. The dissimilarity in the

"secondary" accumulation of aflatoxin suggests that temperature plays

a part in determining not only the amount of metabolite but possibly the

kind of metabolite produced by A. parasiticus. At lower temperatures the

amount of initial spore germination was minimal, hence aflatoxin formation

at $26°C$ was lower in the early storage period than at $37°C$ because aflatoxin

is formed during germination and growth of aflatoxigenic mold spores.

Many possibilities exist for the differences observed at different

temperatures, but these are merely attempts at explaining the trends

observed. The sharp increase of aflatoxin at $26°C$ (Figure 5) after initial

degradation could be a result of more available nutrients present which were not

used for more mycelia formation before the initial decline. It is also

possible that at 26°C the aflatoxin accumulated to a point before a

"breakdown" enzyme was activated, and after the breakdown the products

then inhibit the enzyme, hence continuous accumulation at 26°C. Doyle

and Marth (1978b) reported that as temperature is lowered, so is the rate

at which aflatoxin is degraded and is substantially increased in

fruits when temperatures are increased. At 37°C possibly both the

temperature and the breakdown products inhibit the synthesizing enzyme.

As a result, not much aflatoxin formed after initial decline. There is

also the possibility that both the synthesizing and degrading enzymes were

in different compartments of the cells and degradation did not take

place until the cells lysed. Degradation may then have proceeded to

a point where the amount of healthy spores germinating that were

producing was almost equal to the lysing mycelia; this could result in a

leveling off of accumulation. It may have resulted because the mold

at certain temperatures did not always produce the same amount of

substance(s) needed to degrade aflatoxin and formation of these materials

may also be associated with differences in environmental conditions.

Differences in the amount of degrading substance(s) released from the

mold may also have been a cause of variation, which again could be

temperature dependent. Although the same strain of molds was grown under identical conditions, one grown in one flask may have produced either more or less aflatoxins than another grown in an adjacent flask. Hence the biological variability which was observed in the amount of aflatoxin production is not surprising. It could also be possible that at both temperatures, the mold synthesizes two different enzymes and a temperature of $26°C$ is more favorable for the synthesizing enzyme than for the degrading enzyme. Another possible consideration is that the rate of degradation depends on the age of mycelia and the amount of mycelia produced. Volatile metabolites which may originate from the fungi may inhibit germination of their own spores and inhibition of spore germination may cause a decrease in aflatoxin production. At $37°C$, bacteria found in food may alter the environment as a result of their growth. This alteration of the environment may include a reduction of the oxygen level and an increase in respiratory carbon dioxide or a release of volatile metabolites. Diverse volatile compounds are produced by bacteria and these range from acids to alcohols to other organic solvents. Any of these types of compounds could possibly inhibit spore germination thus affecting aflatoxin production. Greater inhibition would be expected at $37°C$ then at $26°C$. Finally, it should be noted that the ability of the fungi to produce mycotoxins is influenced by physical and nutritional factors. Maximum

concentration of aflatoxin may relate to the exhaustion of fermentable carbohydrate and onset of mycelial autolysis. The complexity of the situation regarding growth of mold, temperature and other environmental effects and aflatoxin production is emphasized by the foregoing discussion.

Since accumulation of aflatoxin at certain temperatures is biphasic in nature, it is important that sampling be done at close regular intervals so as to detect the pattern of aflatoxin peaks in a given substrate. If, for example, in monitoring aflatoxin production in foods such as sausage, the first sample was taken 14 days after storage, to conclude that less than 0.5 ppm (Figure 5) aflatoxin was produced in 14 days at a particular temperature (such as 37°C) would not be wrong from the available data, but it would not account for greater amounts produced earlier. This could lead to error in judgement as to the safety of the product. However, from the work reported here, the lower level (156 ppm) of nitrite with product held at room temperature (26°C) would not produce a similar erroneous conclusion, since storage time was directly related to aflatoxin recovery.

Residual Nitrite and Aflatoxin Production

In addition to temperature, production of aflatoxin can be influenced by several factors including water activity, pH, available nutrients, competitive growth and inhibitory substances. Growth of other

microorganisms may change the available nutrients or produce volatile

and/or nonvolatile end products which may stimulate, inhibit, detoxify

or have no influence on growth of fungi or mycotoxin production. Only

limited studies have been done on how competitive growth influences

aflatoxin production although mold growth and aflatoxin production usually

occur in a competitive environment. The objective of this study was to

determine the effect of nitrite, which inhibits the growth of C. botulinum,

on aflatoxin production by A. parasiticus.

The influence of curing ingredients on aflatoxin production has been

studied by several workers most of whom used laboratory media. Of the

individual ingredients, aflatoxin production was totally inhibited by

potassium nitrate (0.1%) but sodium nitrite (0.05%) stimulated aflatoxin

formation (Strzelecki, 1973). In this work, sucrose and sodium chloride

stimulated the production of aflatoxin B1. However, Meir and Marth

(1977) reported that addition of 2 or 3% NaCl resulted in less aflatoxin

production than produced in sausage containing no salt. Also with no

nitrite, less aflatoxin was produced than observed when 100 ppm nitrite

was added to sausage.

