

AFLATOXIN METABOLITES IN RELATION TO PROTEIN-
ENERGY MALNUTRITION IN ZAMBIA

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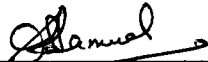
Derina Sara Samuel

A dissertation submitted to the University of Zambia
in fulfillment of the requirements of the degree of
Master of Science in Chemistry

Dedicated to my parents who
have loved and supported me
all through the years.

DECLARATION

I hereby declare that this dissertation is my own work and that it has not been previously submitted for Degree purposes here or at any other University.


Derina Sara Samuel.

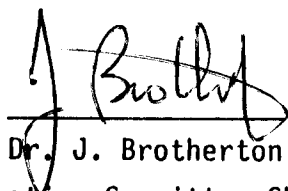
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award of the Master of Science in Chemistry
by the University of Zambia .



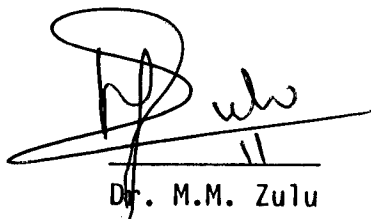
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ABSTRACT

Findings in the Sudan show a possible association between aflatoxins and kwashiorkor, a category of Protein-Energy Malnutrition (PEM). Similarities in the geographical and seasonal prevalence of aflatoxins and kwashiorkor, as well as similarities in the metabolic derangements caused by aflatoxins and observed in kwashiorkor suggest that aflatoxins may play an etiological role in the pathogenesis of kwashiorkor.

Studies in Zambia have shown that aflatoxin contamination occurs in commonly eaten foods, suggesting that the Zambian population is exposed to the toxin. The presence of urinary aflatoxins in patient with and without liver pathology at an incidence which reflects the level of contamination has also been reported. PEM is known to be endemic in Zambia with a seasonal prevalence similar to that observed with aflatoxin contamination of food.

A one-year survey was therefore undertaken to investigate the relationship between aflatoxins and the various categories of PEM. Metabolism of the aflatoxins was determined by analyzing urine and serum samples of PEM cases and appropriate controls from the University Teaching Hospital, Lusaka. As previous studies had shown a higher level of aflatoxin contamination in foods in the Eastern province, urine samples from PEM cases and appropriate controls were obtained from St. Francis' Hospital, Katete, for comparison.

The incidence of urinary aflatoxins in samples from Lusaka and Katete was 6.1 per cent, with a mean concentration of 0.365 ng/ml. Aflatoxin B₁ and aflatoxicol were detected. Aflatoxicol concentrations were highest in control and lowest in kwashiorkor samples, though aflatoxicol was detected slightly more frequently in the latter. Aflatoxin B₁ excretion was observed to follow a similar pattern. Comparing the incidences in Lusaka and Katete, a significant difference was not observed.

In serum samples the incidence of aflatoxins was 2.8 per cent, with a mean concentration of 1.87 ng/ml. Aflatoxin B₁ and aflatoxicol were detected with aflatoxicol only being detected in one case of kwashiorkor. Total aflatoxin concentration was higher in kwashiorkor than controls while the incidence was similar in the two groups.

The incidence of urinary aflatoxin in the rainy season (18.8 per cent) was greater than in the dry season (5 per cent), however, no significant difference was observed in the seasonal variation of aflatoxins in sera. Urinary aflatoxins were detected more often in males than in females but no significant difference occurred in the sera analyses.

The results suggest that there may be certain unusual associations in the incidence, concentrations and type of aflatoxin detected between the different nutritional groups, in particular the role of aflatoxicol in kwashiorkor and the lower excretion levels observed. Possibly this indicates a decreased capacity to deal with aflatoxins. Whether this a cause or an effect of the

kwashiorkor syndrome, is still not clear. These preliminary findings merit an extended study to examine more closely the relationship of aflatoxins and kwashiorkor in Zambian children.

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CHAPTER ONE

INTRODUCTION

I- AFLATOXINS

A. INTRODUCTION

The fungi are a diverse group of eukaryotic, heterotrophic organisms, the latter distinguishing them from plants. The terrestrial group of filamentous microfungi, commonly known as the molds, are the area of this study. Many of the molds produce a wide range of secondary metabolites, some of which are active against micro-organisms (antibiotics), plants (phytotoxins) or animals (mycotoxins). The mycotoxins can thus be defined as secondary metabolites which can cause disease or death in animals, as well as in man, following consumption of contaminated food. The resultant disease is referred to as a mycotoxicosis. Poisoning can also occur on ingestion of poisonous mushrooms (macrofungi), such as Amanita phalloides (commonly called 'Deathcap'), but such incidents are referred to as mycetisms (Smith and Moss, 1985)

Fungal diseases (i.e. diseases caused by the microfungi may be divided into three: mycoses, mycotoxicoses, and allergies (Moreau, 1979). Mycoses are diseases in which the fungus itself invades an organ and causes an inflammation of the organ. The fungus in this case is infectious and quite often contagious. An important distinction between the mycoses and mycotoxicoses is that the fungi associated with the latter are neither contagious nor infectious. Mycotoxicoses can be defined as diseases caused by the ingestion of fungi - infested food and thus the role played by the fungus is indirect. Allergies are specific reactions of a susceptible individual on coming into contact with a particular fungus, usually through inhalation of the spores

The poisonous nature of certain toadstools had been recognised for centuries but it was not until the mid-nineteenth century that a microfungus, Claviceps purpurea, was directly associated with diseases in man. Ergotism, the first mycotoxicoses recorded, was associated with the consumption of cereals, particularly rye. Two distinct forms of ergotism, a nervous /convulsive form or a gangrenous form, could occur with occasional cases of a mixture. Cases of gangrenous ergotism were recorded as early as the ninth and tenth century, and in the eleventh century the disease became particularly frequent in France where it came to be known as St. Anthony's fire. In Russia and Eastern Europe, the second form of ergotism, which affected the nervous system, was more common. Thousands of people died during these outbreaks. However, though the disease was well documented, the involvement of the mold C. purpurea was only noted in 1711 (Barger, 1931) and the presence of the toxic substance, the ergot alkaloids, only identified in the early twentieth century (Purchase, 1974). Due to improvements in food storage, the disease is now rarely observed in humans, though occasional outbreaks still occur in farm animals.

Alimentary toxic aleukia (ATA) is a disease caused by the ingestion of over-wintered grain. This is probably one of the best described human mycotoxicoses and was first reported in Siberia, in 1913. Severe epidemics occurred between 1941 and 1947 in which there was severe loss of life, more than 10% of the population being affected (Joffe, 1962). The fungal toxin was found to be stable with time and heat. Fusarium tricinctum (F.sporotrichioides) was implicated in the disease and it is now suspected that its toxins, the trichothecenes were responsible for

the pathogenesis of the disease (Ueno, 1977).

