AFLATOXIN METABOLITES IN HUMAN URINE AND LIVER IN ZAMBIA

by

Lee Ann Gage Dil

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

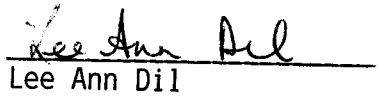
THE UNIVERSITY OF ZAMBIA

LUSAKA

1986

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.



Lee Ann Dil

This dissertation has been examined by

a committee as follows:

Dr. H.P. Van Egmond

National Institute of Public Health and

Environmental Hygiene,

Bilthoven, The Netherlands.

External Examiner

Dr. J.S.N. Kaggwa

Dissertation Supervisor

Cheryl Lovelace

Prof. C.E.A. Lovelace

Co-Supervisor

Prof. A.C. Bayley

Dissertation Chairman

ABSTRACT

Aflatoxins are believed by many investigators to play a major role in the etiology of primary hepatocellular carcinoma (PHC). As PHC is the commonest tumour of men in Zambia, investigation of aflatoxin exposure of the Zambian population is warranted. Previous studies in Zambia have shown that aflatoxins are present in certain foods and confirmation of consumption was obtained from a study demonstrating the presence of aflatoxin metabolites in human urine. In this study urine samples were collected from patients with and without liver disease admitted to the University Teaching Hospital in Lusaka, in an attempt to examine the possible effect of liver pathology on the type and amount of aflatoxin metabolite(s) excreted. No significant differences were observed. The present study was therefore carried out to investigate this relationship in more depth.

Studies have shown that conjugation reactions play an important role in aflatoxin metabolism in animals although the amount and type of aflatoxin conjugates in humans is not known. Thus urine samples, from patients with and without liver disease, were collected for one year and analysed for both free and conjugated aflatoxin metabolites.

The incidence of urinary aflatoxin excretion in liver disease and control patients was 3.7 per cent with a mean concentration of 9.1 ng/100 ml. Aflatoxins B_1, M_1 and aflatoxicol (AFL) were detected. No significant differences were observed between the two groups of patients although aflatoxins were seen more frequently and at greater concentrations in urine of control patients. This may suggest that patients with liver disease have an impaired ability to metabolize and excrete aflatoxin.

aflatoxin studies, serum and liver samples were analysed for HBV markers. The HBV carrier state in this study was observed to be exceedingly high: 32 per cent of control patients were shown to have hepatitis B virus surface antigen in their serum. No relationship between the presence of HBV markers, aflatoxin metabolites excreted, and PHC was observed. It was noted, however, that patients excreting aflatoxin also had evidence of HBV infection. A longitudinal, prospective study should now be carried out to investigate the possible interactions of these two factors more closely.

Glucuronide and sulphate conjugates of aflatoxins M_1, P_1 and AFL were looked for. Surprisingly, no conjugated metabolites were detected, even in patients excreting up to 20 ng of free aflatoxin /100 ml urine. This suggests that these conjugation reactions do not play a major role in aflatoxin metabolism in humans.

The urinary survey was extended to a rural hospital in the Eastern Province. Consumption of aflatoxin was observed to be more of a problem there, where urinary aflatoxins were detected significantly more often (incidence = 15.4 per cent) and at greater concentrations (mean = 16.5 ng /100 ml).

Liver tissues, taken at autopsy from subjects with and without liver disease, were also examined for aflatoxin metabolites. Only aflatoxin B_1 was observed, at concentrations of 0.3-9.5 ng/g. Most of the samples were from controls and therefore the effect of liver pathology on the type and/or amount of aflatoxin metabolite(s) observed could not be assessed. However, the liver of one PHC subject was included and was found to contain over 10 times more aflatoxin than the highest level observed in controls.

The results of the urinary and liver studies were tabulated according to age, sex and season. Aflatoxin was detected significantly more often ($P < 0.05$) in livers obtained from males than females and, in Lusaka, only urine samples collected from males were positive for aflatoxin. It was found that the likelihood of consuming aflatoxin was significantly higher in the rainy season than the dry season ($P < 0.05$).

It is generally believed that both aflatoxin and hepatitis B virus (HBV) influence the incidence of PHC. Therefore, concurrent with

Acknowledgements

I wish to express my sincere gratitude to Dr. J.N. Kaggwa and Professors C.E.A. Lovelace and A.C. Bayley for their invaluable advice and guidance during the course of my research and in the preparation of this dissertation. Many thanks also go to Mr. R. Hamwitala who worked tirelessly for one year, collecting and extracting urine samples. I would also like to acknowledge Dr. N. Nkanza who provided the liver samples.

I would like to express my great appreciation to Dr. Ansary and his staff at Medical Illustration for the expert work they carried out to produce the photographs, figures and tables contained in this dissertation. I am also indebted to Mr. N. Ng'andu for statistical advice and Miss M. Musopelo for typing the manuscript.

Lastly, I would like to thank my family; my husband for his constant love and encouragement, my parents-in-law for their support, both financial and emotional, and also my own parents for the help they gave me during the course of this study.

This work was sponsored by the World Health Organization and the University of Zambia.

TABLE OF CONTENTS

Tables

Tables Continued

Figures

Figures continued

CHAPTER ONE

INTRODUCTION

1. History of Aflatoxins

Mycotoxins, toxic metabolites of certain saprophytic fungi, cover a wide range of chemical compounds. Aflatoxins, produced as secondary metabolites by various strains of *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus*, have been the most intensively studied because of their extraordinary potency as hepatocarcinogens in a wide variety of animal species.

The aflatoxins were first isolated in 1961, subsequent to an outbreak of an apparently new disease in turkey poults occurring in England in 1960, causing an estimated loss of more than 100,000 birds (Blount, 1961). Outbreaks of a disease with similar symptoms were reported simultaneously in other species and in other countries. Reports were received from Kenya and Uganda of severe losses of ducklings and in the United States and an outbreak of liver cancer was noted among hatched rainbow trout (Wolf and Jackson, 1963). Investigations carried out by numerous scientists revealed that the disease resulted from the presence of a toxic factor in the feeds of these animals. Further examination revealed the presence of the fungus *A. flavus*, and subsequently the metabolites of the fungus were identified as the toxic agents (Asplin and Carnaghan, 1961; Sargeant *et al.*, 1961). In view of their origin they were collectively named aflatoxins (*ie. A. flavus toxins*).

In 1963 aflatoxins were demonstrated to be a mixture of four related compounds, using chromatography on thin layer silica gel plates developed in chloroform:methanol (98:2) (Hartley *et al.*, 1963). A single blue fluorescent spot was split into four components. Two of these fluoresced blue under ultraviolet light (365 nm), had R_F values of 0.4

and 0.36, and were designated aflatoxins B_1 (AFB_1) and B_2 (AFB_2) respectively. The other two fluoresced green, had R_F values of 0.34 and 0.31 and were designated aflatoxins G_1 (AFG_1) and G_2 (AFG_2) respectively.

The isolation of these four main aflatoxins was soon followed by the isolation of a number of other analogues. The most important of these is perhaps aflatoxin M_1 which is the principal free metabolite produced when animals and humans ingest AFB_1. It was first discovered by de Iongh *et al.*, (1964) as a toxic factor in cow's milk, hence its name aflatoxin M_1 (AFM_1). Subsequently it was found that the aflatoxin metabolite commonly found in the urine of animals fed AFB_1 was also AFM_1 (Masri *et al.*, 1967).

2. Sources of Aflatoxins

Aflatoxins arise naturally when a toxin-producing strain of *A. flavus* or *A. parasiticus* grows on a substrate in a geographical area where environmental conditions are suitable for the development of the mould. Aflatoxin-producing strains appear to be distributed ubiquitously but in general contamination is a problem of tropical and semi-tropical areas. Optimal production occurs on foodstuffs at temperatures of 25-30^{o}C and a relative humidity of 85 per cent or greater (Heathcote and Hibbert, 1978).

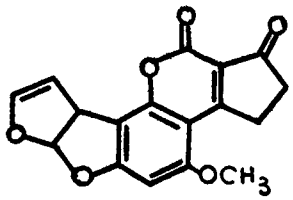
Virtually every foodstuff or food product is potentially susceptible to contamination, which may occur at any stage of food production or subsequent processing (IARC Monographs, 1975). Samples of nearly all major dietary staples have been found to contain aflatoxin at one time or another and commodities commonly reported to be contaminated include groundnuts and groundnut products, maize and other grains such as rice, wheat and millet, and vegetables like beans, soybeans and dried green

leaves (Muhihu, 1978). The highest concentrations of aflatoxins have been found in edible nuts: In Eastern, Central and Southern Africa groundnuts are the most serious source of contamination (*e.g.* Mozambique Van Rensburg *et al.*, 1975).

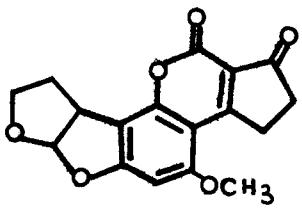
Contamination of a food commodity with aflatoxin is generally believed to occur most often during post-harvest and is associated with poor drying and storage practices. Thus the risk of exposure is increased in rural areas with simple storage methods. Adequate control measures involving especially rapid post-harvest drying of crops and storage at moisture contents of less than 10 per cent can virtually eliminate contamination (IARC Monographs, 1975). There is also evidence that pre-harvest contamination can be a problem and this is usually associated with insect damage. Therefore insect pests should be controlled (Lillehoj *et al.*, 1976; Njapau, personal communication).

3. Chemistry of Aflatoxins

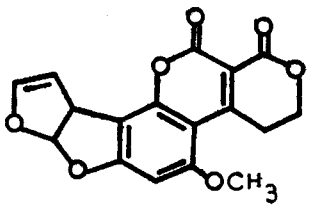
The four naturally occurring aflatoxins, as stated previously, are designated aflatoxins B_1, B_2, G_1 and G_2, according to their fluorescent colour and chromatographic positions. The structures of these parent aflatoxins and the metabolites that have been isolated and characterized are given in Figure 1.1. They all contain a difuran ring structure fused to a substituted coumarin moiety. The reactions of the aflatoxins are derived from the unsaturation in the furan moiety and from the lactone structure and are mediated, in animals, by the hepatic microsomal mixed function oxidase system. AFB_1 is the major fungal metabolite although AFG_1 is likely to occur whenever there is AFB_1. AFB_2 and AFG_2 are the dihydroderivatives of AFB_1 and AFG_1 respectively, hydrogen being added



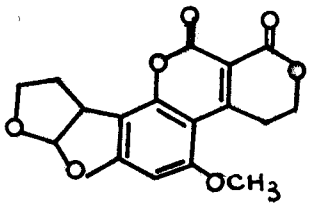
Aflatoxin B_1



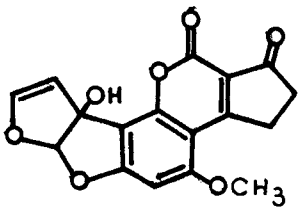
Aflatoxin B_2



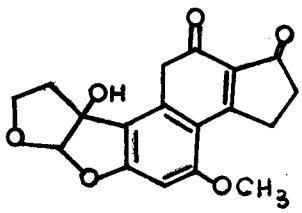
Aflatoxin G_1



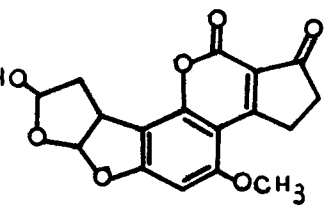
Aflatoxin G_2



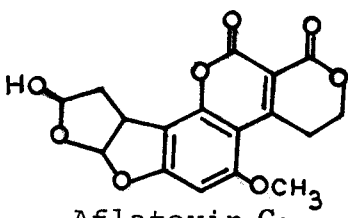
Aflatoxin M_1



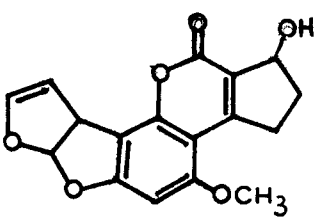
Aflatoxin M_2



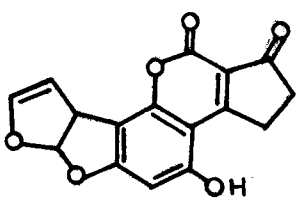
Aflatoxin B2a



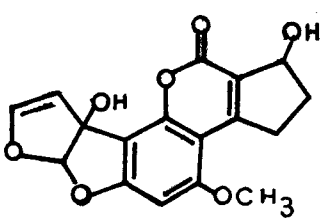
Aflatoxin G_{2a}



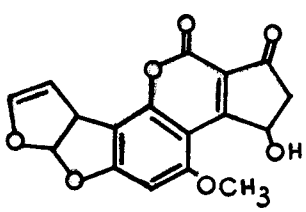
Aflatoxicol



Aflatoxin P_1



Aflatoxicol M_1



Aflatoxin Q_1

Figure 1.1 STRUCTURE OF THE AFLATOXINS

across the double bond in the terminal furan ring. The other metabolites all contain an additional hydroxy group at one of various positions (Figure 1.1). These more polar metabolites are apt to be conjugated and excreted (Adekunle *et al.*, 1977). Each metabolite differs in its biological activity and therefore metabolism plays a prominent role in determining the toxicity and carcinogenicity of these compounds.