The production of aflatoxin in two levels of sodium nitrite at the

same temperature (Figure 7) shows what is considered here to be a

biphasic pattern. This pattern of aflatoxin production with time has

Figure 7. Concentration of aflatoxin B1 in sausage with two
levels of added sodium nitrite and stored at 37°C.
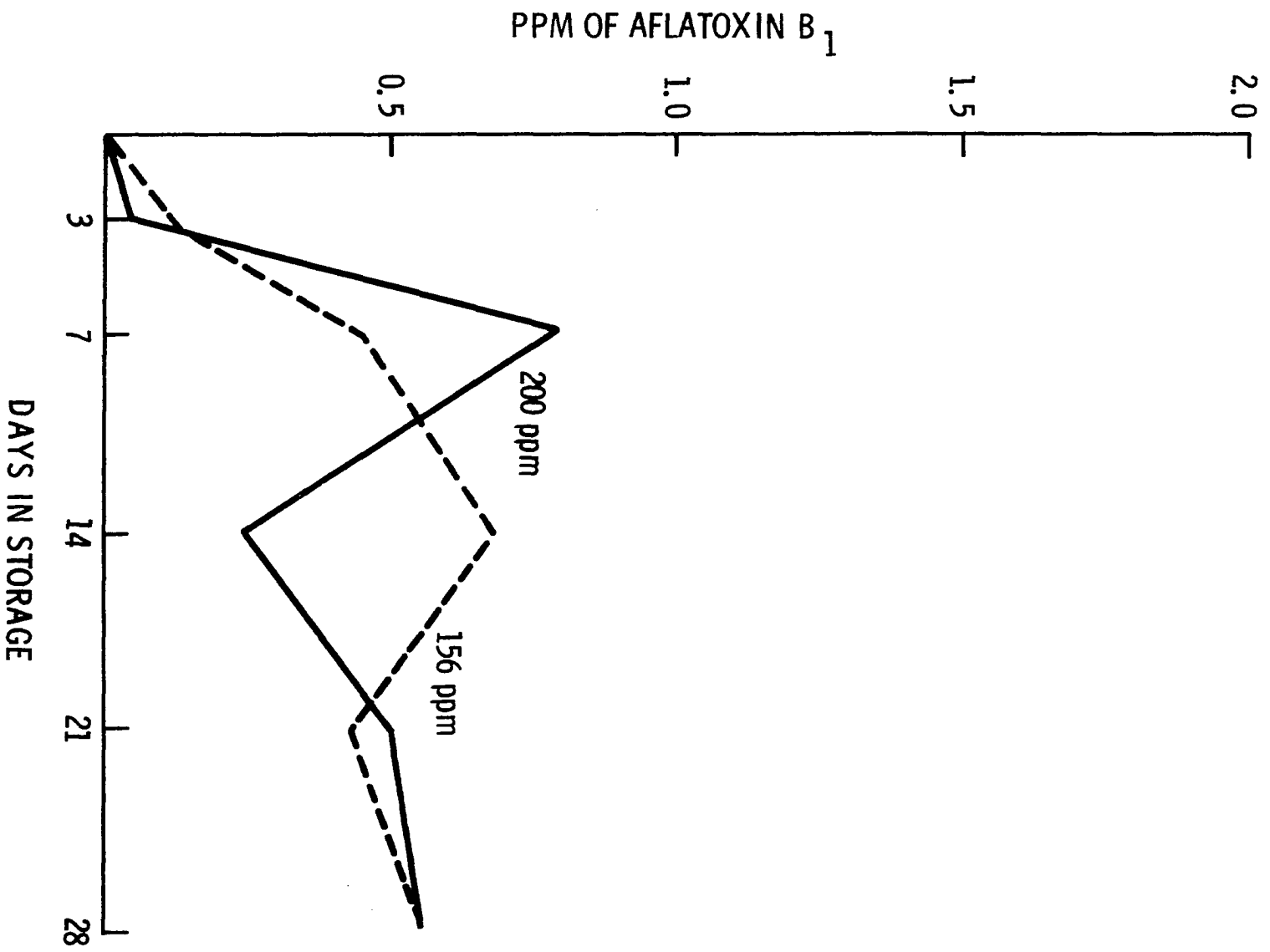
been discussed under the influence of temperature. Differences in time required for maximum production with the two different nitrite levels may be noted; the 200 ppm level sausage showed a peak at 7 days while the 156 ppm concentration samples reached a peak at 14 days. Visual examination of sausages with 156 and 200 ppm sodium nitrite showed approximately, equal amounts of mold growth.

Aflatoxin production was somewhat higher in sausages that were calculated to have 200 ppm sodium nitrite (0.80 ppm aflatoxin) as compared to sausages that were made to have 156 ppm (0.70 ppm aflatoxin) at 37°C. These differences are even more evident with storage at 26°C (Figure 8) where the amount of aflatoxin for samples with 200 ppm sodium nitrite were about 22% higher in 7 days than found for the 156 ppm samples and the difference was even greater (about 40%) at 21 days of storage. At 14 days, aflatoxin production was similar for sausages with both nitrite levels and stored at 26°C. By 14 days, the residual nitrite had decreased to about 14 and 16 ppm for the added 200 and 156 ppm levels respectively (Figures 9 and 10). The mold may not have utilized the nitrite directly but may have used the breakdown products for growth and production of secondary metabolites. High accumulation of aflatoxin coincided with low levels of residual nitrite; in other words, trends were opposite for residual nitrite and aflatoxin production regardless of storage temperature.

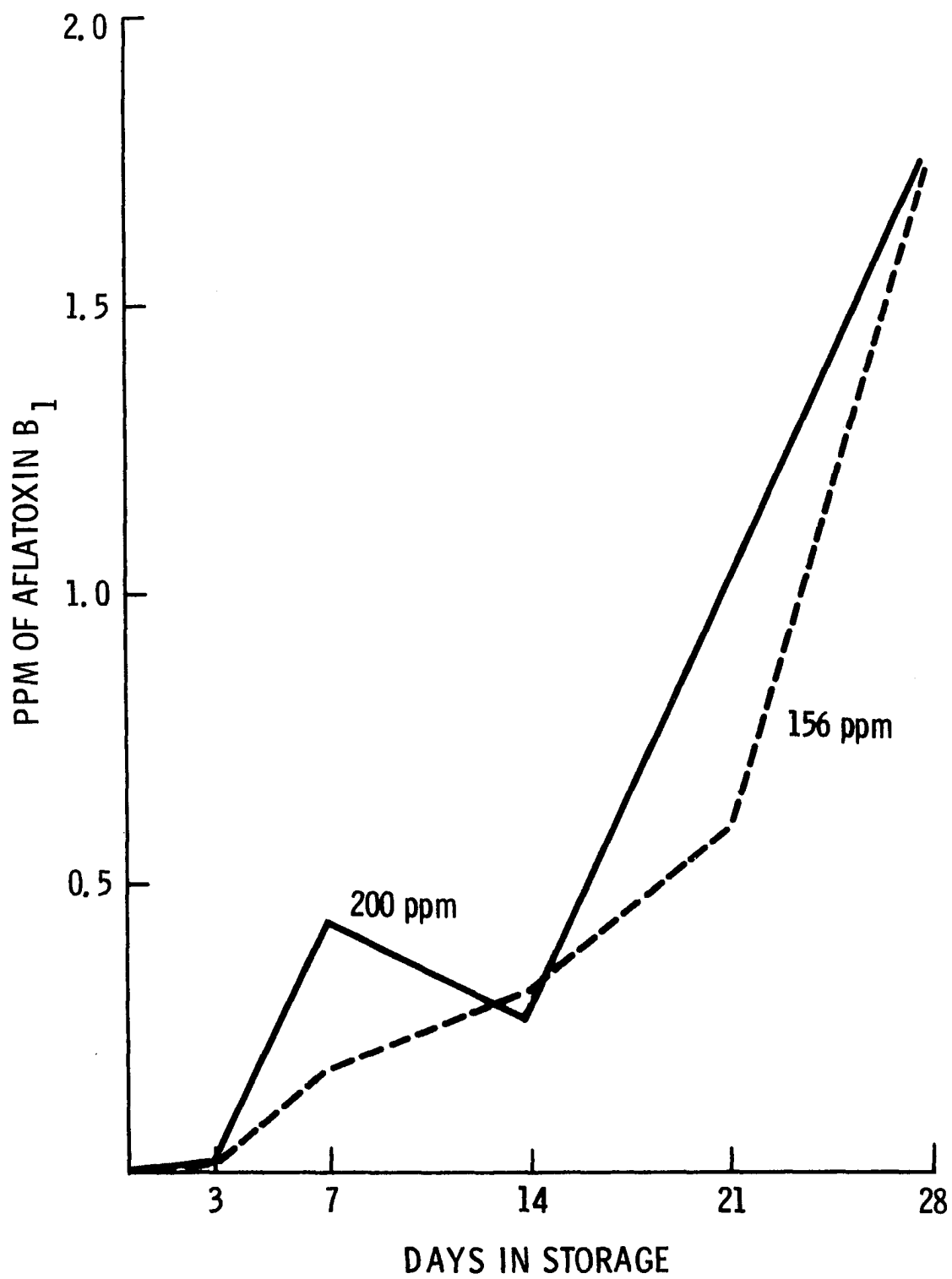Figure 8. Concentration of aflatoxin B1 in sausage with two levels of added sodium nitrite and stored at 26°C.