Another epidemic also occurred in the same period of the second World War, in Japan. Rice imported into Japan was found to be contaminated with a number of molds, mostly of the genus Penicillium, and was considered unfit for human consumption. Normal food supplies were dislocated due to the wartime situation and so the contaminated rice was consumed, causing the death of many people (Uraguchi et al., 1961). The disease was termed the 'yellow rice' disease, as the grains were discolored by fungal growths. The most significant species isolated was P. islandicum from which two hepatotoxins were obtained: luteoskyrin and islanditoxin (= cyclochlorotine). Other species isolated were P. citreo-viride (P. toxicarium) and P. citrinum, from which the toxins citreoviridin (causing cardiac beri-beri) and citrinin (causing nephrotoxicity) were obtained, respectively (Goldblatt, 1969; Smith and Moss, 1985).

Many instances of animal mycotoxicoses have probably passed, unrecorded. However, in 1931, a disease caused by Stachybotris atra was reported to have occurred in Russia, in which large numbers of horses and livestock were lost (Forgacs and Carl, 1962). This occurred in winter and when the animals were fed on hay which was contaminated with S. atra, for in the spring when the animals were returned to pasture the toxicosis disappeared. The toxins produced have now been characterized as belonging to the trichothecene group (Eppley and Bailey, 1973).

Zearaleonone, another of the Fusarium toxins, has been implicated in vulvovaginitis of pigs which was first recorded in the U.S.A. in 1928,

and later in Australia in 1937 (Pullar and Lerew, 1937). It also causes an estrogenic syndrome in pigs, as well as infertility in cattle. However, there have not been any reports of adverse effects of zearaleonone in man, following the ingestion of naturally contaminated food.

Unlike the majority of species of Penicillium that are most abundant in temperate climates, those of the genus Aspergillus are most commonly encountered in the tropics and warmer climates, several of which produce toxic metabolites. This genus is of particular importance since many of its species are capable of growth and metabolism at lower water activities and can therefore contaminate crops that are too dry to be attacked by other microorganisms (Smith and Moss, 1985). The aflatoxins are the most extensively studied of the toxins produced by the various species of Aspergillus, as well as probably the most extensively studied of the mycotoxins. Sterigmatocystin (produced by A. versicolor), the ochratoxins (produced by A. ochraceus) and patulin (produced by A. clavatus) are a few of the other toxins produced by the species of Aspergillus. The three have been found to be carcinogenic in experimental animals (Smith and Moss, 1985). Sterigmatocystin has been of interest due to its structural affinities with the aflatoxins and it is now thought to be an intermediate in the biosynthesis of aflatoxins.

The study of mycotoxins probably began with the discovery of the aflatoxins. Their recognition as carcinogens in animals, and possibly in man, added impetus to the research into other mycotoxins as a possible threat in the environment. For a long time the terms aflatoxin and mycotoxin were synonymous. However, this is not the case any longer since

so much research is being done on the various other mycotoxins found.

B. HISTORY

In the spring and summer of 1960 an outbreak of a mysterious disease occurred in the south and east of England amongst turkeys aged between 3 and 30 weeks. Altogether there were some 600 outbreaks of this disease termed turkey 'X' disease (indicating obscurity of etiological agent), and it was estimated that consequently over 100,000 turkeys had died (Blount, 1961). It was soon realised however, that ducklings and pheasants were similarly affected, with heavy mortalities being experienced. On one farm alone, more than 14,000 ducklings died over a period of 4 - 5 weeks (Asplin and Carnaghan, 1961). Chickens were less susceptible, it seemed, for though the disease stunted their growth, lower mortality-rates were observed (Asplin and Carnaghan, 1961). Around the same time, reports were received from Kenya and Uganda about a disease that was causing heavy mortality rates in ducklings (Asplin and Carnaghan, 1961). Meanwhile, in the United States, an outbreak of trout hepatoma was discovered. Inspecting a shipment of commercially raised trout at the California State border, many were found to have hepatomas (Wolf and Jackson, 1963). Pigs and calves seemed to be affected by a similar disease (Loosmore and Harding, 1961; Loosmore and Markson, 1961)

Deaths on such scale, together with their serious economic implications resulted in a great stimulation of research activities in mycotoxicology. The various incidents reported seemed to indicate that the disease was neither confined to a particular area nor to any particular species.

As a result considerable publicity was generated in the press, both locally (in the English counties most affected) and nationally. Finding the solution to the etiology of this mysterious disease eventually turned out to be a fascinating illustration of multidisciplinary approach. Veterinarians, pathologists, microbiologists, mycologists, as well as organic, analytical, physical and biochemists, based both in industry and various research institutions, pooled their resources to make what must rank as one of the most important and rapid advances in environmental toxicology.

The turkey 'X' disease was characterized by anorexia and resulting retardation of growth, lethargy and weakness of wings. The turkeys soon developed a staggering gait before they collapsed and died. A characteristic position was assumed at death with the head drawn back on an arched neck and the legs extended fully backwards (Goldblatt, 1969). Postmortems performed on the affected birds revealed generalised edema (Swarbrick, 1960), mild cirrhosis of the liver, liver hemorrhages and liver necrotic lesions, pancreatic hemorrhages and kidney congestion (engorged/swollen kidneys) (Wannop, 1960 a).

Histopathological examination showed a degeneration of the liver parenchyma cells accompanied by nodular regeneration with retrogressive changes observed in some of these cells (regenerative and retrogressive processes being indicative of toxicity) (Siller and Ostler, 1961). Bile duct hyperplasia, or extensive proliferation of the bile duct epithelium with the appearance of ascities (Wannop, 1960b), pathological changes in the kidney and heart and enterities of the duodenum were the other findings also reported (Siller and Ostler, 1961). Earlier,

Wannop (1960a) had reported and commented on the three different manifestations observed in the turkey 'X' disease: one affecting the central nervous system, the second involving the liver and kidneys and the third characterized by enteritis ; however, he attributed the causal agent of the three different manifestations as being a single entity rather than several.

That the cause of this disease might be a dietary factor was based on two observations, firstly that a change in diet reduced the mortality rate whilst improving the condition of the affected birds (Smith, 1960) and secondly that the disease was not biologically transmissible. On examining the incidence of the turkey 'X' disease over a number of areas, Blount (1961) reported that the common factor was a Brazilian groundnut meal which he suggested contained the toxic substance. However, in his intensive investigations for a toxic factor, Blount (1961) was unable to disclose contamination of the suspected meal by any known insecticide, solvent, plant toxin or alkaloid. Investigating the death of the 14,000 ducklings, Asplin and Carnaghan (1961) found that Brazilian groundnut meal was part of the rations and on replacing it with soya bean meal and dried milk, there was a dramatic improvement in the condition of the birds. Mortality, however, continued in those fed the Brazilian groundnut meal. Towards the end of 1960, it was generally agreed amongst the investigators that the Brazilian groundnut meal was the cause of the turkey 'X' disease. Furthermore, investigations into unexplained illnesses and deaths amongst calves which had earlier been attributed to ragwort poisoning (Loosmore and Markson, 1961) as well as previous losses amongst pigs (Loosmore and Harding, 1961) showed that the Brazilian groundnut meal had also been used in the rations. Bullocks seemed also affected by the

Brazilian groundnut meal (Clegg, 1961).