4. Toxicity of Aflatoxins

4.1. Animal Studies

The acute and chronic effects of aflatoxin exposure have been well studied and it is known that they are toxic to a large number of animal species. AFB_1 is the most acutely toxic parent metabolite, followed by aflatoxins G_1, B_2, and G_2, in order of decreasing potency. In tests on day-old ducklings, Carnaghan *et al.*, (1963) gave the LD 50 values (in mg/kg body weight) as: B_1, 0.36; G_1, 0.78; B_2, 1.70; G_2, 3.45.

The sensitivity of animals to aflatoxin varies from species to species as shown in Table 1.1. Even within any given species variation in susceptibility is seen among individuals. In a study using rhesus monkeys, Deo *et al.*, (1970) found that among animals of the same sex and age, given the same amount of toxin and maintained with identical dietary intakes, some showed massive haemorrhagic necrosis of the liver and others only minimal alterations.

Susceptibility to aflatoxins is determined to some extent by the sex and age of the animal. Young animals are more susceptible than mature animals and the male (rat) is more sensitive than the female (Table 1.1). Susceptibility is also influenced by the condition of the

TABLE 1.1

ACUTE TOXICITY OF AFLATOXIN B_1 IN VARIOUS SPECIES

SPECIES		LD_{50} SINGLE DOSE (mg AFB_1/kg BODY WEIGHT)
Duckling *		0.3
Trout +		0.5 - 1.0
Pig *		0.6
Sheep +		1.0 - 2.0
Guinea Pig *		1.4
Baboon +		2.0
Chicken +		6.0 - 16.0
Rat *		
	neonate	0.56
	weanling	5.5
	male	7.0
	female	18.0
Mouse*		9.0

(+Coker, 1979; *Newberne and Butler, 1969)

animal and composition of its diet. Most significant is the observation that a protein-deficient diet enhances toxicity (Madhavan and Gopalan, 1965).

The toxic effects of aflatoxins on various animal species were thoroughly investigated by Newberne and Butler (1969) and have been more recently reviewed by Heathcote and Hibbert (1978). The initial manifestations of aflatoxicosis include general malaise, failure to thrive and a decreased resistance to infection. The main acute effects are hepatic cell necrosis, gastrointestinal bleeding, nephritis and lung congestion. Chronic toxicity results in liver lesions and liver tumours.

The liver is the main target organ in all species studied and bile duct hyperplasia has come to be recognized as the most characteristic and easily identifiable result of aflatoxin poisoning although not specific to aflatoxin. The location of liver necrosis varies with the species. The rat and duckling display periportal distribution, in guinea pigs and swine the necrosis is centrilobular, while in rabbits mid-zonal lesions are seen (Newberne and Butler, 1969). This observation, in addition to the results demonstrating that susceptibility to toxicity varies from species to species, suggests that different species metabolize and excrete aflatoxin in different ways and/or at different rates.

Animal experiments which are perhaps most relevant to the question of whether humans are susceptible to aflatoxicosis are those involving primates. A limited amount of work in this area has been published. The majority of studies involve the rhesus monkey.

Deo *et al.*, (1970) fed a partially purified mixture of AFB_1 and AFG_1 to 64 young male rhesus monkeys at dose levels of 1.0 mg/kg body weight daily (Group I), 0.25 mg/kg body weight twice weekly (Group II),

and 62 µg/kg body weight once a week (Group III). All animals in Group I died before the end of the third week. This dose however, is very high and is unlikely to occur in a human situation. Dose levels of Groups II and III are more realistic and could conceivably be consumed by humans (see Table 1.2). Thus it is significant that the animals of Group II exhibited signs of severe liver damage and the animals of Group III showed similar symptoms in a milder form. The authors concluded that the rhesus monkey is a species highly susceptible to aflatoxicosis.

Campbell and Stoloff (1974) have summarized available primate data and have suggested that, for primates in general, a daily dose likely to produce serious liver damage in a short period of time is about 0.05 mg/kg body weight and a single dose resulting in death is about 2.2 mg/kg body weight.

4.2. Human Studies

The possible acute and chronic effects of aflatoxin in humans have been estimated by observing suspected cases of aflatoxicosis and investigating the diet associated with these cases and/or examining tissue and body fluid specimens for evidence of aflatoxin consumption. Human incidents in which aflatoxin involvement is claimed or postulated are given in Table 1.2.

In order for the reported aflatoxin contamination levels to have meaning an estimation of the dose required to produce a toxic effect in humans must be made using experimental animal data. Campbell and Stoloff (1974) have summarized the data on toxicosis in primates and computed the

TABLE 1.2

HUMAN CASES OF AFLATOXICOSIS

YEAR	INVESTIGATORS	COUNTRY	EVIDENCE FOR AFLATOXIN CONSUMPTION	CONSEQUENCES
1966	Payet *et al.*	Senegal	peanut meal 0.5-1.0mgAFB_1/kg	chronic hepatitis
1970	Serek-Hansen	Uganda	Cassava 1.7mgAFB_1/kg	death of one boy sib-lings ill
1970 and 1974	Torres *et al.* VanRensburg	Mozambique	900 meals analyzed -9.3%AF positive range 0-1.5mgAF/kg	toxic hepatitis commonly seen in a histological study
1971	Amla *et al.*	India	peanut meal 0.3mgAFB_1/kg	Indian Childhood Cirrhosis
1971	Bourgeois *et al.*	Thailand	rice 6mgAFB_1/kg	EFDV/Reye's Syndrome
1971	Shank *et al.*	Thailand	tissue specimens at autopsy-AFB_1 present in 22 out of 23 cases liver - 93ngAFB_1/kg stomach - 1270ngAFB_1/g	23 cases of EFDV/Reye's Syndrome
1972	Bosenberg	Germany	liver specimen at autopsy-AFB_1 present	sudden death of one sub-ject with liver sclerosis
1974	Dvorackova *et al.*	Czechoslavakia	liver specimens at autopsy-AFB_1 present in 2 out of 3 cases	EFDV/Reye's Syndrome

TABLE 1.2 cont'd

YEAR	INVESTIGATOR	COUNTRY	EVIDENCE FOR AFLATOXIN CONSUMPTION	CONSEQUENCES
1975	Krishnamachari *et al.*	India	maize -2mgAF/kg; estimated people consuming 2-6mgAF daily for about 1 month	toxic hepaitis outbreak -397 cases 105 deaths
1976	Richir *et al.*	France	liver specimens at autopsy - AFB_1 present	liver disease cases
1982	Ngindu *et al.*	Kenya	maize -3.2-12.0mgAF/kg liver specimens at autopsy -89ngAFB_1 /g	toxic hepatitis outbreak -20 cases, 12 deaths

necessary level of contamination of human food to ensure a toxic dose. They concluded that humans eating food containing 1.7 mg/kg daily would develop serious liver damage in a short period of time. This value is within the range of a number of reported incidents (Table 1.2). It is also important to consider that populations usually exposed to aflatoxins may be expected to have a higher rate of concurrent disease and malnutrition, thus the primate data may overestimate minimum hazardous levels (Van Rensburg, 1977). In addition, the majority of suspected cases concern children, who are likely to have a lower tolerance for aflatoxins than adults.

Encephalopathy and fatty degeneration of the viscera (EFDV) or Reye's syndrome is an acute disease of children characterized by cerebral edema and fatty degeneration of the viscera, particularly the liver. Becroft (1966) suggested that aflatoxins may cause Reye's syndrome based on pathological indications that the liver lesions were toxic in origin, the similarity of the lesions to those of experimental aflatoxicosis, the special sensitivity of young animals to aflatoxins, and indications from case histories that the disease is likely to result from ingestion of a toxin present in food (Becroft and Webster, 1972).

This syndrome has been recognized in a number of countries but is especially common in Thailand where it affects hundreds of children. A study of aflatoxin contamination of Thai foods has shown that it corresponds with seasonal and geographical patterns of EFDV (Bourgeois *et al.*, 1971). In two cases of EFDV samples of the main food consumed prior to the fatal illness were found to be heavily contaminated with aflatoxin. One of the food samples, steamed rice, contained 6 mg/kg, one of the highest known intakes by humans (Campbell and Stoloff, 1974).

Aflatoxins have been observed in tissue specimens of Thai children who suffered from EFDV. In a study of 23 EFDV cases AFB_1 was found in one or more tissues from 22 of the 23 cases studied. Although aflatoxin was also detected in tissue specimens from some of the control subjects, only cases of EFDV had more than trace amounts of AFB_1 (Shank *et al.*, 1971).

Aflatoxin has also been incriminated in the etiology of Indian Childhood Cirrhosis, a condition of obscure etiology which is the third most common cause of death in hospitalized children under the age of five years in India (Chandra and Seth, 1972). Amla *et al.* (1971) reported on 20 kwashiorkor patients that had received a supplementary diet of peanut flour later found to contain AFB_1 at a level of 0.3 mg/kg. Three of the children displayed signs of subacute toxicity and had hepatic lesions identical to those seen in Indian Childhood Cirrhosis. The level of aflatoxin contamination reported in this study is relatively low but may still be significant as the toxic effect of aflatoxin has been shown to be enhanced in a protein-deficient state (Madhavan and Gopalan, 1965).

Recently aflatoxins have been linked with kwashiorkor. Hendrickse and co-workers (1982) analyzed aflatoxins in serum and urine samples from Sudanese children and observed that aflatoxin serum levels were, on average, 10 times higher in children with kwashiorkor than in other malnourished and control groups. In contrast, aflatoxin levels in urine were lower in children with kwashiorkor. These findings possibly indicate that the ability of children with kwashiorkor to transport and excrete aflatoxins is impaired by the metabolic derangements associated with kwashiorkor. The authors, in turn, point out that

several metabolic effects of aflatoxins produced in animal studies, including hypoalbuminemia (Tung *et at.*, 1975), fatty liver (Hamilton, 1977) and immunosuppression (Richard, 1978), are similar to derangements consistently found in kwashiorkor. They go on to describe a possible vicious cycle in which the liver, after suffering initially from the symptoms of kwashiorkor, is impaired in its ability to handle and excrete aflatoxins, causing serum aflatoxin levels to rise as the ability to handle these substances progressively declines.

In addition to being implicated in the diseases of children described above, aflatoxins have been reported to cause illness in adults. Serck-Hansen (1970) described a single fatal case of apparent aflatoxicosis in Uganda. Cassava collected from the home of the subject was found to contain 1.7 mg/kg aflatoxin and no other cause of death was visible.