Figure 9. Residual sodium nitrite from sausage with 200 ppm added $NaNO_2$ and aflatoxin production by A. parasiticus at two temperatures.
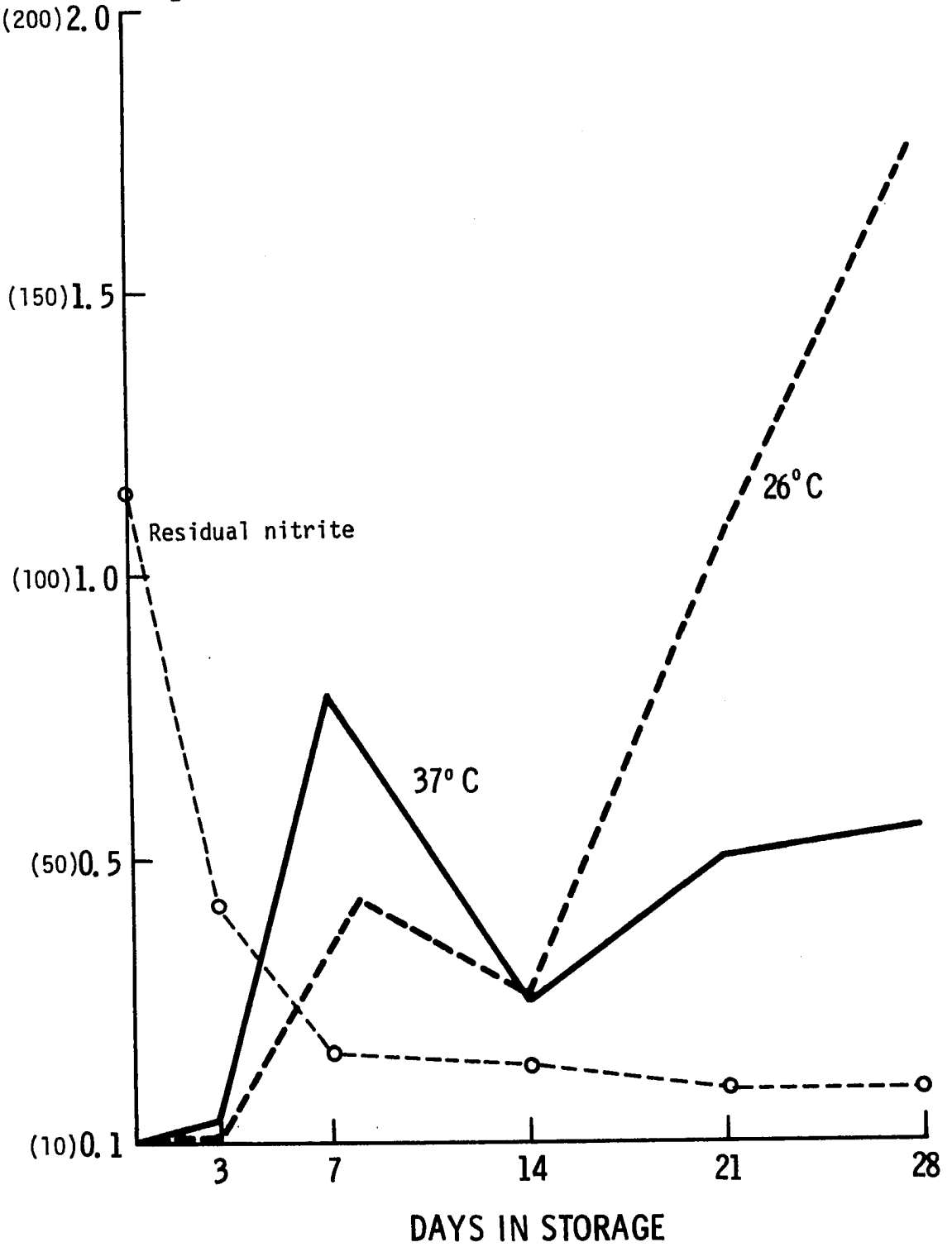
PPM AFLATOXIN
RESIDUAL NaNO$_2$
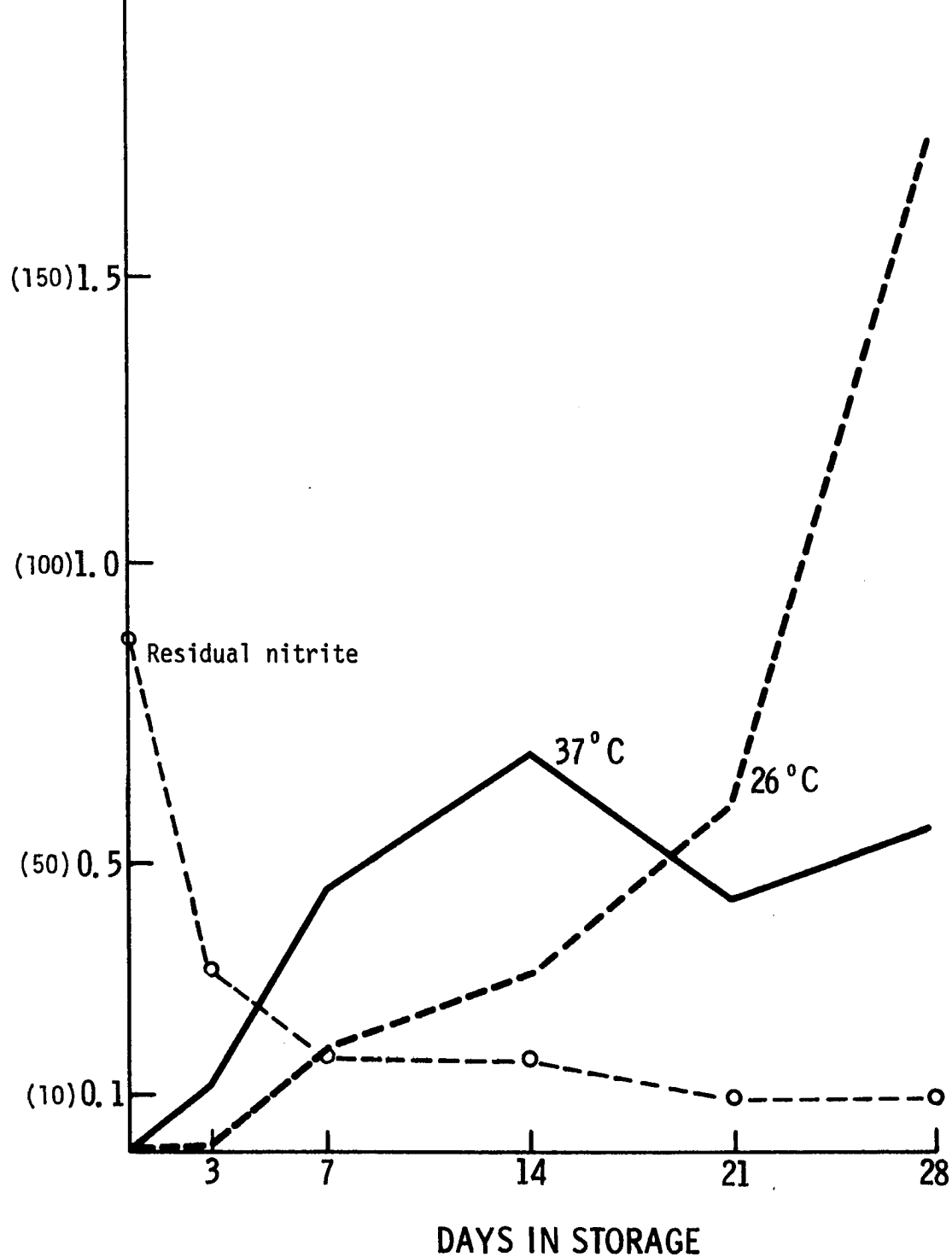
Figure 10. Residual sodium nitrite from sausage with 156 ppm added $NaNO_2$ and aflatoxin production by A. parasiticus at two temperatures.