Asplin and Carnaghan (1961) reported that the outbreak of disease in Kenya and Uganda, similar to that produced by the Brazilian groundnut meal, was associated with the feeding of expeller-processed groundnut meal. It had been grown in Uganda and Tanzania and processed locally. An extract of the meal reproduced the characteristic liver lesions in ducklings. Groundnuts from West Africa showed the presence of a toxic compound, similar to that found in Brazilian groundnut meal (Sargeant et al., 1961a). Furthermore, Sargeant et al. (1961b) reported that analyzing various samples from different parts of the world, they found a toxic factor similar to that in Brazilian groundnut meal present in some groundnut meals from India, Uganda and Tanzania, French West Africa, Nigeria, Gambia and Ghana. In the U.S., the etiologic agent of the trout hepatoma outbreak was found to be in the cotton seed component of the feeding ration and it was suggested that a carcinogen was present in the meal (Wolf and Jackson, 1963). This therefore meant that the toxic factor was not confined to the Brazilian groundnut meal, or for that matter to just groundnut meal.

A methanol and chloroform extract prepared from samples of Brazilian groundnut meal was shown to be toxic to ducklings (Allcroft and Carnaghan, 1960) and a toxic factor was obtained from a concentrated chloroform extract (Allcroft et al., 1961). Heating the meal seemed to decrease the toxicity of the extract and the toxic factor, on analysis, was determined as being neither a pyrrolizidine alkaloid, nor the nitrogen oxide of such an alkaloid (Allcroft et al., 1961).

Asplin and Carnaghan (1961) proposed the use of day old ducklings for the screening of suspected meals since they seemed highly susceptible to the toxic factor, as a specific response was obtained, the proliferation of bile duct epithelium occurring within a few days. A biological test was then developed in which an extract prepared from the suspected meal was administered by oral intubation (Sargeant et al., 1961 a). The dosing of ducklings with the concentrated extracts afforded a basis for a sensitive and relatively rapid method for the detection of toxicity, enabling the screening of many samples of groundnut meal as well as other samples.

A lot of speculation arose as to the origin of the toxic factor. This included a suggestion that it could be derived from a fungus (Sargeant et al., 1961a). Austwick (1960 pers. comm in Allcroft, 1969) on examining a sample of toxic Brazilian groundnut meal had observed, under the microscope, the presence of hyphae in the groundnut cotyledon tissue but this was not observed in the non-toxic meals. Attempts at culture failed, proving that the hyphae were dead (Austwick and Ayerst, 1963). However, a consignment of highly toxic groundnut meal from Uganda was found to be heavily contaminated with fungi and Sargeant et al. (1961b) succeeded in producing pure cultures of the fungal species present. The fungus was subsequently identified as Aspergillus flavus Link ex Fries by J.J. Elphick (Sargeant et al., 1961b). To prove that this indeed was the causal agent, Aspergillus flavus was grown on sterile non-toxic groundnuts as well as on synthetic media and chloroform extracts prepared of the cultures were found to produce the histological lesions characteristic of the turkey 'X' disease (Sargeant et al., 1961b). However, though the etiological agent had finally been pinpointed, it was far from the end of the story.

In view of its origin, the toxic factor isolated from cultures of A.flavus and responsible for the turkey 'X' disease was named 'aflatoxin' (Interdepartmental Working Party on Groundnut Toxicity Research, 1962). At that time it was not known that this toxic factor was not just a single entity but a mixture of several structurally similar components. Neither was it realized that the composition of the mixture depended on the particular strain of A.flavus as well as the growth conditions present.

In retrospect, it is now realized that the turkey 'X' disease was most probably not the first reported outbreak of aflatoxicosis. Records indicate that toxic groundnut meal had been imported from time to time into England even in the 1950's (Allcroft, 1969). Paget, in 1954, had described a fatal, though non-infectious, disease in guinea pigs which he called 'exudative hepatitis'. The symptoms were gross edema of the subcutaneous tissue, ascites and lesions on the liver, pancreas and lymphoid tissues but at the time the cause was attributed to a nutritional deficiency. The disease occurred sporadically in many laboratories and was associated with a particular diet which contained 15 per cent groundnut meal. In 1957, Stalker and McLean reported another outbreak of the disease and they suggested that there might be a toxic factor in the diet. Later, in 1962, Paterson et al. demonstrated that the diet (a sample of which had been saved) contained a high aflatoxin content of 5 parts per million (5 $\mu\text{g}/\text{kg}$).

Hepatomas in the rainbow trout (Salmo gairdnerii) had been reported as early as the beginning of this century (Halver, 1969). The liver lesions described were identical to those observed in the outbreak of trout hepatoma in 1961 (Wolf and Jackson, 1963). It is now suspected that the aflatoxins probably played quite a significant role in the etiology of the cancer.

However the first significant reports of what now appears to be quite likely, cases of aflatoxin toxicity in domestic animals, were by Newberne et al. (1955) and Burnside et al. (1957). In 1955 a disease in dogs, referred to as 'hepatitis X', was investigated by Newberne et al. (1955) and they traced the cause to a commercial dog food, which was found to contain groundnut meal. An extract of the meal reproduced the symptoms of the disease but the etiologic agent was not discovered. A few years later, Burnside et al. (1957) reported that they had isolated pure cultures of the toxin-producing strains of A.flavus and Penicillium rubrum from moldy maize. The moldy maize had already been shown to have caused an outbreak of toxicosis in swine and cattle fed on the maize as part of their rations. On innoculating sterile, non-toxic maize with the two cultures and subsequently feeding it to the animals, the disease was reproduced. This confirmed the cultures as being toxic. However, the emphasis at the time was placed on the P.rubrum culture. The same moldy grain fed to dogs caused symptoms similar to those observed in the swine and also corresponded to the symptoms of the 'Hepatitis X' disease previously observed in dogs (Bailey and Groth, 1959). The etiological agent of the two outbreaks seems, therefore, to have been most probably one and the same. In 1961, Loosmore and Harding described

a disease syndrome in pigs which was similar to that described by Burnside et al. (1957) and the aflatoxins were implicated in the etiology. Similarly, Newberne et al. (1966) studied the effects of various doses of aflatoxin on dogs and reported that the lesions induced were similar to those observed in cases of 'hepatitis X'.

As has been mentioned before, there had been even earlier incidents in which mold contamination of foods had caused various disease syndromes in animals as well as in man. Once the chemical and biological nature of the aflatoxins was determined, it became evident that several instances of aflatoxicosis had previously been observed in both experimental, as well as farm animals. However, the causes were either wrongly assigned or remained speculative. Why then had the turkey 'X' disease, principally involving turkeys and ducklings, have the impact it had, on the scientific community, at that particular time?