An extensive outbreak of toxic hepatitis occurred in Western India in 1974, affecting 397 people, 106 of whom died (Krishnamachari *et al.*, 1975). The outbreak was associated with consumption of maize heavily contaminated with *A. flavus* and maize samples taken from the homes of those affected showed aflatoxin levels ranging from 6.25 to 15.6 mg/kg. The population involved here, as in most cases of aflatoxicosis, was rural and poor and virtually dependent on a single crop produced by themselves.

A more recent outbreak of possible aflatoxicosis occurred in Kenya in 1981 (Ngindu, et al.,1982). Twenty patients with hepatitis were admitted to hospitals, 12 of whom died. The illness tended to occur in family groups, family clusters accounting for 80 per cent of cases and 83 per cent of fatal cases. Two families, from which 8 of 12 sick members died, were eating maize containing as much as 12 mg/kg aflatoxin

and specimens of liver tissue obtained at autopsy contained up to 89 ng/g AFB_1.

There are perhaps many more cases of aflatoxicosis than those reported. Van Rensburg (1977) has stated that there is a lack of awareness of the potential problem of toxicosis among clinicians even though the hepatocarcinogenic character of aflatoxins has received wide recognition. This is likely due to the similarity of aflatoxicosis to other common diseases, especially hepatitis, and the fact that treatment for both diseases would be similar.

5. Carcinogenicity of Aflatoxins

5.1. Animal Studies

The carcinogenicity of aflatoxin has been demonstrated in many animal species. The most frequently studied animals have been the trout and rat. In rainbow trout a diet containing 0.1 µg/kg AFB_1 gave a 100 per cent tumour yield (Halver, 1965) and in rats dietary AFB_1 levels of 0.1 mg/kg, given for up to 80 weeks induced liver carcinoma at an incidence of greater than 50 per cent (Wogan, 1973), making AFB_1 the most potent hepatocarcinogen known. There is, however, wide species variation and there are species relatively resistant to the induction of hepatic tumours, for example the mouse (Wieder *et al.*, 1968). In addition, factors such as strain, age and sex that contribute towards the acute toxicity of aflatoxin also affect its chronic toxicity. The results of feeding trials in various animals are shown in Table 1.3.

TABLE 1.3

LEVEL OF AFLATOXIN B_1 IN DIET RESULTING IN LIVER TUMOURS IN IN VARIOUS SPECIES

SPECIES	B_1 IN DIET (µg/kg)	PERIOD FOR TUMOUR INDUCTION (MONTHS)
rainbow trout	2.0	20
sockeye salmon	12.0	?
rat	15.0	24
duck	30.0	14
pig	1000	30
marmoset	2000	2.25
guppy	6000	11
rhesus monkey	500 mg over 6 years	No tumour after 18 months

(IARC, 1975)

A limited amount of work with primates has been published. Gopalan *et al.* (1972) administered a mixture of aflatoxins in a sub-acute dose to a male and female rhesus monkey for five and a half years. Two and a half years after administration had been discontinued the male animal died from a hepatoma. Three years later the female died after developing a metastasizing intrahepatic bile-duct carcinoma (Tilak, 1975). Adamson *et al.* (1973) carried out a study using a mixed sex and species group of 40 rhesus and cynomologous monkeys. Primary liver carcinoma developed in one female rhesus monkey after administration of a sub-acute dose of AFB_1 for six years.

Aflatoxin B_1, administered at sub-acute, has also been shown to produce hepatic tumours in marmosets (Lin *et al.* (1974) and tree shrews (Reddy and Svoboda, 1975).

Hepatomas are not the only tumours resulting from aflatoxins. Butler and Barnes (1966) noted the induction of carcinomas of the glandular stomach in rats fed aflatoxin. The same authors (1968) also described oncogenic effects on the kidney. In addition, it has been shown that AFB_1 induces colonic cancer in rats (Wogan and Newberne, 1967) and pulmonary tumours in mice (Wieder *et al.*, 1968).

Since aflatoxins are most frequently found to contaminate foodstuffs in regions where there is a high prevalence of malnutrition, experimental attempts have been made to determine the effect of diet, particularly protein, on aflatoxin carcinogenicity. Interestingly, a high protein intake has consistently been shown to increase AFB_1 carcinogenicity but depress its acute toxicity (Madhavan and Gopalan, 1965, 1968; Adekunle *et al.*, 1977; Temcharoen *et al.*, 1978). These studies gave

evidence that dietary protein deficiency affords moderate protection against AFB_1 carcinogenicity in rats and monkeys.

In addition to the carcinogenic effect of chronic administration of aflatoxin, immunosuppressive actions have been observed (reviewed by Lutwick, 1979). For instance, diets containing 250-500 μg/kg aflatoxin, when fed to poultry, predisposed them to attack by viruses and bacteria (Edds *et al.*, 1973). If this decrease in resistance to infectious diseases is applicable to humans, it may represent an important feature of the chronic effects of aflatoxins.

5.2. Human Studies

Epidemiological studies carried out in Swaziland (Keen and Martin, 1971; Peers *et al.*, 1976), Uganda (Alpert *et al.*, 1971), Thailand (Shank *et al.*, 1972), Kenya (Peers and Linsell, 1973), and Mozambique (Van Rensburg *et al.*, 1974) offer statistical support for the association between the incidence of hepatocellular carcinoma and consumption of foods contaminated with aflatoxin. In these studies it was assumed legitimate to compare current exposure to aflatoxin with present cancer rates as observations were limited to stable rural populations with diet, storage and cooking habits that had not changed over the recent past.

Keen and Martin (1971) analyzed market samples of groundnuts, known to be a main source of aflatoxin, and the frequencies of contamination were related to cancer registration in three geographical areas of Swaziland, which could be expected to yield different exposures to aflatoxin. In the high veld the average intake of aflatoxin was 5.1 ng/kg body weight/day and the incidence of hepatoma was 2.2

/100,000/year. In the middle veld intake was 8.9 ng/kg body weight/day and the rate of hepatoma was 4.0/100,000/year, while in the low veld intake was 43.1 ng/kg body weight/day and the hepatoma rate was 9.7/100,000/year. Although this study measured only one dietary item, the correlation of level of contamination with frequency of liver cancer is clear.

The study by Alpert *et al.* (1971), in Uganda, extended the range of food examined to include other major dietary staples. Again an association between levels of aflatoxin contamination and distribution of liver cancer was demonstrated.

The studies from Mozambique, Thailand, Kenya and a further study in Swaziland, attempted to evaluate aflatoxin exposure using 'food from the plate' surveys and then to relate this to cancer incidence. Van Rensburg *et al.* (1974) reported a mean dietary aflatoxin intake of 222.4 ng/kg body weight/day in the Inhambane district of Mozambique. The liver cancer incidence of this area was calculated to be 13.0/100,000/year (Van Rensburg, 1977). Thus the area with the highest reported aflatoxin consumption level had the greatest recorded hepatoma rate.

The results of some of these studies are summarized in Table 1.4. It can clearly be seen that cancer rates increase as consumption of aflatoxin increases. Over a wide range there is a linear relationship between cancer incidence and the logarithm of the level of aflatoxin intake (Figure 1.2).

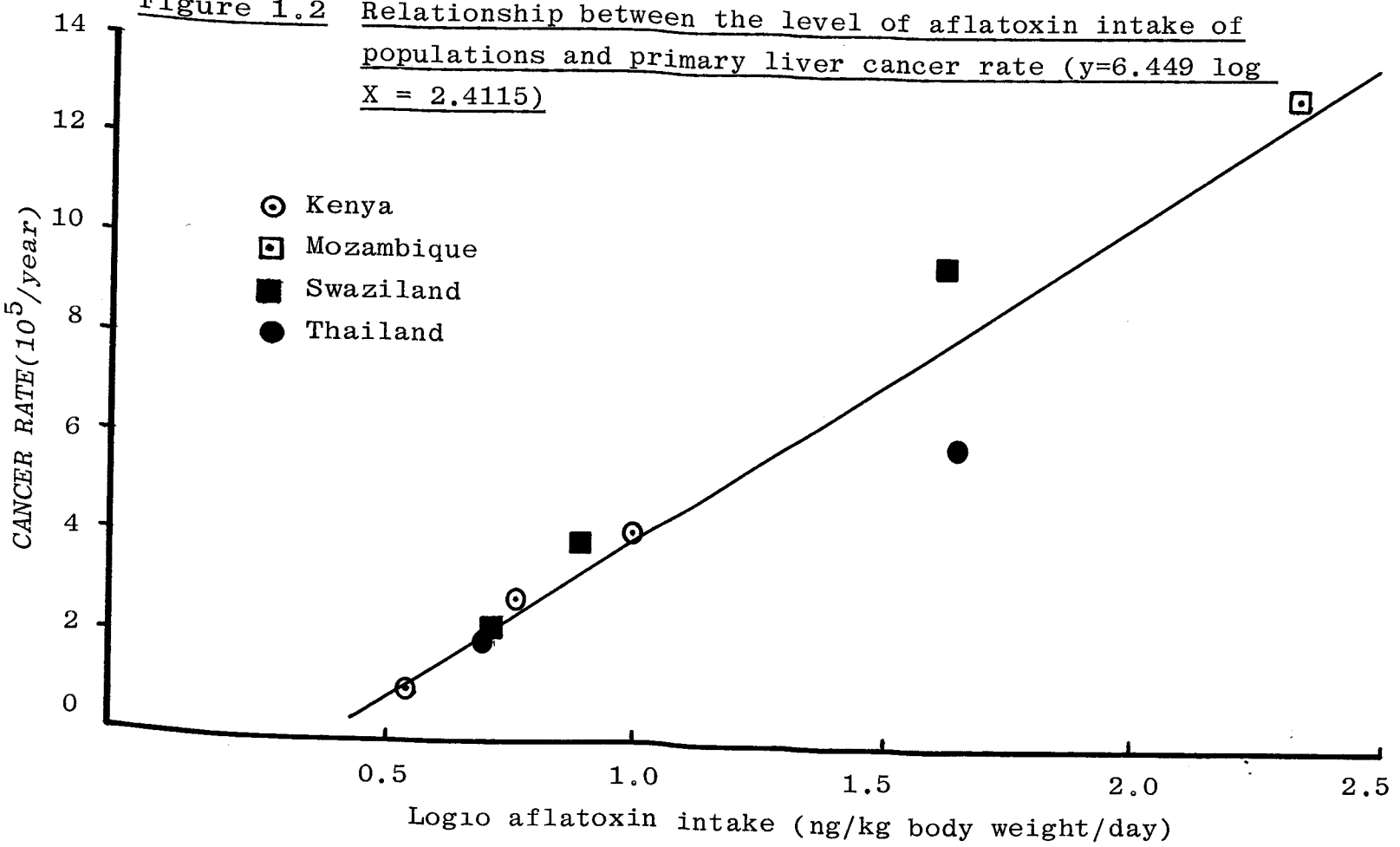
TABLE 1.4

SUMMARIZED RESULTS OF EPIDEMIOLOGICAL STUDIES MEASURING PRIMARY LIVER CANCER INCIDENCE RATE AND AFLATOXIN INTAKE

STUDY	SUB-AREA	AFLATOXIN INTAKE (ng/kg/day)	CANCER RATE (105/year)
Kenya	high altitude	3.5	0.7
Thailand	Songkhla	5.0	2.0
Swaziland	high veld	5.1	2.2
Kenya	mid altitude	5.8	4.0
Swaziland	mid veld	8.9	4.0
Kenya	low altitude	10.0	4.2
Swaziland	Lebombo	15.4	4.3
Thailand	Ratburi	45.0	6.0
Swaziland	low veld	43.1	9.7
Mozambique	Inhambane	222.4	13.0

(Van Rensburg, 1977)

Figure 1.2 Relationship between the level of aflatoxin intake of populations and primary liver cancer rate (y=6.449 log X = 2.4115)



(Van Rensburg, 1977)

The data from these epidemiological studies indicate a highly probable causal relationship although they do not prove a cause-and-effect situation because of the possible involvement of some other factors. Van Rensburg (1977) has concluded that the best approach now would be to concentrate on accumulating exclusive evidence to show that no other plausible factor can be demonstrated to have a similar relationship.