PPM AFLATOXIN
RESIDUAL NaNO$_2$

(200)2.0

(150)1.5

(100)1.0

Residual nitrite

37°C

26°C

(50)0.5

(10)0.1

3 7 14 21 28

DAYS IN STORAGE

As stated earlier, from this study, it seemed that the amount of aflatoxin present has a bearing on the amount and/or rate of aflatoxin degradation. While it is possible that the presence of nitrite enhances the growth of the mold and possibly the production of aflatoxin, from what is known about the ability of the mycelia to degrade aflatoxin, nitrite either inhibits or slows down the rate of degradation of aflatoxin present. Aspergilli are nitrifiers capable of converting ammonium ions to nitrate (Shih et al., 1974). It is possible that one of the intermediates of the nitrification process has an inhibitory effect on the degrading enzymes while it may or may not enhance the production of aflatoxin. Nitrite salts have been reported to inhibit microbial growth (Perigo and Roberts, 1968). The nitrous acid form is assumed to interfere with the dehydrogenase enzyme system of the bacteria and yeasts. However, addition of nitrite to the sausage might also serve as an additional source of nitrogen which could permit more mold growth and as a result more aflatoxin production (Meir and Marth, 1977). Permeability of the mycelium may have been altered so that direct stimulation of enzyme systems by sodium nitrite might have occurred. It has been reported that low concentrations of sodium chloride (1-5%) stimulated aflatoxin production in A. parasiticus at 35°C (Shih and Marth, 1972) and the combination of both sodium chloride and sodium nitrite might have a stimulatory effect in the production of aflatoxin B1.

On the other hand, Bullerman et al. (1969a) found that 2.2% sodium chloride

caused less synthesis of aflatoxin in glucose-ammonium-nitrate broth

than that obtained when no salt was added. Meir and Marth (1977) indicated

that aflatoxin production was less in sausage made with sodium chloride

present compared with sausage containing sodium nitrite. These

investigators stated that it was difficult to separate these two variables with

regard to their stimulatory or inhibitory effects when compared with control

sausage containing both salts.

Effect of Nitrite on the Growth and Sporulation of A. parasiticus

A. parasiticus was plated on Sabouraud glucose agar with four

different levels of sodium nitrite and incubated for 28 days. After 4 days

of incubation, it was noticed that growth and sporulation were taking place

in the control and at low levels of nitrite (Plate 5) but no sporulation occurred

at higher levels. By 8 days of storage, the trend seemed to reverse, there

was more growth and spore formation at the higher levels of nitrite than

was seen at the low levels (Plate 6). After 8 days, there was no change

in the amount of growth and sporulation but evidence of more sporulation in

the higher levels of nitrite could be seen as the plates with more spores

get darker with time (Plate 7).

Plate 5. Growth of A. parasiticus on Sabouraud glucose agar
with nitrite added. 4 days incubation at 24° C.
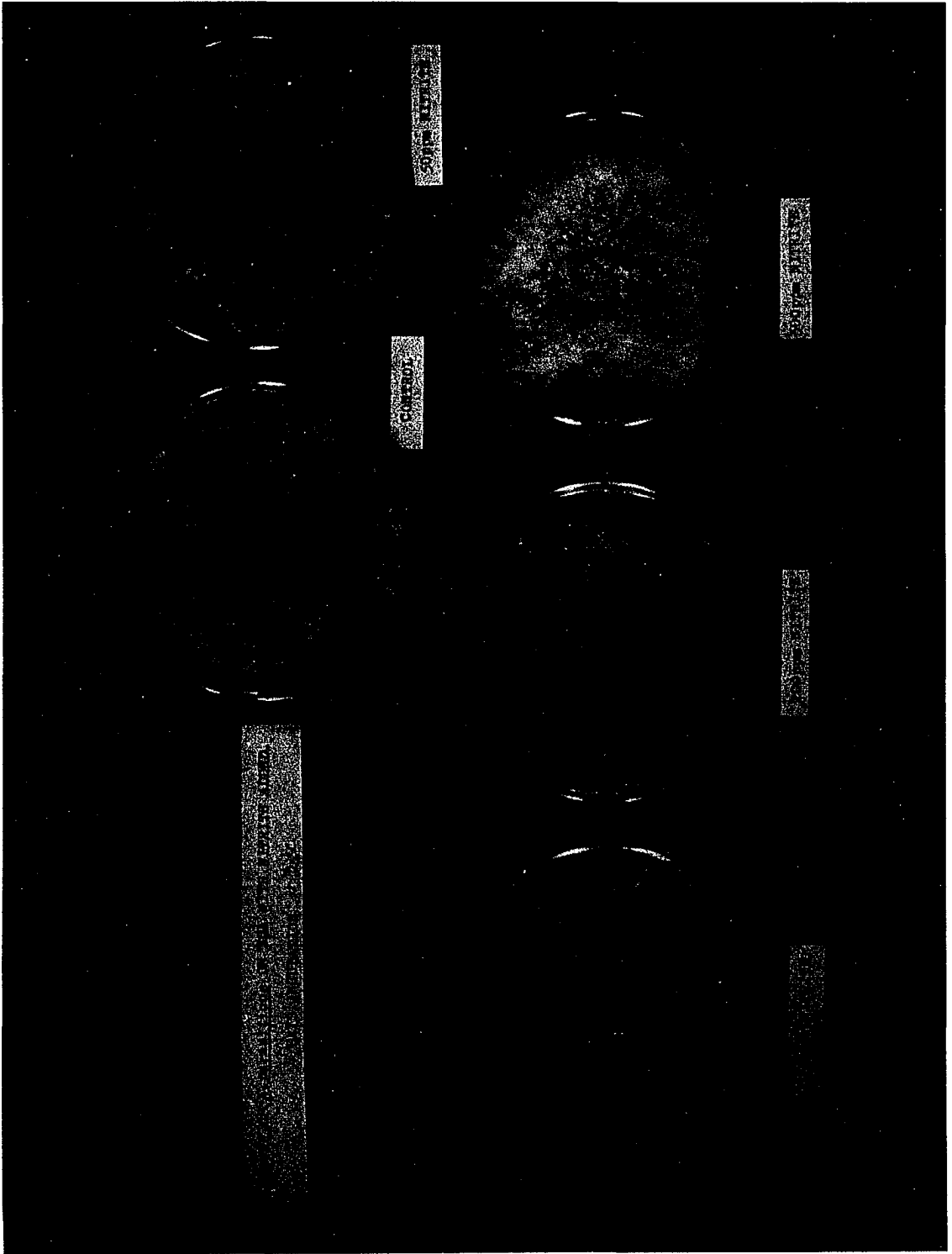Nitrite levels: 0, 50, 156, 200 and 500 ppm.