The two most important reasons are that firstly groundnuts are an important and cheap source of protein supplementation in the developing countries, thus the possibility that humans could also be affected by the consumption of aflatoxin-contaminated foods intensified research into this aspect. Secondly, it was shown that aflatoxins were toxic to most species of animals and at levels of contamination which could quite easily occur in foods intended for human consumption. Therefore 'safe' levels of aflatoxin had to be determined (Campbell and Stoloff, 1974).

C. ISOLATION AND CHARACTERIZATION OF THE AFLATOXINS

The term aflatoxins, as we now know them, refer to a group of bisfuranocoumarin metabolites isolated from strains of Aspergillus flavus and A. parasiticus groups of fungi. A single toxic factor, as it was initially thought to be, turned out later to be a complex mixture of various metabolites of different toxicities.

Sargeant et al. (1961 a and 1961 b) were the first group to demonstrate that the toxic principle in the Brazilian groundnut meal could be extracted and resolved by paper chromatography. They managed to concentrate the extract 250 times and then using paper chromatography were able to purify, the not-so-pure crystallized toxin, still further. Development on the Whatman No. 1 paper was done in a solvent system of n-butanol-5 percent acetic acid which showed the toxic principle as a single spot at a Retardation factor (R_f) of 0.7. The spot exhibited a bright blue fluorescence under ultra-violet (UV) light (wavelength of 365nm) and as little as 0.1 μ g of the crystalline toxin was sufficient for detection by this method. A routine chemical assay of the toxin was thus provided and other investigators set out to improve the extraction and resolution methods.

Nesbitt et al. (1962) soon showed that the single blue-fluorescent spot, observed earlier by Sargeant et al. (1961 b) could be further

resolved into two spots on alumina chromatoplates that had been developed in chloroform-methanol (98.5 : 1.5,v/v). One spot had an R_f value of about 0.6, which fluoresced blue-violet under UV light whilst the other fluoresced green at a slightly lower R_f value. These were given the trivial names aflatoxin B and aflatoxin G, respectively.

Using chromatography on thin layer silica gel (Kieselgel, E.Merck) plates, Smith and McKernan (1962) obtained 12 fluorescent compounds from extracts of cultures of toxigenic strains of A. flavus, of which five caused liver lesions using the biological assay, earlier developed by Asplin and Carnaghan (1961). Two of these spots fluoresced blue-green compared to the dark-blue fluorescence of the other three. It was concluded that the toxic activity was related to several of the fluorescent compounds and that possibly with improved separatory procedures, it would be discovered that the activity was even more complex than first suspected. The authors also reported a confirmatory test for the aflatoxins whereby the aflatoxins changed color, when observed under UV light, from a blue or blue-green color to a bright, yellow color after being sprayed with a solution of 50 percent sulphuric acid.

In the Netherlands, another group of investigators reported having isolated a crystalline substance responsible for the turkey 'X' disease (van der Zijden et al., 1962). The crystalline substance obtained was designated FB but it was concluded that impurities were still present. It was de Iongh et al. (1962) who derivatized the extract with Girard's T reagent, subjected it to thin layer chromatography (TLC) and obtained a complex pattern of fluorescent spots. Of these

2 were toxic to ducklings and were designated FB_1 and FB_2 , the former having a blue-violet fluorescence.

In the United Kingdom, Hartley et al. (1963) reported the isolation and characterization of four closely related toxins on silica gel plates using chloroform-methanol (98 : 2, v/v) as developing solvent. two of them had R_f values of 0.4 and 0.36, fluoresced blue-violet and were designated aflatoxins B_1 and B_2 , respectively. The other two had slightly lower R_f values at 0.34 and 0.31, fluoresced green and were designated aflatoxins G_1 and G_2 , respectively. Aflatoxin B_1 appeared to be identical with the FB_1 earlier reported by the Dutch investigators. What had previously been designated aflatoxin G by Nesbitt et al. (1962), was found to be identical to aflatoxin G_1 while the aflatoxin B appeared in actuality to be aflatoxin B_1 , contaminated with some B_2 .

Relatively small amounts of the toxins were all that was available at the time and so their structural elucidation relied heavily upon the interpretation of ultraviolet, infrared (IR), nuclear magnetic resonance (NMR) and mass spectral (MS) data. The molecular formulas as determined by elementary analyses and mass spectrometry were $B_1 - C_{17}H_{12}O_6$ and $G_1 - C_{17}H_{12}O_7$. Aflatoxin B_2 was shown to be the dihydroderivative of aflatoxin B_1 by catalytic hydrogenation of the latter with the uptake of one molar equivalent of hydrogen (van der Merwe et al., 1963). This South African group also showed that aflatoxin G_2 was the dihydroderivative of aflatoxin G_1 , using the same

method (van der Merwe et al., 1963). Structures were suggested but these differed slightly from the structures elucidated and reported in a preliminary communication (Asao et al., 1963). Further studies were carried out and the final structures were confirmed (see Fig. 1.1)

The isolation, identification and characterization of these four main aflatoxins in the short time that had elapsed since its discovery must be regarded as quite a major achievement in the chemistry of natural products. Research continued and this was soon followed by the isolation of numerous other metabolites of the main aflatoxins. The discovery of these toxins was facilitated greatly by their ability to fluoresce under UV light and to be extracted into a variety of organic solvents.

Allcroft and Carnaghan (1963) observed that cows that had ingested aflatoxin-contaminated rations excreted a toxic factor in their milk which had a biological effect (i.e. it produced liver lesions identical to that caused by aflatoxin B₁). The Dutch group of investigators showed that the toxic factor which had been named 'milk toxin', in view of its origin, was a compound that fluoresced blue-violet at an R_f of 0.4 after development in a solvent system of chloroform-methanol (97 : 3, v/v) (de Iongh et al., 1964). The R_f value was well below that of aflatoxin B₁ which indicated that it definitely was a different compound. De Iongh et al. (1964) reported chromatographic evidence that a lactating rat was able to convert aflatoxin B₁ into this 'milk toxin' and that an extract of A. flavus grown on crushed groundnut contained this component as well. The presence of a similar component in the liver of rats fed aflatoxin B₁ was also reported (Butler and Clifford, 1965). Soon after it was reported (Allcroft et al., 1966) that on