Additional human studies which support the aflatoxin-cancer hypothesis are summarized in Table 1.5. It is interesting to note that not all of the cases cited involve liver cancer. Aflatoxin has also been implicated in cancers of the colon (Deger, 1976) and lung (Dvorackova, 1976, 1981).

6. Metabolism of Aflatoxins

Metabolism plays a prominent role in determining the toxicity and carcinogenicity of AFB_1. It is generally believed that AFB_1 requires metabolic activation to exert its toxic and carcinogenic effects and that there are several competing pathways existing in the hepatocyte, in which ultimate toxicity or carcinogenicity is determined by the partitioning of the toxin among these pathways. The mixed function oxidase (MFO) system, located in the microsomal membrane, is responsible for the formation of a number of less toxic metabolites of AFB_1, including AFM_1, AFQ_1, AFP_1 and AFB_{2a}. The cytoplasmic reductases reduce AFB_1 to aflatoxicol (AFL). These metabolites are generally considered as detoxification products although to varying degrees they are still considerably toxic and carcinogenic (Table 1.6).

TABLE 1.5

HUMAN CASES WHERE AFLATOXIN HAS BEEN IMPLICATED AS THE ETIOLOGICAL FACTOR

YEAR	INVESTIGATORS	COUNTRY	EVIDENCE FOR AFLATOXIN INVOLVEMENT
1973	Van Nieuwenhuize *et al.*	Netherlands	A study of men who had inhaled aflatoxin-contaminated particles while working in a mill. Estimated dose: 160-395 μgAF/M^3/man/week. Of 55, 12 Fatal liver disease cases 11 various cancer cases. Of 55 matched controls, no liver disease cases, 4 various cancer cases.
1976	Babunmi *et al.*	Nigeria	A study of urinary samples of Nigerians showed increased levels of aflatoxin in those with hepatomas and other liver diseases.
1976	Deger	United States	Two men aged 42 and 28 years, developed colonic carcinoma after spending 3 years and 1 year respectively, scraping aflatoxins from chromatographic plates.
1976	Dvorackova	Czechoslavakia	A study of a man who developed alveolar carcinoma after working on aflatoxin contaminated peanut meal. Lung tissue obtained at autopsy contained a compound with the same chromatographic properties of AFB_1.
1976	Phillips *et al*,	United States	A study of a man with carcinoma of the rectum and liver reported liver tissue to contain 520ngAFB_1/g liver.

TABLE 1.5 cont'd

YEAR	INVESTIGATORS	COUNTRY	EVIDENCE FOR AFLATOXIN INVOLVEMENT
1980	Onyemelukwe *et al.*	Nigeria	A study of liver tissues obtained at autopsy from 8 liver disease subjects, 5 of whom had died of liver cancer, and 15 control subjects. AFB_1 was detected in the tissue of 4 of the 5 liver cancer cases (2-15ng/g) and none of the remaining cases.
1981	Dvorackova	Czechoslavakia	A study of pulmonary tissue obtained at autopsy from 2 subjects revealed AFB_1 to be present in both cases. One of the subjects had been exposed daily for 3 months to dust of aflatoxin - contaminated peanut meal.
1981	Stora *et al.*	Czechoslavakia	In a study of liver biopsy tissues obtained from 15 liver cancer cases it was shown that 5 contained AFB_1, at concntrations of 0.36 - 5.2 ng/g.
1982	Bulatao-Jayme *et al.*	Philippines	A study of dietary intakes of 90 primary liver cancer cases and 90 matched controls revealed that the mean aflatoxin load per day of the liver cancer cases was 440% that of controls.

TABLE 1.6

AMES' SALMONELLA MUTAGENIC ASSAY OF AFLATOXINS

COMPOUND	NUMBER OF REVERTANTS PER ug	MUTAGENIC POTENCY RELATIVE TO AFB_1
AFB_1	8527 ± 1434	1.000
AFL	1940 ± 337	0.228
AFG_1	285 ± 50	0.033
AFM_1	275 ± 41	0.032
AFQ_1	99 ± 29	0.012
AFB_2	18 ± 13	0.002
AFP_1	10 ± 5	0.001
AFG_2	9 ± 9	0.001
AFB_{2a}	2 ± 2	0.000

(Wong and Hsieh, 1976)

The above metabolites are hydroxy metabolites which can potentially be further metabolized by Phase II enzymes into glucuronide and sulphate conjugated metabolites rendering them easily excretable. However, Autrup (1982) has pointed out that conjugation of primary metabolites could also be used as a transport mechanism to transfer metabolites to a different cell type, where they could be deconjugated and reactivated into metabolites that bind to cellular macromolecules. Glucuronides of carcinogens have been postulated to be important in kidney, bladder and colon carcinogenesis.

The MFO system is also responsible for converting AFB_1 into a highly reactive chemical species capable of binding to DNA, AFB_1-2,3-epoxide, believed to be the "ultimate carcinogen." The various pathways of AFB_1 metabolism are shown in Figure 1.3.

AFB_1-2,3-epoxide is formed from AFB_1 by AFB_2-2,3-oxygenase. This metabolite, because it is highly reactive, has not been isolated. However, metabolism of many xenobiotic compounds proceeds via the formation of epoxides, which is frequently followed by their hydration by epoxide hydrolase enzymes, yielding corresponding dihydrodiols. Therefore, the formation of a dihydrodiol is evidence for the prior existence of an epoxide. The proposal that the unstable electrophile, AFB_1-2,3-epoxide, is formed metabolically is based on the identification of its dihydrodiol, 2,3-dihydroxy-2,3-dihydroaflatoxin B_1 (AFB_1-dhd), the major product of hydrolysis of AFB_1-DNA and AFB_1-protein adducts formed *in vivo* or *in vitro* (Swenson *et al.*, 1974). By forming adducts with endogenous or added nucleophiles (DNA, RNA or protein) AFB_1-2,3-epoxide can be trapped *in situ* and determination of AFB_1-macromolecule adducts is now routinely used to indicate the existence of AFB_1-2,3-epoxide (Garner *et al.*, 1979).

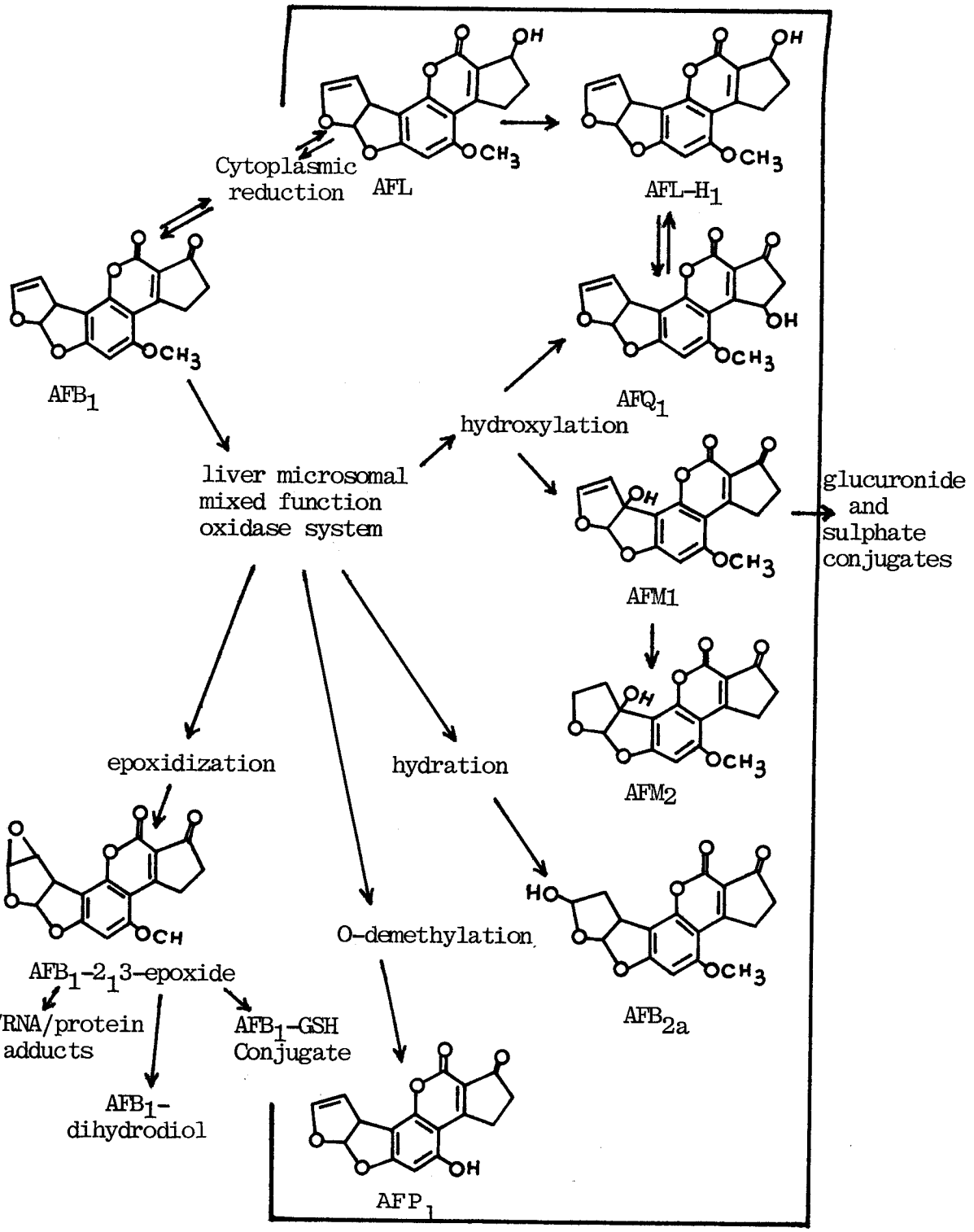


Figure 1.3 Pathways of AFB_1 Metabolism

(Wong and Hsieh 1980; Essigman *et al.*, 1982)

Considerable work has been carried out in an attempt to understand the interaction of this activated form of AFB_1 with DNA. The major aflatoxin-DNA adduct formed *in vivo* in liver or other animal tissues, and *in vitro* in the presence of DNA and a metabolic activation system, is 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy aflatoxin B_1 (AFB_1-N^7-Gua). The second most abundant adduct is 2,3-dihydro-2-(N^5-formyl(-2^1,5^1,6^1-triamino-4^1-oxo-N^5-pyrimidyl-3-hydroxyaflatoxin B_1 (AFB_1 formamidopyrimidine) (Essignmann *et al.*, 1982). The absolute structures of these adducts have been established (Figure 1.4). Further DNA-bound derivatives of AFB_1 and other aflatoxin metabolites are known to form but have not been fully characterized. All available data indicate that it is always the N^7 atom of guanine that is the principal, if not exclusive, target in DNA.

The deactivation of AFB_1-2,3-epoxide involves the formation of the polar metabolite, AFB_1-dhd, as well as AFB_1-glutathione (GSH) conjugate, catalyzed by epoxide hydrolase and GSH-epoxide transferase respectively (Figure 1.4).

The glutathione conjugate of AFB_1 has been shown to be a major metabolite in rat bile (Emerole, 1981). The ability to conjugate AFB_1 with GSH parallels species susceptibility to AFB_1 and the formation of AFB_1-GSH is an important detoxification mechanism in resistant species, such as the mouse (Degen and Neumann, 1981; O'Brien *et al.*, 1983; Raj and Lotlikar, 1984).