Plate 6. Growth of A. parasiticus on Sabouraud glucose agar
with nitrite added. 8 days of incubation at 24° C.
Nitrite levels: 0, 50, 156, 200 and 500 ppm.

GROWTH OF A. parasiticus ON SGA WITH NITRITE ADDED
8 DAYS OF INCUBATION AT 24°C

CONTROL

50 ppm NITRITE

156 ppm NITRITE

200 ppm NITRITE

500 ppm NITRITE

Plate 7. Growth of A. parasiticus on Sabouraud glucose agar
with nitrite added. 28 days of incubation at 24°C.
Nitrite levels: 0, 50, 156, 200 and 500 ppm.

GROWTH OF A. MARSEILONS ON SGA WITH NITRITE ADDED. 28 DAYS OF INCUBATION AT 24°C

From this one could conclude that high levels of nitrite temporarily restrict the profuse growth and sporulation of A. parasiticus. But as the level of residual nitrite becomes low, growth of A. parasiticus may be enhanced and as a result more aflatoxin production results. This lends support to the finding that more aflatoxin was produced by A. parasiticus in sausage samples with 200 ppm sodium nitrite at 26°C than sausage samples with 156 ppm sodium nitrite at the same temperature.

Samples stored at 5, 26 and 37°C had average water activity values of 0.96, 0.95 and 0.93 respectively. Available moisture for growth did not seem to be influential in this study since the water activities of samples at the three temperatures were within the levels reported for optimum growth and aflatoxin production by Aspergillus species (Diener and Davis, 1967; Northholt et al., 1976).

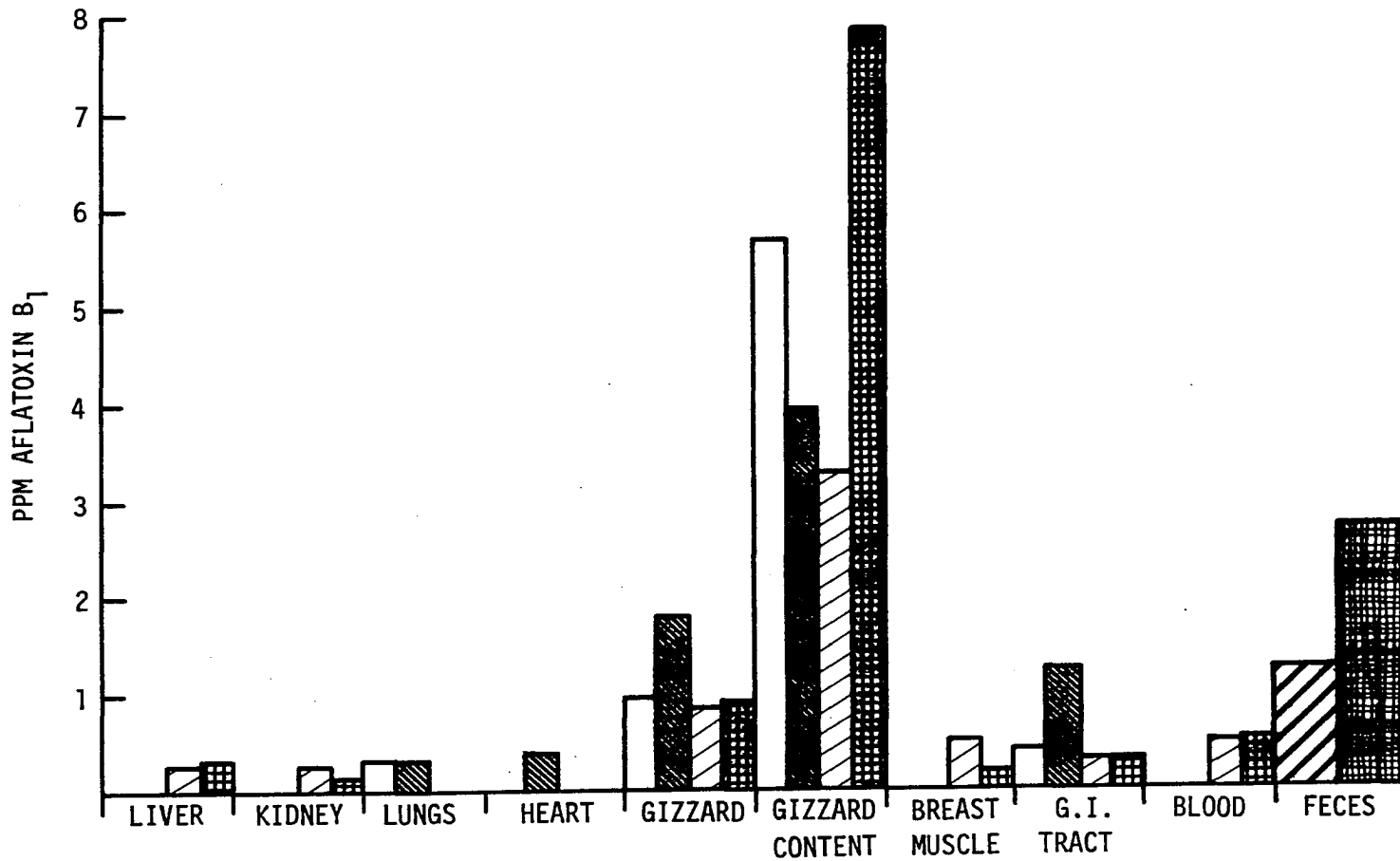## Feeding Studies With ^{14}C-aflatoxin B1

The purpose of this study was a) to determine the modes of absorption, distribution and elimination of dietary aflatoxin b) to quantitate aflatoxins in various edible portions of chicken. A single oral administration of 1 mg of ^{14}C-aflatoxin B1, with 1600 or 3200 cpm in a capsule, to three week old chickens did not seem to have any physical effects. No toxicological lesions were observed in any of the tissues of the tested chicken when postmortem examination was conducted.