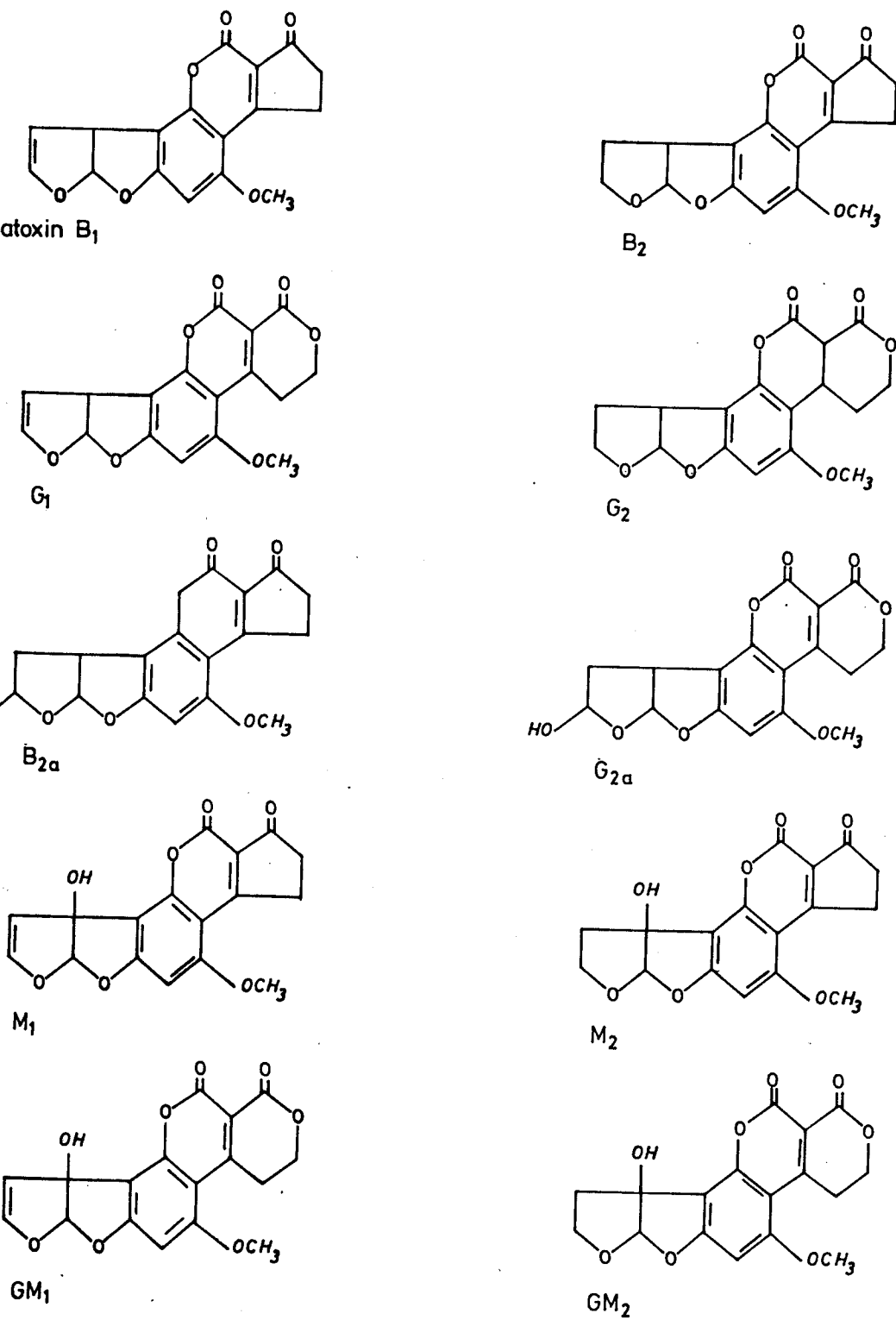
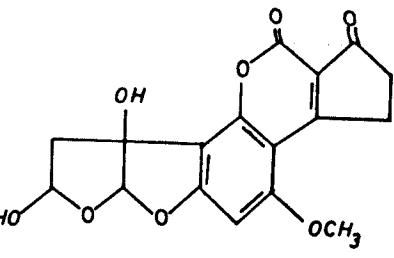
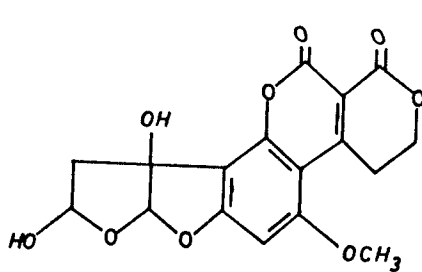


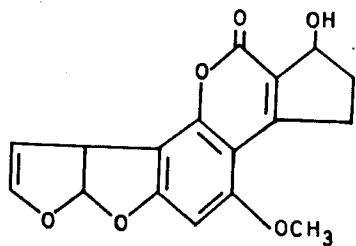
Fig. 1.1. AFLATOXIN STRUCTURES



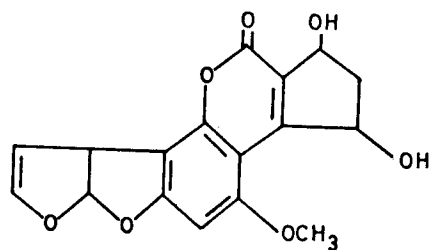
M_{2a}



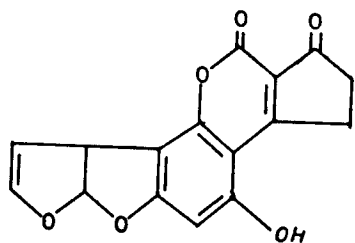
GM_{2a}



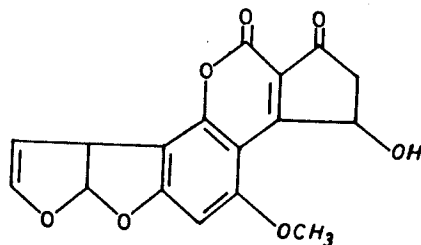
Aflatoxicol



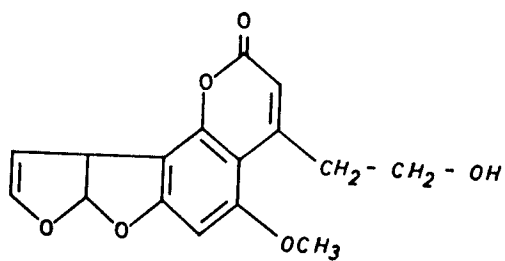
Aflatoxicol H₁



P₁



Q₁



Parasiticol (B₃)

Fig. 1.1. (contd.)

administration of a single dose of mixed aflatoxins (B_1 , B_2 , and G_2) to sheep a substance identical to the 'milk toxin' was found two hours later in the liver, kidney and urine. Since the toxin was not confined to milk, it was suggested by the authors that the trivial name aflatoxin M be given, to indicate its original isolation from milk but at the same time permitting more general applicability.