O'Brien *et al.* (1983) examined the *in vitro* ability of cytosol prepared from the liver of various species, including the male human, to conjugate epoxide with GSH. Large differences in conjugating ability were observed, ranging from 0 to 72 per cent for quail and mouse respectively. Human cytosol produced AFB_1-dhd but little or no AFB_1-GSH, suggesting that

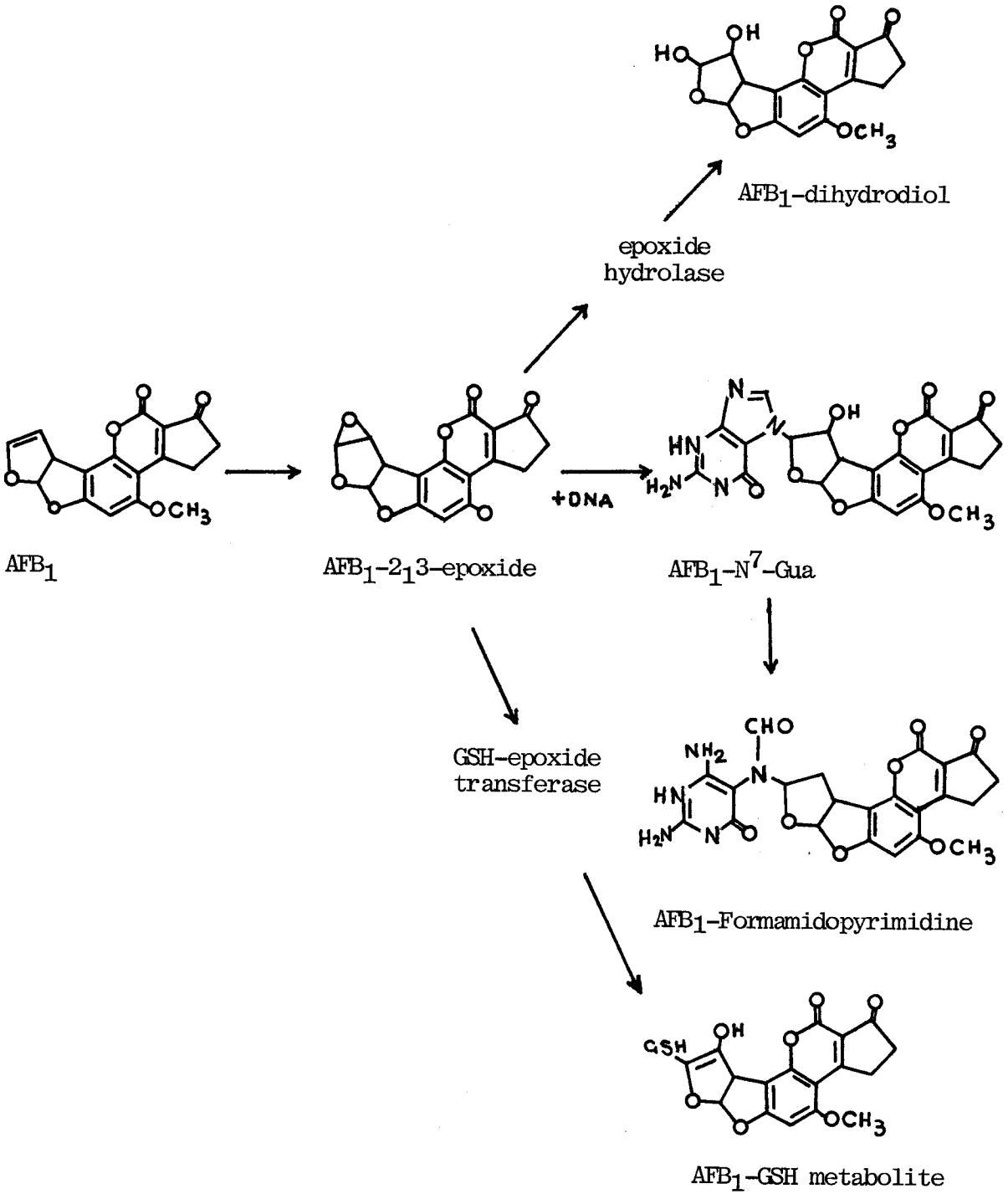


Figure 1.4 Metabolism of Aflatoxin B_1 - 2,3-epoxide

(Emerole, 1981; Essigmann *et al.*1982)

humans may be relatively highly susceptible compared to other species if, in fact, GSH conjugation is an important deactivation mechanism.

Ch'ih *et al.* (1983), on the other hand, using rat hepatocytes, concluded that AFB_1-2,3-epoxide is primarily deactivated by epoxide hydrolase and that the depletion of epoxide by GSH was insignificant. This is in agreement with Emerole *et al.* (1979), who suggested that GSH is a less efficient nucleophile for AFB_1 metabolism than for other chemical carcinogens.

The activities of GSH-transferase and epoxide hydrolase have been shown to be sex-dependent. Female rat liver preparations have higher levels of GSH conjugation activity than male preparations (Degen and Neumann, 1981) and Kamden *et al.* (1982) demonstrated that GSH-transferase activity is inducible by 40 per cent in females administered AFB_1, whereas in males its activity may decrease by 50 per cent. These authors also showed that AFB_1 administration increased the activity of epoxide hydrolase in female rats by more than five-fold compared to only a two-fold increase in males.

The extent of formation of AFB_1-epoxide correlates well with species susceptibility to the toxic and carcinogenic effects of aflatoxin (Garner and Wright, 1975; Swenson *et al.*, 1977; Neal *et al.*, 1981). Ueno *et al.* (1980) reported that the level of *in vivo* binding of metabolized AFB_1 to DNA appears to reflect species susceptibility to the carcinogenic action of the toxin, and the level of protein binding the susceptibility to the acutely toxic action of AFB_1. From these findings, Bennett *et al.* (1981) suggested that measurement of the extent of formation of these adducts in the liver of AFB_1-treated animals would allow estimation of the risk presented to these animals as a result of their exposure. These

investigators showed that administration of AFB_1 to rats resulted in urinary excretion of AFB_1-N^7-Gua in a dose-dependent manner. Thus, by measuring this metabolite in urine an estimation of species ability to form and bind AFB_1-epoxide to macromolecules, and ultimately the potential risk of developing toxic and carcinogenic symptoms of aflatoxin poisoning as a result, can be made.

Recently this method has been applied to humans. Autrup *et al.* (1983), working in the Murang'a district of Kenya where food samples are known to be contaminated with aflatoxin (Peers and Linsell, 1973), analyzed human urine samples by high performance liquid chromatography (HPLC) and found six of 81 samples to contain AFB_1-N^7-Gua. These results indicate that the active form of AFB_1 does interact with cellular nucleic acids in humans and lends further support for the hypothesis that aflatoxin plays an important role in the etiology of liver cancer.

The metabolites of AFB_1 given in Figure 1.3 have all been isolated as *in vivo* and/or *in vitro* metabolites in numerous species. Aflatoxin M_1 has received the most attention because it was the first metabolite to be identified and because it is the predominant metabolite found in urine and milk. However, it represents only about 3 per cent of the ingested AFB_1 dose in various species studied (*e.g.* 1-4 per cent of AFB_1 dose in humans, Campbell *et al.*, 1970; 4 per cent of AFB_1 dose in pigs, Luthy *et al.* **1980).**

AFM_1 toxicity data has been reviewed by Campbell and Hayes (1976) who concluded that the acute toxicity is equivalent to that of AFB_1 and the carcinogenicity and mutagenicity are considerably less than that observed for AFB_1. However AFM_1 still possesses the 2,3-epoxidizable double bond and thus could potentially contribute to aflatoxin carcinogenicity (Essigmann *et al.*, 1982).

AFM_1, possessing a hydroxyl group, can be conjugated with glucuronide and sulphate to yield water-soluble metabolites which are likely to be excreted easily. However, it appears that this is not a major route as Dalezios *et al.* (1973) were unable to detect AFM_1 conjugates in monkeys administered AFB_1. Recently, Wong and Hsieh (1980) reported conjugates of AFM_1 to account for only 1.2 and 0.4 per cent of urinary aqueous aflatoxin metabolites in the monkey and rat, respectively.

AFB_{2a} is a second hydroxylated metabolite of AFB_1 although it has rarely been observed in animal studies. Dann *et al.* (1972) found AFB_{2a} in urine of rats dosed with AFB_1 and Hsieh *et al.* (1977) reported the *in vitro* conversion of AFB_1 to AFB_{2a} by various species, including humans. Some investigators (Patterson and Roberts, 1970; Gurtoo and Dahms, 1972) have considered AFB_{2a} to perhaps represent a major metabolite as it is possible that AFB_{2a} is quickly removed by protein adsorption through Schiff's base formation, a reaction favoured by the slightly alkaline pH of most body tissues (Heathcote and Hibbert, 1978). Toxicity studies of AFB_{2a} indicate that it is essentially non toxic (Wogan, 1973) and it is generally regarded as a detoxification product of AFB_1.

AFL, whose formation is catalyzed by a reversible cytoplasmic reductase(s) has been shown to be produced *in vitro* by numerous species (Hsieh *et al.*, 1977) and has been identified as an *in vivo* metabolite in rat plasma (Wong and Hsieh, 1978) and more recently in human plasma (Hendrickse *et al.*, 1982), urine (Hendrickse, 1984), and liver samples (Lamplugh and Hendrickse, 1982). In these human studies AFL was detected mainly in samples taken from children suffering from kwashiorkor or marasmic kwashiorkor.

AFL has been reported to be the most potent mutagenic and carcinogenic metabolite of AFB_1 isolated (Table 1.6). It has been suggested that metabolic conversion of AFB_1 to AFL plays a significant role in the carcinogenicity of AFB_1 and that AFL-forming activity may be used as a parameter to estimate species susceptibility to aflatoxin-induced carcinogenesis (Wong and Hsieh, 1978). In a comparative metabolism study carried out by these authors (1980) AFL was identified as the major *in vivo* metabolite in the plasma of rats, a species highly susceptible to aflatoxin carcinogenesis, but not in the plasma of similarly dosed mice, a species resistant to aflatoxin carcinogenicity.

In addition to the highly toxic nature of AFL *per se*, the reversibility of the enzymatic formation of AFL from AFB_1 has led to the theory that AFL may serve as an intracellular reservoir of AFB_1 and thus possibly enhance the action of AFB_1. Wong *et al.* (1979) found that AFL was rapidly metabolized to AFB_1, both in the rat *in vivo* and in primary hepatocyte culture. In addition, AFL is believed to be lipophilic and this could presumably facilitate intracellular migration of AFB_1 to target sites, *i.e.* the liver. As AFB_1-epoxide is highly reactive and since no protein carrier systems have been identified to suggest cellular transport of the epoxide, the reservoir of AFB_1 created by the reversible formation of AFL may increase the intracellular bioavailability of AFB_1 for nuclear activation (Wong and Hsieh, 1978).

AFP_1, a further hydroxy metabolite of AFB_1, is formed by O-demethylation and was so named because it is a phenol (Dalezios *et al.*, 1971). Dalezios *et al.* (1973) showed that in rhesus monkeys, AFP_1 accounted for about 5 per cent of an orally administered dose of AFB_1 and was present mainly in a conjugated form (3.3 per cent as glucuroride and 1.2 per cent

sulphate conjugate). Merrill and Campbell (1974b) demonstrated that small quantities of AFP_1 could be produced by human liver microsomal preparations taken at autopsy. However, their attempts to detect its excretion as either a free or conjugated metabolite in the urine of humans ingesting 9.6-40.3 µg AFB_1 over 48 hours were unsuccessful (Merrill and Campbell, 1974a). It appears that AFP_1 has never been reported as a metabolite in human urine.

The acute toxicity of AFP_1 has been shown to be much less than that of AFB_1 in animal studies (Buchi *et al.*, 1973) and, using the Ames' *Salmonella* assay, it was found to be essentially non-mutagenic (Table 1.6). In addition, AFP_1 excretion has been shown to parallel species susceptibility to aflatoxin. Species relatively resistant to aflatoxin, such as the mouse, were more active in converting AFB_1 to AFP_1 (Wong and Hsieh, 1980).