In order to account for the total amount of ^{14}C-aflatoxin retained in

the chickens, it was necessary to determine the amount excreted. Since fecal

samples were collected each time chickens were sacrificed during the course

of the experiment, patterns of excretion of aflatoxin as measured by

radioactivity could be determined. The pattern of excretion in feces and

retention in other organs and tissues is presented in (Table 1). The data

presented is for net radioactivity detected per gram of feces, tissues or

organs of treated chickens. The results show that pattern of excretion

could increase or decrease with time, emphasizing that differences exist

among chickens in the rate of excretion of aflatoxin. From these data,

it is not possible to make a definite statement as to the rate of excretion

because of the differences that exist among chickens. These differences

also indicate the need for several replications for statistical analysis to be

done. With a group of chickens, some will have fast metabolic rate while

others may be slow and this will be the determining factor in the pattern

of excretion.

Histograms representing the distribution of aflatoxin B1 per gram of

tissue at 12 and 24 hours are presented in Figures 11 and 12. Table 2

gives data on percent distribution of aflatoxin B1 in tissues of chickens at

intervals up to 72 hours following administration of a single oral dose of

Figure 11. Concentration of aflatoxin B1 detected in tissues, organs, blood and feces 12 hours after oral dosing with 1 mg aflatoxin B1 (four chickens; each bar represents results from one chicken).

12 HOURS

PPM AFLATOXIN B₁

LIVER   KIDNEY   LUNGS   HEART   GIZZARD   GIZZARD   BREAST   G.I.   BLOOD   FECES
                                                CONTENT   MUSCLE   TRACT

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Figure 12. Concentration of aflatoxin B1 detected in tissues, organs, blood and feces 24 hours after oral dosing with 1 mg aflatoxin B1 (four chickens; each bar represents results from one chicken).
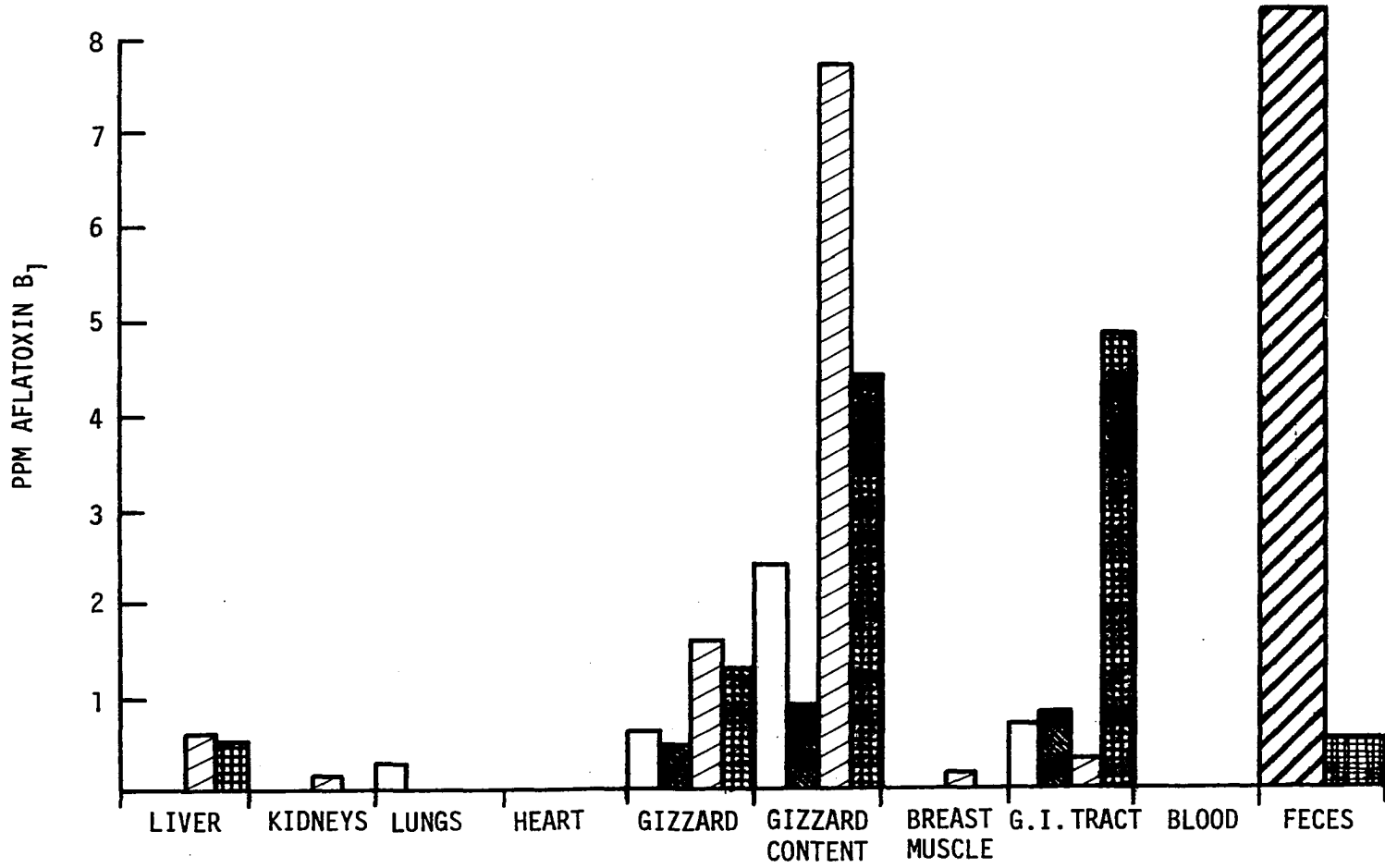
24 HOURS

Table 1. Radioactivity counts on tissues, organs, blood and feces of chicken at different time intervals.

SAMPLE	COUNTS PER MINUTE														
	REP I								REP II						
	12 HRS		24 HRS		48 HRS		72 HRS		12 HRS		24 HRS		48 HRS		72 HRS
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7
Liver	438	373	421	443	179	189	156	86	2073	2567	1333	1423	2025	1851	1736
Kidney	55	395	79	57	44	69	43	31	260	245	282	352	126	188	238
Lungs	60	189	40	40	37	23	16	12	122	149	77	68	62	50	83
Heart	67	25	37	33	15	4	3	19	60	< 1	40	<1	< 1	< 1	< 1
Gizzard	244	113	131	306	<1	5	40	<1	187	434	613	122	50	30	49
Gizzard Content	2272	3881	869	587	67	159	60	47	2852	6016	6688	4078	2491	2360	1718
Breast Muscle	10	11	< 1	4	7	5	< 1	13	59	128	25	22	27	197	23
G. I. Tract	77	239	228	204	101	143	69	18	419	195	163	445	255	268	247
Blood	46	35	38	35	77	8	10	5	116	81	140	79	70	105	70
Feces	1647		1980		592		162		1854		2971		5391		* (no feces available)

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Table 2. % distribution of aflatoxin B1 in tissues.