Holzappel et al. (1966), in South Africa, repeated the experiments of Allcroft et al. (1966). They administered a single dose of mixed aflatoxins and 48 hours later, urine was collected and extracted, with chloroform. The extract was further concentrated using silica gel (Kieselgel, Merck) plates. The concentrated extract was then resolved using paper chromatography, on filter paper impregnated with formamide-water (85 : 15, v/v) and then developed in a solvent mixture of ethyl acetate-benzene (9 : 1, v/v). Two fluorescing components were consequently obtained, a blue-violet one with an R_f of 0.34, designated aflatoxin M_1 and a violet one at a lower R_f of 0.25, designated aflatoxin M_2 . These designations were made assuming the urine factor and milk toxin were one and the same compound. It is interesting to note that whereas the first aflatoxins, B_1 , B_2 , G_1 and G_2 , were resolved by silica gel chromatography, the resolution of aflatoxins M_1 and M_2 was performed on filter paper. Holzappel et al. (1966) were also able to isolate aflatoxins M_1 and M_2 from extracts of moldy groundnuts, in addition to the other four aflatoxins. On the basis of UV, IR, NMR and MS data, this group of workers determined aflatoxins M_1 and M_2 as being the

4-hydroxyderivatives of aflatoxins B_1 and B_2 , respectively (Fig. 1.1)

Two other hydroxylated aflatoxins, one fluorescing blue and the other green were isolated and characterized by Dutton and Heathcote (1966), from cultures of A. flavus. The characterization was unambiguous and the compounds were shown to be 2-hydroxy derivatives of aflatoxins B_2 and G_2 . In accordance with the existing nomenclature, these compounds were designated as aflatoxins B_{2a} and G_{2a} , respectively. These are formed by the hydration of aflatoxin B_1 and G_1 , with the free -OH groups in a different position from that found in the milk toxins. It was quite surprising, at the time, to find that these aflatoxins were relatively non-toxic (Dutton and Heathcote, 1966).

Another blue-fluorescing compound was isolated from a yeast culture inoculated with A. flavus, by Heathcote and Dutton (1969). In keeping with the nomenclature being followed, it was designated aflatoxin B_3 . When these findings were reported, it was discovered that in the United States, Stubblefield et al. (1970) had isolated a compound identical to aflatoxin B_3 , from cultures of A. parasiticus and it had been named parasiticol. From its structure, it has been deduced that aflatoxin B_3 /parasiticol is formed by the hydrolytic cleavage and decarboxylation of the δ -lactone ring of aflatoxin G_1 .

Heathcote and Dutton (1969) detected, at the same time as the discovery of aflatoxin B_3 , a green fluorescent compound in extracts from cultures of A. flavus, with an R_f value slightly less than that of aflatoxin M_1 . Its structure was established and it turned out to be the 4-hydroxy derivative of aflatoxin G_1 . Conforming to the existing nomenclature the compound was designated aflatoxin GM_1 . A minor

metabolite fluorescing green was separated from aflatoxin GM₁ with some difficulty. This new component was shown spectrally to be related to aflatoxin GM₁ and found to be the 4-hydroxy derivative of aflatoxin G₂, it was therefore named aflatoxin GM₂. These aflatoxins must be regarded as minor, natural metabolites of both molds and animals.

Two groups, Detroy and Hesseltine(1970) and Robertson et al. (1970), independently isolated a degradation product of aflatoxin B₁ produced by various micro-organisms. This compound retained the blue-fluorescent property of aflatoxin B₁ and was shown to have the molecular formula C₁₇ H₁₄ O₆. Aflatoxicol, or aflatoxin R₀ as it was named, has become of considerable biological significance ever since it was observed as a metabolite of aflatoxin B₁ in animals, and also because of its high toxicity in certain animal species.

Aflatoxin P₁ was reported by Dalezios et al. (1971) as the principal (accounting for about 60 percent) urinary metabolite of aflatoxin B₁ in rhesus monkeys. Of this 50 percent was found to be present as glucuronide conjugate, 10 percent as the sulphate conjugate and about 3 percent as unconjugated/free phenol. Together, this accounted for more than 20 percent of an intraperitoneal dose of aflatoxin B₁. Dalezios and his colleagues designated the name of aflatoxin P₁ in reference to its phenolic nature and not, as some may have suspected, because it was found in urine. Aflatoxin P₁ is formed through the O-demethylation of aflatoxin B₁, a metabolism step which had been earlier shown by Wogan et al. (1967) and Bassir and Emafo (1970), though the metabolite had been unidentified at the time. It has been shown to be produced by the mixed-function oxidase

system, in the microsomal fraction of homogenized liver (Dalezios et al., 1973). The only phenol amongst the aflatoxins, it has as yet not been isolated from mold cultures.

Another hydroxylation product of aflatoxin B₁ was isolated by Masri et al. (1974; Buchi et al., 1974). It was given the trivial name aflatoxin Q₁ and found to be the oxidation product of aflatoxin B₁. Generally considered to be a detoxification product (Hsieh et al., 1977), aflatoxin Q₁ possesses a hydroxyl group in the β -position to the carbonyl group of cyclopenteno-ring in aflatoxin B₁. So far aflatoxin Q₁ has only been shown to be produced by the microsomal fraction of monkey (Masri et al., 1974) and human livers (Buchi et al., 1974) in vitro.

The isolation and characterization of two new dihydroxy aflatoxins, which belonged to the M series too was reported at the same time as aflatoxin GM₂, by Heathcote and Hibbert (1974). To conform with the existing nomenclature, these were named aflatoxins M_{2a} and GM_{2a} as they were found to be the 2-hydroxy derivatives of aflatoxins M₁ and GM₁, respectively. Aflatoxin M_{2a} had a high R_f value and fluoresced blue in comparison with aflatoxin GM_{2a}, which fluoresced green.

Aflatoxicol H₁, another dihydroxy aflatoxin, is the product of the combined action of oxidases and reductases and is most likely to be another detoxification product (Salhab and Hsieh, 1975). It has an R_f value lower than aflatoxicol, fluoresces blue and is generally considered non-toxic. This metabolite was isolated from human and monkey liver microsomal preparations, in vitro.

There are at least four additional aflatoxin metabolites that remain to be identified but which have been shown to be toxic. These are the metabolite(s) of (i) Schoenhard et al.(1973); (ii) Friedman and Yin (1973); (iii) Moule and co-workers (Moule and Frayssinet, 1968; 1972; Sarasin and Moule, 1973); and (iv) Scaife (1971). These metabolites may after all be aflatoxicol or a closely related derivative (Campbell and Hayes, 1976) but they remain to be isolated and characterized.

The mold-produced aflatoxins are remarkably stable compounds in normal substrates but at either end of the pH scale (very acidic/very basic) they are highly reactive. A similar situation occurs on exposure to UV light in the presence of oxygen. The reactions of the aflatoxin is mainly due to the degree of unsaturation in the furan moiety, as well as from the lactone structure.

D. PREVALENCE

Aspergillus flavus is known to be ubiquitous and as a consequence the aflatoxins produced by certain of its strains, occur almost everywhere in the tropics. Though A. flavus was the first mold species identified with the original veterinary problem, some confusion exists as to whether it was A. flavus or closely-related A.parasiticus which was identified by J.J. Elphick and reported in the paper by Sargeant et al.(1961b)

Both species are capable of producing the aflatoxins and are the only species that have so far been isolated from aflatoxin-contaminated commodities or commodities associated with cases of overt aflatoxicoses (Stoloff, 1977). However, there is some evidence that A.parasiticus is the species most likely to be encountered in the warmer environments and that it is the species more likely to produce the greatest variety of aflatoxins (Stoloff, 1977). On the other hand, it has been noted that A.flavus has the greater capacity for aflatoxin production (Kulik and Holaday, 1967).