The most recently identified hydroxy metabolite of AFB_1 is AFQ_1, reported to be a major *in vitro* metabolite in numerous species (Hsieh *et al.*, 1977; Shank, 1977), including humans (Buchi *et al.*, 1974; Hsieh *et al.*, 1977). However, it does not appear to have been reported as an *in vivo* metabolite. In a study of the excretion patterns of the monkey, rat and mouse, AFQ_1 was not detected in the urine of animals administered AFB_1, either in its free or conjugated form (Wong and Hsieh, 1980). Campbell and Hayes (1976) were unable to detect free or conjugated AFQ_1 in urine of humans known to have ingested 11.7 to 30.1 µg of AFB_1 during a 48 hour collection period.

AFQ_1 has been shown to be approximately 18 times less toxic than AFB_1 using the Chicken Embryo Test (Hsieh *et al.*, 1974) and was essentially non-mutagenic in the Ames' *Salmonella* mutagenic assay (Table 1.6). It

was however, found to be carcinogenic to rainbow trout (Hendricks *et al.*, 1980). AFQ_1 does retain the 2,3-double bond believed to be responsible for the carcinogenic activity of AFB_1 but the introduction of the hydroxyl group should make it susceptible to conjugation with glucuronide or sulphate and render it excretable.

Recently, Rohring and Yourtee (1983) showed AFQ_1 to be metabolized at significant turnover by rat liver post-mitochondrial fractions to yield the glucuronide conjugate as well as three unidentified chloroform-soluble metabolites. Further analyses showed a notable percentage was strongly bound to proteins and/or undergoing metabolic degradation to "metabolic fragments" that were nonultraviolet-absorbing molecules. These findings may help to explain why AFQ_1 has not been observed as an *in vivo* metabolite.

There have been a few reports observing unmetabolized AFB_1 in urine although it appears that the percentage of an AFB_1 dose remaining as AFB_1 is always very small. For example, in monkeys administered AFB_1, 0.05-0.02 per cent of the dose remained as free as AFB_1 in urine (Dalezios *et al.*, 1973). AFB_1 was detected in urine of children suffering from Indian Childhood Cirrhosis (Amal *et al.*, 1970) and recently Hendrickse and co-workers (1982) reported AFB_1 in urine of African children. These reports contrast those of Cambpell *et al.* (1970) in the Philippines and Lovelace *et al.* (1983) in Zambia who found only AFM_1. Campbell and Stoloff (1974), after comparing the results of the Indian and Philippine studies, suggested that different populations may metabolize aflatoxin in different ways.

Water-soluble conjugates of aflatoxins have been shown to account for a significant percentage of total aflatoxin excreted in a number of species. In turkeys Gregory *et al.* (1983) reported that 55 to 91 percent of total aflatoxin residues excreted were conjugated metabolites and, in rats and hamsters, conjugates accounted for 60 percent of total excretion (Raj and Lotlikar, 1984). In a comparative study Wong and Hsieh (1980) showed that levels of urinary water-soluble conjugates correlated with species susceptibility to aflatoxin, the more susceptible rat and monkey producing less water-soluble metabolites than the mouse, a resistant species. It appears that very few attempts have been made to detect water-soluble aflatoxin conjugates in the urine of humans. Merrill and Campbell (1974a) and Campbell and Hayes (1976), who looked for conjugates of AFP_1 and AFQ_1 respectively, both failed to detect any conjugates in urine of humans known to have ingested AFB_1.

In addition to urinary metabolites two further excretory routes have been investigated to a lesser extent.

Firstly, aflatoxin metabolites have been observed in milk. Most of this work has been done with cows in order to assess the possibility of human exposure through this important food. It has been established by several surveys that cow's milk often contains trace amounts of AFM_1 (reviewed by Patterson *et al.*, 1980). Rodricks and Stoloff (1977) calculated that the ratio of the concentration of AFB_1 in feed to that of AFM_1 in milk was approximately 300:1 and Patterson *et al.* (1980) reported that 2.2 per cent of ingested AFB_1 appeared in milk as AFM_1. In a more recent report (Trucksess *et al.*, 1983) aflatoxins B_1 and M_1 and AFL were detected in cow's milk in the ratio of approximately 1:10:100.

Of greater interest are the reports of aflatoxin in human breast milk. A study carried out in India (Robinson, 1967) described the presence of AFB_1 in human milk, although this finding was not confirmed. Recently, in Zambia AFM_1 was detected in human milk at levels ranging from 0.1 to 1.0 ppb (Bayley, personal communication). AFM_1 and AFM_2 were also detected in one-third of milk samples collected from 99 Sudanese women (Coulter *et al.*, 1984).

Biliary excretion of aflatoxin is also an important route to consider. AFB_1 and its metabolites have been observed in faeces samples from various species, including humans. Shank *et al.* (1971) reported AFB_1 in human stool samples from Thai children who had died of EFDV at concentrations as high as 123 ng/g. Hendrickse (1984) has also observed AFB_2 and AFG_1 in faeces of Sudanese children suffering from PEM. The faeces has been found to constitute the major route of excretion in rhesus monkeys (Dalezios *et al.*, 1973) and pigs (Luthy *et al.*, 1980), accounting for 42 and 58 per cent of an AFB_1 dose respectively. The results of this limited number of studies may indicate that faecal excretion of aflatoxins is important in humans.

The rate and pattern of excretion of aflatoxin metabolites have been shown to differ among species and have been related to variation in species susceptibilities. More resistant species excrete aflatoxin at a greater rate: the mouse excreted 54 per cent of an AFB_1 dose within 24 hours, the rat 47 per cent and the monkey only 32 per cent. Renal excretion was prominent in the monkey, while biliary excretion was more active in the rodents. It was also shown that the biological half-life of aflatoxins in the plasma and liver decreased in order of species susceptibility, namely rat, monkey and mouse.

Several additional studies, based on administration of radioactive AFB_1, have looked at the rate of clearance of aflatoxins in various animals. Gregory and Edds (1983) reported that aflatoxin residues were rapidly cleared from turkeys fed AFB_1, with a half-life of 1.4 days for total aflatoxin clearance from the liver. In pigs, Luthy et al. (1980) found the biological half-life of aflatoxin metabolites in the plasma to be approximately six days. An interesting observation was that most of the aflatoxin in the plasma (70 per cent) was bound to proteins. After 10 days the plasma still contained significant radioactive aflatoxin because of the very slow elimination of the high molecular weight aflatoxin metabolites. Nassar et al. (1982), after noting the small percentage of total aflatoxins normally recovered as identifiable metabolites from urine, faeces and milk (i.e. only 1-4 per cent of dose seen as AFM_1 in human urine - Campbell et al., 1970), carried out a study to reveal the form in which aflatoxin metabolites are delayed in treated animals. Using rats, they found that AFB_1, AFG_1, and their metabolites existed in the systemic blood as protein conjugate. More specifically, this was an aflatoxin-albumin conjugate which was irreversible and permanent. Thus, the conjugate could act as a continuous endogeneous source of the toxin to the liver within the rate of plasma albumin catabolism, explaining why a single dose of AFM_1 given to the rat will result in acute liver damage. They concluded that this persistent aflatoxin - albumin conjugate may be considered as a factor for low excretion levels seen in the urine of animals and humans who have ingested aflatoxin.

With respect to tissue distribution the liver has quite consistently been reported as the organ containing the highest concentrations of aflatoxin. Again, like plasma, most of this seems to be in a bound form. Luthy *et al.* (1980), using pigs, found that less than 0.2 per cent of liver aflatoxin was soluble in methylene chloride and less than 7 per cent was water-soluble and dialysable. Using rhesus monkeys Dalezios *et al.* (1973) demonstrated that the liver contained appreciable levels of aflatoxin and retained up to 5.1 per cent of a dose one week after administration and 1 per cent even after five weeks.

Information on tissue distribution and retention time of aflatoxins in humans can only be speculated upon from data obtained from animal studies and the limited number of studies reporting the presence of aflatoxin in human tissue specimens. Some of these studies are cited in Tables 1.2 and 1.5 and show that aflatoxin has been reported numerous times, mainly in the liver, at concentrations as high as 520 ng AFB_1/g (Phillips *et al.*, 1976). Other human tissues that have been found to contain aflatoxin include lung (Shank *et al.*, 1971; Dvorackova, 1976, 1981), kidney and brain (Shank *et al.*, 1971).

7. Biochemical Effects of Aflatoxins

It has clearly been established that AFB_1 forms covalently linked adducts with nucleophilic sites in target cell DNA, RNA and protein *in vivo* and that formation of these adducts accounts largely for the biological effects of aflatoxin (Swenson *et al.*, 1974, 1977; Garner and Wright, 1975). DNA is the major site of AFB_1 binding and the major

adduct formed is AFB_1-N^7-Gua comprising 80 per cent of DNA adducts formed. The second most abundant adduct is AFB_1-formamidopyrimidine, a derivative of AFB_1N^7-Gua formed by the imidazole ring of guanine opening up. An interesting consequence of this is that the bond linking AFB_1-formamidopyrimidine to the deoxyribose of DNA backbone becomes chemically stabilized, a property of potentially great biological significance (Essigmann *et al.*, 1982). Thus this adduct is likely to be very stable in DNA whereas AFB_1-N^7-Gua would have a tendency to depurinate. The findings of Croy and Wogan (1981) support this concept. These authors, by examining patterns of covalent modification of DNA produced in rat liver after AFB_1 dosing, found that AFB_1-formamidopyrimidine was removed very slowly, if at all, during a 72 hour period after a single dose had been administered and that multiple dosing caused accumulation in liver DNA over a 14 day period. At least 10 additional minor adducts are known to form and these include AFP_1-N^7-Gua and AFM_1-N^7-Gua (Essigmann *et al.*, 1982).

DNA in eukaryotic cells is complexed with chromosomal proteins and organized into transcriptionally active and inactive chromatin and because of this the question of whether AFB_1 binding is selective or random has been raised. Recently Yu (1983) demonstrated that AFB_1, after metabolic activation, binds preferentially to the physiologically active regions of rat liver nucleolar chromatin. Furthermore, it was shown that this binding specificity was largely lost after the removal of chromosomal proteins from the nucleoli.

This finding supports the concept that chromosomal proteins have important structural and regulatory roles in eukaryotic gene expression, conferring features that help to distinguish active genes from inactive ones. Thus the work of Groopman *et al.* (1980), showing that histones represented major protein targets for AFB_1 binding in rat liver nuclei may be of significance. Within the histone fraction, histone H1 contained 30 to 50 per cent of the bound AFB_1 and histone H1 is believed to be responsible for stabilizing chromatin structure. In contrast, Prince and Campbell (1982) found that aflatoxin binding to nonhistones was greater than to histones. They did however, report that binding was influenced by the sex of the animal, females binding less AFB_1 than males.

Numerous investigations have been carried out to evaluate the possible relationships between qualitative and quantitative features of adduct formation and differences in species susceptibility to aflatoxin. Lutz *et al.* (1980) demonstrated that, although the type of adducts formed was the same, the level of *in vivo* covalent binding of AFB_1 and AFM_1 to liver DNA differed widely in the rat and mouse. DNA binding per dose, expressed in units of a 'Covalent Binding Index' (CBI), (mole aflatoxin /mole DNA nucleotides)/mmole aflatoxin/kg animal), was 10,400 for rats and only 240 for mice. This difference reflects the varying susceptibility of these two species to the hepatocarcinogenic activity of AFB_1 which is at least 60 times lower in mice than rats (Table 1.1). Similar results were obtained by Ueno *et al.* (1980). Booth *et al.* (1981) also showed that the level of binding correlated with carcinogenic susceptibility using the rat, hamster, and mouse. These authors went on to examine AFB_1 binding to

liver DNA obtained from human biopsy samples of six individuals. The data showed considerable variation, the values being of the order of those found for the mouse and hamster, species relatively resistant to AFB_1 carcinogenesis. The authors suggested further studies be carried out to examine any genetic differences in human populations in their ability to activate and bind AFB_1. It appears from the literature that no attempts have been made to look for the integration of AFB_1 into DNA of the liver of humans suffering from hepatocellular carcinoma as has been done for hepatitis B virus (Section 9.2). This would, of course, be of great value.