TISSUES	REP I								REP II						
	12 HRS		24 HRS		48 HRS		72 HRS		12 HRS		24 HRS		48 HRS		72 HRS
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7
Liver	—	—	—	—	—	—	—	—	.12	.15	.28	.24	.36	.33	.13
Kidney	—	—	—	—	—	—	—	—	.06	.02	.04	—	.07	—	—
Lung	.06	.04	—	—	—	—	—	—	—	—	—	—	—	—	—
Heart	—	.04	—	—	—	—	—	—	—	—	—	—	—	—	—
Gizzard	.60	1.20	.42	.26	—	—	—	—	.50	.87	.90	.70	.67	.36	.14
Gizzard Content	.83	.44	.80	.16	.08	.03	—	.04	1.00	1.70	1.10	.60	.33	.02	.06
Breast Muscle	—	—	—	—	—	—	—	—	.65	.19	.26	—	—	—	—
G. I. Tract	.38	1.40	.68	1.0	—	—	—	—	.34	.32	.30	4.70	—	—	.87
Blood	—	—	—	—	—	—	—	—	.22	.23	—	—	—	.37	.08
Feces	.86		3.32		1.92		—		2.76		.15		.09		* (no feces available)

^{14}C-aflatoxin B1. Data are given as percentage of the total aflatoxin a administered that appeared in the entire tissue. The levels in liver appeared to increase for the first 48 hours. Digestive tract levels decreased during that time. When 10 mg of aflatoxin B1 were fed, the variation was ± 5% RSD. The variation among livers was ± 25%. These results could not be compared with the work done by Mabee and Chipley (1973 a and b) in that they reported only radioactivity counts and also fed chickens continuously for 14 days. The present study showed that most of the aflatoxin administered to the birds was excreted through the feces.

Aflatoxin B1 is translocated to some tissues within 12 hours of administration. A significant finding here is the detection of aflatoxin B1 in the tissues of breast muscle. It is not surprising to find aflatoxin in the gizzard or its content or in the intestinal tract or liver because these are part of the normal route of digestion of feed and with the liver as an active metabolic site. Being absorbed from the intestine to the liver, to the heart, and from the heart to the lungs and kidneys before being absorbed into the muscles within 12 hours is evidence of the speed at which aflatoxin can be translocated in chickens. The intestinal tract and its contents contained

the largest amount of aflatoxin of any tissue after the feed had gone through the gizzard. Again, this observation tends to support the fact that most of the aflatoxins are excreted through the feces. Aflatoxin is primarily metabolized in the liver but the metabolic pathways are not fully understood. The relatively higher amount of both radioactivity and aflatoxin B1 observed in the liver demonstrated that this organ retained aflatoxin and/or its metabolite to a much greater extent than did other organs.

Six week old chickens were each fed 10 mg of aflatoxins to determine the pathological effect on the liver. The chickens were sacrificed at intervals up to 72 hrs following oral administration of a single dose of 10 mg of aflatoxins. Both normal livers and livers with pathological lesions were detected at each interval. These results again indicate individual differences among chickens in resistance to aflatoxins. About 12 to 18 hours after feeding, the chickens were showing signs of abnormal reflexes. They stopped feeding probably due to loss of apetite and this was confirmed by the presence of very little feed in both the crop and the gizzard and a virtually empty intestinal tract. Pathological examination of the liver showed vacuolization of the hepatocytes, hemorrhages, necrosis and proliferation of the bile duct (Plates 8 and 9) compared with normal liver (Plate 10).
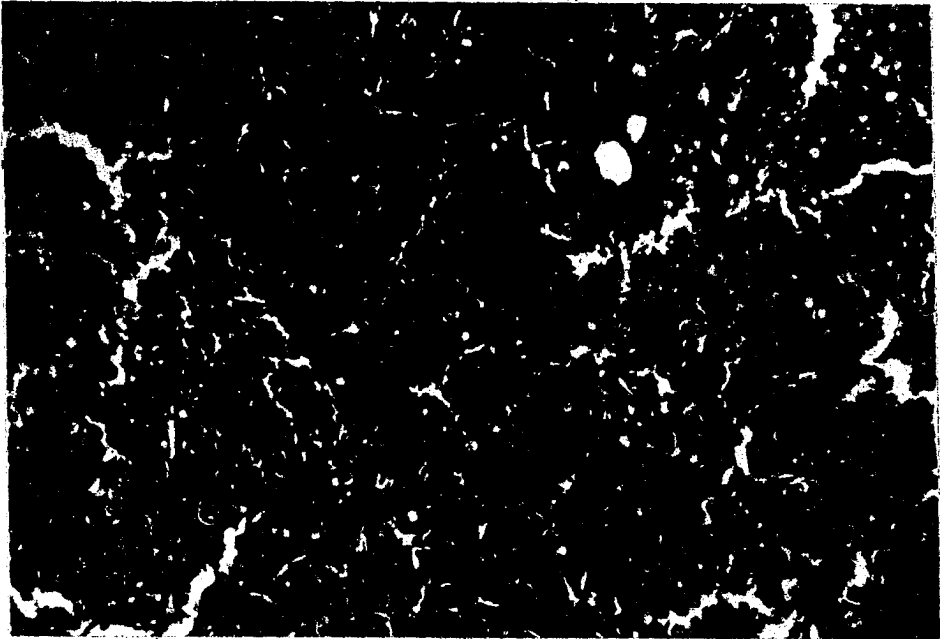
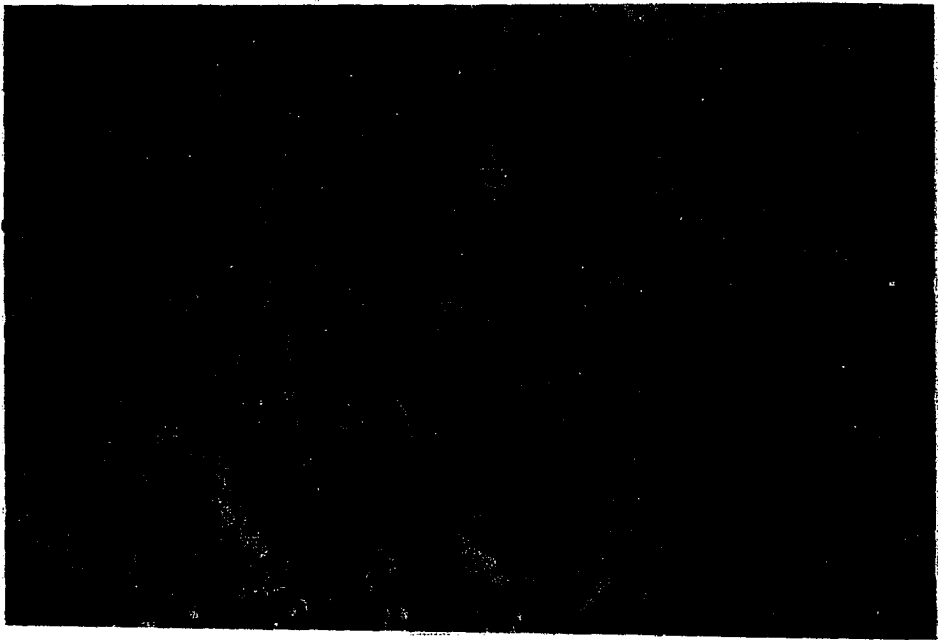Plate 8. Effect of aflatoxins on chicken hepatic tissue 12 hours after oral administration 50X