The mere presence of moldiness is not of itself indicative of toxin production as many of the strains of A.flavus and A.parasiticus do not produce the aflatoxins as their secondary metabolites. (In the rest of the discussion only A.flavus will be mentioned but the comments apply to A.parasiticus too, unless specified differently). From experience and taste, moldy food is usually rejected by man except in starvation situations or in exceptional cases where the staple food or certain components of the diet are deliberately infected with the mold. In this way toxin levels high enough to produce an immediate response in humans are avoided but quite often the low levels that go past unnoticed can cause chronic toxicity and this is the aspect of aflatoxin toxicity in humans that is still subject to speculation.

Contamination can occur in a given locality with great variability with regard to the types of food that can be infected, the frequency with which they are infected and the levels of aflatoxin present in the foods. Due to the amount of publicity

given to the turkey 'X' disease, the aflatoxins are generally considered only a groundnut problem but this is unduly restrictive, for both oilseeds and cereals can provide excellent substrates for the growth of A.flavus. Virtually any foodstuff (or food product) is potentially susceptible to aflatoxin contamination, given the right conditions. Generally, aflatoxin contamination is a problem of the tropical and semi-tropical areas, as would be expected from the temperature preference of the producing mold. From the limited number of surveys carried out, maize is the dietary staple in which aflatoxins are most likely to be encountered, Brazil nuts and pistachio nuts are the dietary titbits in which there is the greatest risk of aflatoxin contamination, while cottonseed and copra are the oilseeds most likely to be contaminated. Groundnuts fit into all the preceding categories, depending on the region involved (Stoloff, 1977). In Eastern, Central and Southern Africa, dietary surveys have revealed that groundnuts are the principal source of the aflatoxins (Van Rensburg et al., 1975). However, under suitable conditions cottonseed can match groundnuts as a source of aflatoxin, a single cottonseed has been known to contain up to 6 000 000 µg/kg (Feuell, 1969). The only fruit in which aflatoxins have been detected are figs though the detected levels and incidences are low (Buchanan et al., 1975). It is worth noting that spices could provide a means of indirect contamination of otherwise uncontaminated foods, if the spice is added after cooking (Coker, 1979).

Groundnuts and maize are used extensively as both human and

animal feed and, as such, the two crops have been intensively studied in relation to the aflatoxin problem. The thought that groundnuts could give rise to both a toxic and carcinogenic substance caused a lot of concern especially in the U.S. where peanut butter consumption is extremely high. This resulted in a lot of research aimed at determining at which stage of food production aflatoxin production was highest. Originally considered to be predominantly a storage problem, it was soon realized that some contamination of a number of commodities could be traced back to the field. As Stoloff (1977) put it, 'the battle against the mold, once thought confined to commercial storage practices, can now be seen as extending from preharvest to household storage of prepared foods.'

Groundnuts

Experimental work on groundnuts has shown that aflatoxin production occurs at temperatures ranging from 13°C - 41°C, when the kernel moisture content is between 10 and 30 percent and the relative humidity is 85 percent or greater (Diener and Davis, 1967). It has also been observed that the kernels are especially susceptible to fungal invasion once mature since the moisture content is usually over 30 per cent while they are still developing. Quite frequently the kernels are over-mature when they are taken out of the soil and this increases the chances of contamination. Insect damage to the groundnut pods, while still in the soil or once harvested

allows for the entrance of fungal spores. This is quite prevalent in unusually dry seasons, possibly because the insects are attracted to the moisture that is retained in the groundnuts. This is further confirmed by the fact that groundnuts from irrigated lands are invariably free of damage. Mechanical damage can cause even greater damage to the pods, and thus greater contamination, if specially designed equipment is not used (Van Rensburg et al., 1975). It has also been confirmed that groundnuts can be contaminated by aflatoxin in the field, before harvest (Patterson, 1977).

Levels of aflatoxin found in groundnuts can vary from one geographical area to another and from one batch to another as well. In a survey carried out of groundnuts imported into the U.K. it was observed that the highest contamination occurred in samples from Malawi, India and Gambia (Smith and Moss, 1985). It should be noted, though, that the difference in sample sizes within a particular survey can result in discrepancies in the evaluation of levels of contamination in the various samples.

Maize

Aflatoxin contamination of maize, as with the other crops, was always assumed to be a storage problem though there were indications that it could occur earlier. Lillehoj et al. (1975a) proved that contamination was possible even in the field. It was shown that A.flavus spores introduced into test ears resulted in the production of aflatoxin within the first 30 days (Lillehoj et al., 1975b). An explanation for the pre-harvest contamination has

been put forward and this could also apply to post-harvest contamination (Hamilton, 1982). The spores of A.flavus being ubiquitous in both the air and soil of the maize fields, settle on the maize silk, germinate and then grow down the silk without causing any damage to the plant. However, when stresses occur in the plant such as during a drought or soil nutrient depletion over three weeks and the limitations placed on the fungus are lifted, allowing for rapid growth, production of aflatoxin and its accumulation. After harvest the same could occur with the limiting factors being moisture, humidity and temperature. The problem of aflatoxin production in the field was thus established and in the U.S.A. there has been so much data proving economic losses from pre-harvest contamination that the problems of storage have been eclipsed by far (Hamilton, 1982). However, in the developing countries, the storage aspect of the problem is still quite serious. Therefore, storage of inadequately-dried agricultural products or re-wetting of the stored products possibly still has the greatest potential for aflatoxin production. The duration of storage could also be a factor since the longer it is left, the better the chances of building an environment conducive to the growth of A.flavus with the possibility of aflatoxin contamination in the crop.

Decontamination

Prevention is probably the best approach to the control of aflatoxins. Broadly speaking, the control could be divided into

pre-and post-harvest measure. The former would include adequate water supply, correct fertilization, proper cultivation, early harvest, use of specially designed implements if mechanically harvested, and rapid drying of the crop. A long-term possibility is the development of crop varieties which are genetically resistant to the growth of Aspergillus in the field. At the post-harvest stage probably the best measure would be to keep the moisture content of the stored crop as low as possible. However, at times aflatoxin contamination is simply unavoidable and in such cases clean-up procedures need to be used. These can be divided into three : physical removal, biological removal or chemical inactivation.