It is known that one feature of AFB_1 carcinogenicity is its high degree of specificity with which it induces tumours and toxic lesions in the liver. This led some workers to compare the liver with non-target organs with respect to the formation of AFB_1-DNA adducts. One non target organ is the kidney and Essigmann *et al.* (1982), after dosing rats with radioactive AFB_1 and analyzing their livers by HPLC, found levels of adducts in kidney DNA to be 10 per cent of those in liver.

The formation of covalent adducts with DNA and chromatin proteins induces direct chromosomal damage. However, chromosomal damage can also be brought about indirectly through the formation of reactive secondary agents upon interaction with non-DNA targets. These secondary agents which are mostly active oxygen species, lipid-hydroxperoxides and their degradation products, then bring about chromosomal damage. One mechanism by which these species can do this is through their clastogenic activity on chromosomal membranes which can lead to numerous chromosome aberrations such as chromatin fragmentation. Xenobiotics which act both by direct and indirect mechanisms are believed by some investigators to represent potent complete carcinogens. Recently,

AFB_1 was shown to have a membrane-active character, acting as a potent clastogen at very low levels of covalent AFB_1-DNA adducts, indicating AFB_1 may be a complete carcinogen (Amstad *et al.*, 1984).

AFB_1 has also been shown to produce dramatic inhibition of liver nuclear RNA polymerase II activity in rats. This enzyme is responsible for cellular mRNA synthesis and its malfunction may thus affect genetic information transfer and be related to AFB_1 carcinogenesis. Yu *et al.* (1982) attempted to relate RNA polymerase II inhibition to AFB_1 carcinogenesis by examining tissue, sex and species specificities known to be characteristic of AFB_1 carcinogenesis. Their results showed that AFB_1 inhibited RNA polymerase II activity only in the target tissue, liver, and not in non-target tissues, *e.g.* lung and brain, it inhibited activity preferentially in male over female rats, and had no effect on mouse liver RNA polymerase II activity.

It is believed by some investigators that while the hepatocarcinogenic effects of AFB_1 are mostly related to DNA binding, susceptibility to the hepatotoxicity of AFB_1 is related to the level of protein binding (Ueno *et al.*, 1980). In guinea pigs, a species relatively susceptible to the acute toxicity of AFB_1 but quite resistant to the hepatocarcinogenic effects of AFB_1 when compared to the rat, AFB_1 was shown to accumulate more in liver protein and RNA and less in DNA.

The key intermediate metabolite in the formation of AFB_1-protein adducts is believed to be AFB_1-dhd, formed by hydrolysis of AFB_1-2,3-epoxide (Swenson *et al.*, 1977, Ueno *et al.*, 1980). Neal *et al.* (1981) showed that protein synthesis was extremely sensitive to AFB_1-dhd and that the relative production of AFB_1-dhd by microsomes from various species paralleled their susceptibility to acute AFB_1 poisoning. AFB_1-dhd has been observed to react covalently with proteins at physiological pH,

presumably by the spontaneous formation of aldehyde derivatives which can then react with protein amino groups by Schiff base formation (Swenson *et al.*, (1975).

In addition to the formation of aflatoxin-protein adducts, AFB_1 has been shown to inhibit protein synthesis at numerous stages by affecting various cell organelles, including the disaggregation of polysomes and the degranulation of the endoplasmic reticulum (cited in Groopman *et al.*, 1980). Furthermore, aflatoxin has been reported to affect additional organelles. For instance, AFB_1 decreases lysosome integrity and this may be related to its carcinogenicity. Lastly, the mitochondria are considered to be one of the major intracellular targets for AFB_1 (Niranjan *et al.*, 1982) and AFB_1 has been shown to inhibit oxidative phosphorylation.

8. Primary Hepatocellular Carcinoma

Primary hepatocellular carcinoma (PHC) has a characteristic geographical distribution in sub-Saharan Africa and the Far East and has for many years been a major problem in the countries of Southern and Eastern Africa. In Zambia, PHC is the commonest tumour of men (Zambia Tumour Registry Records) and the risk is estimated to be at least ten cases per 100,000 men over the age of 30 years (Bayley, 1979). The mean age at onset is 42 years, with a range from 18 to over 70 years. PHC affects men more than women, the sex ratio being 8:1 in Zambia. Thus, the majority of patients are men in the prime of life with heavy family and social responsibilities (Bayley, 1979).

The tumour originates in the parenchymal cells of the liver and grows very rapidly: 58 out of 110 patients seen in Lusaka during the years 1971-1978 were dead within 60 days of diagnosis (Bayley, 1979). The typical clinical picture in Zambia can be described as follows: A male patient in his early forties, previously well, has developed right sided abdominal pain and has evident weight loss. An easily visible, hard and irregular lump can be felt in the upper abdomen. Surgery is rarely possible because the tumour usually involves the whole liver. Cytotoxic drugs help some patients, but only for a few weeks or months. Survival beyond four months is exceptional (Bayley, 1979).

Several features of liver cell carcinoma vary between low and high incidence areas and these are summarized in Table 1.7. In addition to the marked geographical distribution of PHC there are distinct ethnic and cultural variations. The high incidence in Southern Africa affects the negriform race chiefly, and not Whites or Asians living alongside them (Oettle, 1965). Within Negro groups the risk is not uniform. For instance, the Shangaan tribal people living amongst Swazis in Swaziland have a much higher incidence of liver cancer (Keen and Martin, 1971). In Uganda, a greater frequency of PHC is seen in poor immigrant tribes from Rwanda and Burundi than in the local Baganda tribe (Alpert *et al.*, 1969). Extraordinary fluctuations in incidence have also been noted between relatively adjacent areas where tribal differences are not significant. For example, the people of the low veld region of Swaziland stand a much greater risk of developing liver cancer than those living in the high veld (Table 1.4).

TABLE 1.7

COMPARISON OF THE FEATURES OF HEPATOCELLULAR CARCINOMA IN LOW AND HIGH INCIDENCE AREAS

	U.S.A, WESTERN EUROPE	SUB-SAHARA AFRICA, S.E. ASIA
Incidence	low - 2 to 3% of all cancers[1]	high - as great as 20 to 40% of all cancers[1]
Age and sex	middle to old, 60+ years linear increase with age male predominance	young, 30-40 years do not have a linear increase with age[2] male predominance
Previous state of health	often develops as a complication of cirrhosis of long duration - alcohol is a common feature	Perfectly well prior to onset
Incidence of cirrhosis	Common	Common
Morphology	Micronodular	Macronodular
Relative proportions of hepatocellular carcinoma and cirrhosis	Cirrhosis outnumbers carcinoma	Carcinoma outnumbers cirrhosis

1 Szmuness, 1978
2 Figure 1.6

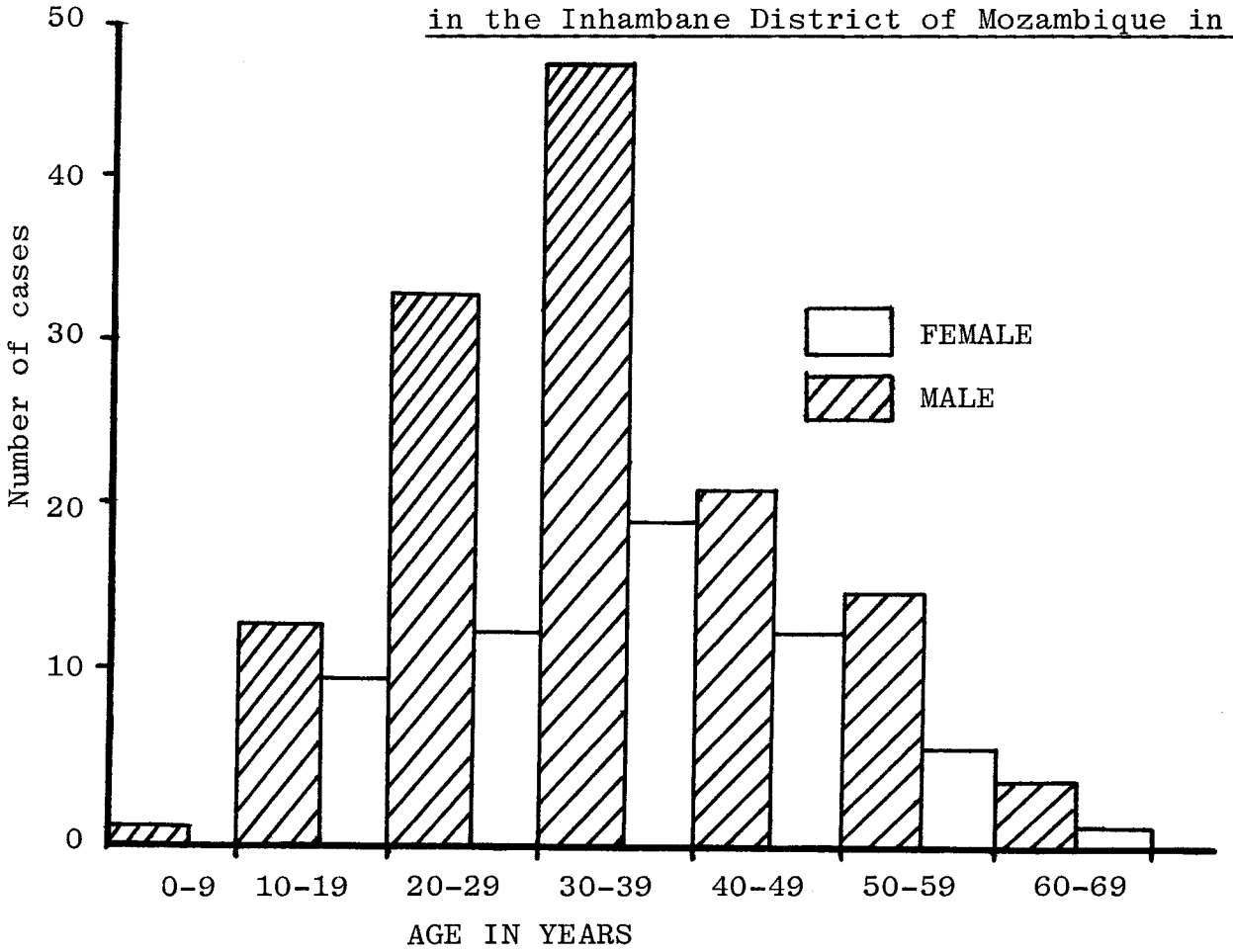
The clinical picture of PHC varies considerably between low and high incidence areas (Table 1.7). The course of the disease described for Zambians is typical of all Africans, where the presentation is acute and deterioration is rapid. In the West, liver cancer tends to develop at the end of a long illness and may be discovered unexpectedly at *post mortem* as a complication of underlying chronic disease (Oettle, 1965).

Excessive alcohol consumption is a common feature among liver cancer patients in Western countries. In Africa, although alcoholism is prevalent, studies have shown that it does not seem to be a major factor in liver cancer. A study in Uganda (Alpert *et al.*, 1969) showed that the rate and type of alcohol ingestion was similar in controls and cases.

Cirrhosis is a common feature of all liver cancer cases although Anthony (1977) has made an interesting observation in that, whereas in Western countries liver cancer often develops as a complication of cirrhosis of long duration (Kew *et al.*, 1971), in tropical countries the two diseases arise almost simultaneously: most patients present and die with their tumour without any preceding symptom or sign of cirrhosis (Alpert *et al.*, 1969).

A further feature of PHC which varies between low and high incidence areas is the age at which the disease occurs. Patients in the United States and Western Europe tend to be 60 years of age or older and a linear increase with age is observed. In Africa and Asia liver cancer patients are more often in their thirties or forties and a linear increase with age is not observed, but rather an increase up to 40 years of age followed by a decline in incidence with age (Figure 1.5).