Plate 9. Effects of aflatoxin on chicken liver tissue 72 hours
after oral administration 100X

Plate 10. Normal structure of chicken liver 25X.

Effect of Scirpene Toxins on Chicken Embryos

Scirpene toxins injected into eggs were found to be toxic. A tenth of a microgram of the following toxins: T-2, diacetoxyscirpenol (DAS) and deoxynivalenol (vomitoxin) were injected into eggs as previously described and incubated for 21 days. Also, a group of eggs was injected with an equal volume of ethanol as a control for the solvent. After seven days the eggs were candled to check for dead embryos. There were no dead embryos among the control and the vomitoxin groups; however, 9 of 30 (30%) and 100% of the embryos were dead in T-2 and DAS group respectively. At the end of 21 days, 19 of 30 (63%), 3 of 30 (10%) and 2 of 30 (7%) of the embryos were dead in the T-2, vomitoxin and ethanol groups respectively.

These results agree with work reported by Chi et al. (1978) on the order of toxicity of 12, 13-epoxytrichothecenes. In this study, DAS and T-2 proved to be toxic to chicken embryos. DAS was more toxic and more potent than T-2. Vomitoxin did not seem to be very toxic when compared with the control group.

Another check was performed using lower dose levels. Eggs that were injected with 0.1 microgram of the above toxins were incubated until they hatched. The chicks were raised to three weeks of age and were then necropsied. Their liver and kidneys were normal. There was no observable physical defects. From this work it is possible to conclude that

transmission of low levels of scirpene toxins is not lethal to the embryo and does not have any pathological or physical effects on the chick that hatched from the egg.

SUMMARY

Aflatoxin B1 was produced when A. parasiticus (NRRL 2999)

was grown in a liquid medium for 72 hours at 28°C. Chlamydospores

were formed instead of a normal mycelial mat. Sodium acetate (2-^{14}C)

was incorporated efficiently on addition to cultures growing in the

liquid medium in two stages. Aflatoxins were separated on thin layer

chromatography, quantitated with a spectrophotometer and radioactivity

of aflatoxin was determined by scintillation counting and confirmed by

autoradiography.

A methanol-water extraction method was used for the extraction and

analysis for aflatoxin in all tissues as well as blood and feces. By means

of this method, internal standards were recovered. Charcoal was used

to partially purify the methanol-water extract when necessary.

In a study on effects of nitrite on mold growth and aflatoxin production

in cured meats, pork sausage was inoculated with a saline spore suspension

of A. parasiticus and stored for varying lengths of time up to 28 days at

5, 26 and 37°C. Examinations were made for aflatoxin production and

growth of the mold. The sausage had nitrite added to provide calculated

levels of 156 and 200 ppm of sodium nitrite and also 2.5% sodium chloride

was added as part of the curing salts mixture. No mold growth was observed

at 5°C; likewise, no aflatoxin was found at this temperature. Mold growth

was about equal at 26° and 37°C and aflatoxin B1 was produced at both temperatures. More mold growth; i.e., more mycelia and spores were observed with 4 days of storage at lower levels of added sodium nitrite and the trend reversed to more mycelia and spore formation with increase in added sodium nitrite up to 500 ppm after 4 days of storage in Sabouraud glucose agar.

The effects of administering a single oral dose of 1 mg aflatoxin B1-^{14}C to three week old chicks was determined as a test for determining distribution of aflatoxin in animal tissues. Analyses were made of the distribution of ^{14}C in liver, kidney, lung, heart, gizzard, gizzard content, breast muscle, gastrointestinal tract, blood and feces. No toxic effects were observed in the chickens during the 72 hours of the experiment. Most of the ^{14}C-aflatoxin B1 was excreted in the feces within 48 hours after dosing and most of the amount retained in the body was found in the liver. Chemical assay for the parent compound in the above tissues, organs and feces demonstrated the same trend as that of the radioactive recoveries. Effects of administering a single oral dose of 10 mg aflatoxins to six week old chickens were also determined. Studies of the effects on the liver was conducted. Within 12 to 18 hours after dosing, chickens had difficulty with their reflexes, they stopped feeding and pathological examination of the liver showed hemorrhaging, vacuolization of the hepatocytes, necrosis and proliferation of the bile duct.

Investigation of other mycotoxins was performed by inoculating scirpene toxins into chicken embryos. Diacetoxyscirpenol (DAS) proved to be more toxic (lethal to embryos) followed by T-2 and vomitoxin. However, chicks that hatched from the inoculated eggs had no observable defects or any pathological defects.

CONCLUSIONS

1. Extraction of aflatoxin B1 from tissues may be accomplished with methanol-water as an extracting solvent and removal of interfering pigments was improved in this work by the use of activated charcoal in chloroform.

2. No mold growth or production of aflatoxin was detected on sausage samples stored for up to 28 days at 5°C; this low temperature, therefore, is not conducive to aflatoxin formation.

3. With inoculated sausage held at 26°C or 37°C, aflatoxin may form within three days. Therefore, the practice of removing mold growth from sausage kept at room temperature or at higher temperatures for several days will not insure freedom from aflatoxin. In considering storage of sausage, the most favorable temperature for aflatoxin production and the most hazardous was 26°C, as compared with 5 or 37°C holding temperature.

4. Animal tissue such as sausage meat will support high yields of aflatoxin if conditions favoring the growth of A. parasiticus are present.

5. Production of aflatoxin shows a biphasic pattern and as such sampling and analysis should be done at close intervals in tests to determine when aflatoxin is first produced, or for determining trends in concentration over time.

6. When chicken consumes aflatoxin in feed, most of the aflatoxin is excreted through the feces within 48 hours and of the amount retained, most is found in the liver.

7. When the chick embryo test is used, of the three scirpene toxins, DAS, T-2, and vomitoxin, the most potent is DAS, followed by T-2 and vomitoxin in that order.

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