Physical removal of damaged seeds/kernels or moldy food is something that has been practised in most cultures, even before the discovery of the aflatoxins. This most certainly results in a significant reduction of aflatoxin levels in the remainder of the crop. Roasting of nuts would provide a further 40 - 60 per cent reduction in aflatoxin levels (Stoloff, 1977). Aflatoxin can be extracted from crude oil obtained from oilseeds using various chemicals e.g. 10 per cent aqueous sodium chloride, 1 per cent aqueous calcium chloride or organic solvent systems (Goldblatt and Dollear, 1977)

Biological detoxification of aflatoxin-contaminated foods is possible using domestic animals as a 'filter'. Since only a small percentage of the ingested aflatoxin is recovered in animal tissues/products such as meat and eggs, a conversion of this nature does reduce human exposure to the toxin without wastage of valuable

food. The exception to this possibility is milk which can still contain a relatively high percentage of the aflatoxin consumed.

Chemicals tested include acids, alkalis, aldehydes, oxidizing agents and various gases. The main problem in the use of chemical inactivation is that although the aflatoxin is destroyed to quite a large extent, there are very few chemicals that do so in the absence of leaving deleterious residues or significantly reducing the nutrient content. Treatment of cottonseed and maize with ammonia is probably the most promising of the detoxifying methods available. Under alkaline conditions, the unsaturated lactone ring in the aflatoxin structure opens up in ammoniation and the result is rearrangement leading to carbon-carbon bond scission (Hamilton, 1982). In theory the reverse should occur, the aflatoxin reformed when put into acidic conditions, but in practice this does not occur.

Animal Residues

Human exposure to aflatoxins can occur through the edible products (in general, meat, milk and eggs) of animals that have consumed aflatoxin in their feed. The residue in the tissue could either be the aflatoxin consumed (aflatoxin B₁ has been the one most extensively studied) or one of the metabolites (surveillance studies concentrate on aflatoxin M₁ while experimentally aflatoxicol, aflatoxicol H₁, aflatoxins P₁, Q_a and B_{2a} have been identified as well).

Aflatoxin M₁ in milk was the first metabolite to be observed (Allcroft and Carnaghan, 1963) and it was found that the acute LD₅₀ value of AFM₁ was similar to that of AFB₁, in ducklings (Holzapfel et al., 1966) making it toxic so it has been the metabolite most extensively studied. Taking the average of 8 herds of dairy cattle studied, 300 µg/kg AFB₁ (dry weight) in the daily ration showed up approximately 1 µg/L AFM₁ in milk (Rodricks and Stoloff, 1977). Some aflatoxin B₁ does find its way into the rations of dairy cattle, however stringent the rules are, since aflatoxin M₁ (AFM₁) has been detected in commercial milk or its derived products in the United States (Rodricks and Stoloff, 1977). In a 1977 survey done in several south-eastern states of the United States, contamination levels of 0.1 ng/ml (µg/L) to over 0.7 µg/L were detected in 60 to 80 per cent of market milk samples tested (Stoloff, 1980). This caused some concern and so the Food and Drug Administration (FDA) expanded their regulatory program to include milk. The action level decided upon was 0.5 µg/L (0.5 ppb) for AFM₁ in fluid milk products entering interstate commerce (FDA, 1977).

According to some investigators, AFM₁ appears to be stable in milk and the pasteurization process seemingly has no effect on the levels (Stoloff et al., 1975; van Egmond et al., 1977). However, others have reported that the processing of milk can reduce the amounts of detectable residues of AFM₁ quite significantly : up to 65 per cent with pasteurization at 72°C for 45s, 81 per cent with sterilization at 115°C and 86 per cent when spray-dried (Smith and Moss, 1985). It has been agreed, though, that during the separation

of the components of milk, in the preparation of the various dairy products, there is a very specific distribution of AFM_1 , due to its insolubility in milk fat (being a hydrophilic aflatoxin) and resultant absorption into curd. Considering the various steps involved in cheese-making, it could be expected that cheese would have 3.5 - 5 times the AFM_1 level found in the original milk whilst butter would have 0.5 - 0.7 times the original amounts (Stoloff, 1980). Prior to the restrictions on the aflatoxin levels permitted in dairy feeds it was not uncommon for fairly high levels to be detected in milk products such as cheese, ice-cream, yogurt, etc., in Europe and USA. Following the introduction of the action level in the USA, the levels detected have declined significantly. It therefore seems quite clear that the best approach to the control of AFM_1 in milk is through the control of the levels of AFB_1 permitted in animal feeds.

At this point it should be mentioned that though ammoniation of aflatoxin-contaminated feeds has been shown to considerably reduce the toxic levels, caution needs to be exercised in the distribution of this feed. A study was done to investigate this in which cows were fed ammoniated aflatoxin-contaminated cottonseed meal (Sinnhuber et al., cited in Rodricks and Stoloff, 1977). Strangely enough, though the milk from the cows showed no detectable aflatoxin levels, when the milk was fed to rainbow trout (known to be very sensitive to the toxic effects of aflatoxin) it produced significant liver damage as well as a statistically significant incidence of hepatomas. Market milk was used as a control and this produced a lower incidence of hepatomas. This observation is rather

interesting in that the feed considered to have been detoxified in actuality was apparently still quite toxic. Possibly, there could be a reversion from the reaction products to aflatoxin (though this would not explain the absence of detectable aflatoxin in the milk fed to the trout), or more probably the residues themselves may be toxic. Further investigations would be required, however, before any conclusions can be drawn.

Studies to determine the transfer ratios ($\mu\text{g}/\text{kg}$ aflatoxin in feed : $\mu\text{g}/\text{kg}$ aflatoxin in animal tissue) in which the common meat animals were intentionally dosed with aflatoxin, predict that there is little danger of carry-over since the ratios are quite high. The transfer ratios in the liver (where the highest levels of aflatoxin are found) are as follows : cattle - 14 000; swine - 800; broiler chicken - 1 200. In layers the transfer ratio is 2 200 in eggs (Rodricks and Stoloff, 1977). In the liver aflatoxin B₁ is found together with approximately equal amounts of aflatoxin M₁. As the toxic level to humans is considered as approximately 1.7 mg/kg daily (Campbell and Stoloff, 1974), such ratios means that the amount the animal would need to consume would cause pathological symptoms in the animal or even death and therefore the chance of these animals entering the food chain is hardly a threat. It therefore appears that such a conversion would probably be the best way to use food that is too highly contaminated with aflatoxin for human consumption, though not so high that they find a way into the edible animal tissues (except for milk).

Regulatory guidelines on levels of aflatoxin permissible in foods is extremely variable in different parts of the world. In most

developing nations, severe restrictions may lead to unnecessary hardship due to loss of food, or loss of income. The permitted levels of aflatoxin if specified, are often only applied to commodities for export, but internal foods are not subject to any screening. This is the situation in Zambia. An example of permitted levels are as follows : Belgium and Netherland - 5 $\mu\text{g}/\text{kg}$ (aflatoxin B_1), Japan - 10 μg (aflatoxin B_1) and the United States - 20 $\mu\text{g}/\text{kg}$ (total aflatoxin). Generally 10 $\mu\text{g}/\text{kg}$ (total aflatoxin) is taken as the maximum permissible concentration in Zambia (Food and Drugs Control Laboratory, pers. comm.).