Figure 1.5 Number of cases of Liver Cancer According to Age in the Inhambane District of Mozambique in 1968



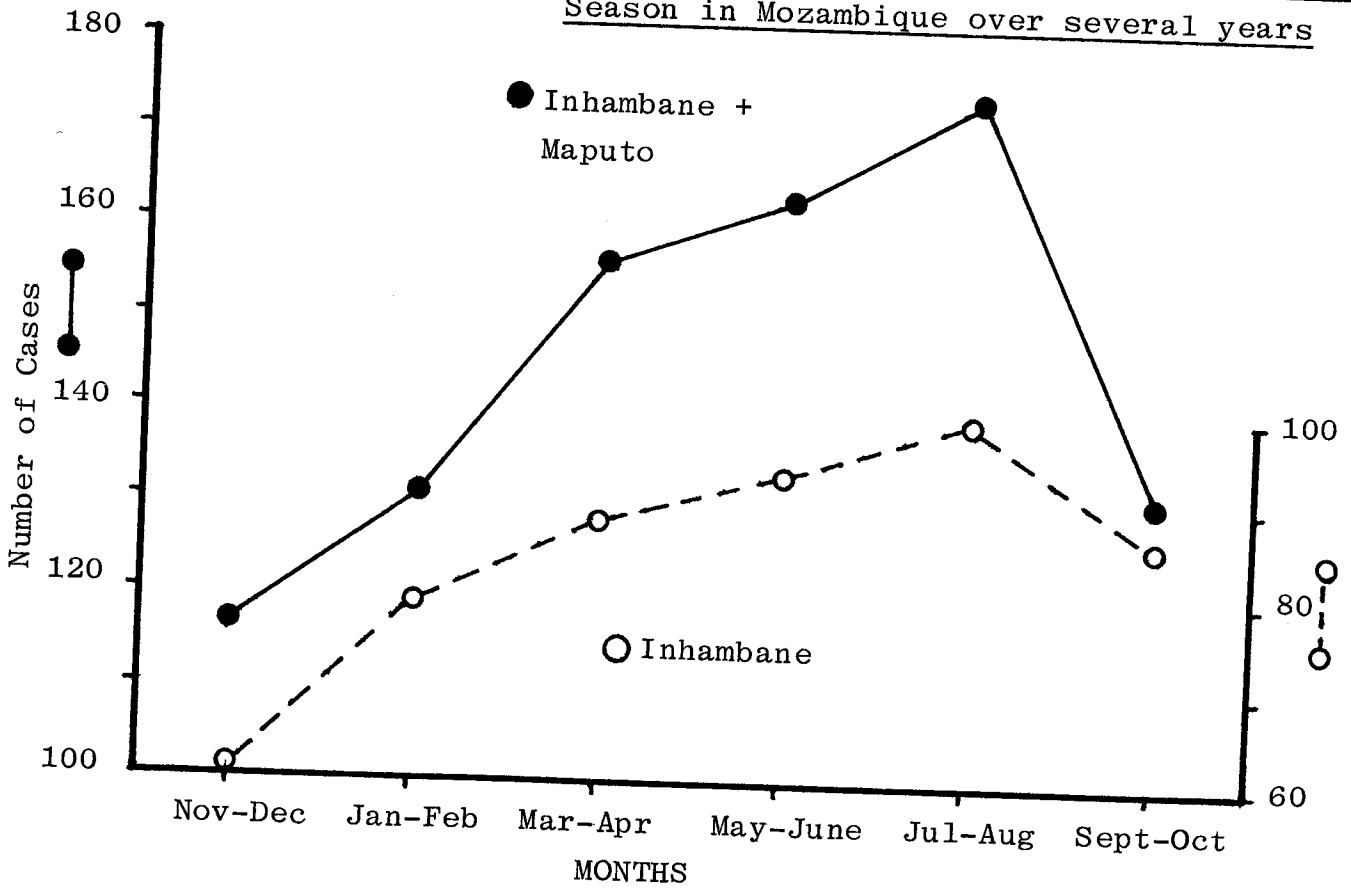
(Purchase and Goncalves, 1971)

This could possibly be due to increased exposure to the carcinogen during adulthood or differences in age susceptibility to a standard dose. Alternatively, the disease may be precipitated at an earlier age in individuals having a particular predisposition when exposed to higher levels of the carcinogen (Van Rensburg, 1977). Furthermore, this observation may be explained, in part, by failure to register older age-groups adequately (Oettle, 1965) and also by differences in life expectancy figures between the countries of Africa and North America and Europe, making the percentage of the population which is elderly vary a great deal.

An interesting observation is the seasonal fluctuation in occurrence seen in Africa, notably Mozambique (Purves, 1973; Van Resburg, 1977) and Nigeria (Okonkwo and Obionu, 1981). The Mozambique data show a remarkable seasonal variation (Figure 1.6), suggesting the carcinogenic stimulus varies seasonly in its intensity and implying a very short latent period for the predisposing factors (Oettle, 1965; Van Rensburg, 1977).

A further point of interest is that there is a lowering of the risk in high incidence groups who move to a low incidence area. Japanese immigrants to Hawaii do not carry with them the high risk of liver cancer which the Japanese population in the homeland display (Quisenberry, 1960). This observation, together with the marked geographical and cultural distribution of PHC, suggest strongly an environmental etiological factor such as aflatoxin, although the role of hepatitis B virus is viewed with equal or greater importance.

Figure 1.6 Number of Cases of Liver Cancer according to Season in Mozambique over several years



(Van Rensburg, 1977)

9. Proposed Etiological Factors of Primary Hepatocellular Carcinoma

Factors which have been implicated in the etiology of PHC include aflatoxins, hepatitis B virus, genetic factors, alcohol, malnutrition, cirrhosis, parasites, iron overload and environmental chemical carcinogens. Evidence from a number of epidemiological and laboratory studies has suggested that there is an interplay of two or more of these factors and thus evidence for one factor does not necessarily discredit another.

9.1. Aflatoxins

The possible role of aflatoxins in the etiology of PHC has been discussed in Sections 5 to 7. There is some evidence that aflatoxins may act in combination with hepatitis B virus in the pathogenesis of PHC and this is discussed in Section 9.2.

9.2. Hepatitis B Virus (HBV)

The hypothesis that a hepatitis virus may play a role in the etiology of PHC was put forward in the mid 1950's (Paget *et al.*, 1956; Steiner and Davis, 1957), five years before aflatoxins had even been discovered. The evidence for the implication of HBV in the pathogenesis of liver cancer is based on epidemiological and geographical observations of a strong correlation between HBV infection and PHC; the observed significant increase in the prevalence of HBV markers in serum of PHC patients compared to the general population; observations that indicate HBV infection precedes the development of the tumour; the presence of hepatitis B antigens in malignant tissue; evidence of covalent

integration of the HBV genome into the DNA of tumour cells; the observations that several cell lines derived from PHC cases secrete hepatitis B virus surface antigen; and finally, the finding of chronic liver damage and PHC in animal species infected with a virus phylogenetically related to human HBV.

The first three lines of evidence came after the development of specific serologic assays, most importantly radioimmunoassay (RIA) for markers of HBV infection. Various markers appear in the plasma of infected individuals at different stages. These are summarized by Zuckerman (1982). The first marker is hepatitis B virus surface antigen (HBsAg) which appears in the plasma two to eight weeks before biochemical evidence of liver dysfunction or the onset of jaundice. This antigen persists during the acute illness and usually disappears from circulation during convalesence. Next to appear is a specific hepatitis B DNA polymerase associated with the core of the virus and at approximately the same time another antigen, the *e* antigen, becomes detectable. Antibody to the core antigen (anti-HBc) is present two to four weeks after the appearance of HBsAg and is always detectable during the early acute phase of the illness. Core antibody of the IgM class is not detectable within a few weeks of uncomplicated acute infection, but core antibody of the IgG class persists after recovery for many years, possibly for life. Next to appear in the circulation is antibody to *e* antigen. Antibody to the surface antigen component (anti-HBs) is the last marker and appears late in convalesence.

The presence of HBsAg in the blood for more than six months constitutes the HBV carrier state (WHO Report, 1977) and it has been estimated conservatively to number about 200 million people. Surveys conducted in many areas of the world have shown the prevalence of HBsAg in healthy persons to vary from 0.1 per cent or less in North America and Australia, to 20 per cent or more in some tropical countries of Africa and South East Asia (cited in Zuckerman 1982). In the latter areas over half of the population carry anti-HBs, indicating a past infection. Moreover, many studies have shown that the frequency of serological markers of HBV infection parallels the incidence of PHC in various parts of the world. It has also been clearly demonstrated that the presence of HBsAg in the serum of PHC cases is significantly much greater than in control cases (Table 1.8).

In Zambia, patients with liver cancer have been investigated for evidence of past or present HBV infection. Chronic infections were found in 68 per cent of the cases compared to 8 per cent of controls (Tabor *et al.*, 1977). In a more recent study by Zumla and Voller (1982), using the enzyme linked immunosorbent assay (ELISA), HBsAg was found in 60 per cent of Zambian patients with PHC compared to 16 per cent of matched tumour controls and 14 per cent of healthy controls, again revealing a highly significant difference ($p < 0.01$).

A case - controlled study of PHC cases and their families in Senegal (Larouze *et al.*, 1976) showed that 97 per cent of PHC patients and 93 per cent of controls had evidence of HBV infection but that the patients were less likely to have surface antibody. Seventy-one per cent of mothers of PHC cases were chronic carriers of HBV compared with only 14 per cent of mothers of controls. The chronic infection rates of the fathers of patients and controls were identical.

TABLE 1.8

FREQUENCY OF HEPATITIS B SURFACE ANTIGEN (HBsAg) AND ANTIBODY TO HEPATITIS B CORE (ANTI-HBc) IN BLOOD OF PATIENTS WITH PRIMARY HEPATOCELLULAR CARCINOMA AND IN CONTROLS IN SELECTED COUNTRIES

(based only on studies using RIA or a test of equivalent sensitivity and in which controls were included)

Country	Serums of patients with PHC		Serums of Controls	
	Number tested	Percent positive	Number tested	Percent positive
	Hepatitis B surface antigen			
Greece	189	55.0	106	4.7
Japan	260	37.3	4,387	2.6
Mozambique	29	62.1	35	14.3
Senegal	291	51.9	100	12.0
Singapore	156	35.3	1,516	4.1
South Africa	138	59.5	200	9.0
Spain	31	19.3	101	2.0
Taiwan	84	54.8	278	12.2
Uganda	47	47.0	50	6.0
United States	34	14.7	56	0
Vietnam	61	80 .3	94	24.5
Zambia	19	63.1	40	7.5
	Antibody to hepatitis B core antigen			
Greece	80	70.0	160	31.9
Hong Kong	37	70.3	58	36.2
Senegal	291	87.3	100	26.0
South Africa	76	86.0	103	31.7
Spain	31	87.0	101	14.8
United States	33	48.5	56	0

(Blumberg, 1980)

However, none of the fathers of PHC patients had anti-HBs, while 48 per cent of fathers of controls were anti-HBs positive. These results suggest that chronic HBV infections which are important for the development of PHC are passed from mother to child early in life and also reveal a paternal effect, the failure of fathers of PHC patients to produce anti-HBs even though heavily exposed to HBV. This suggests a child may be at greater risk of developing PHC by inheriting from his father a relative incapacity to produce protective antibody. Alternatively, it may indicate that an environmental factor(s) in the household is responsible for suppressing the antibody response to HBsAg. The authors proposed that aflatoxin could be this factor.

The hypothesis that the role of aflatoxin in liver cancer may be immunological rather than directly carcinogenic was proposed by Lutwick (1979), based on the calculation by Prince (1978) that the risks of hepatoma development in HBV carriers in Mozambique and the United States were similar. Aflatoxin, by suppressing the cellular immune response, may increase the HBV carrier rate and hence the risk of PHC. Lutwick further postulated that supressed cell-mediated immunity may also, or alternatively, act by failing to limit beginning malignancies, permitting cells to grow into clinically overt hepatomas. Numerous papers have demonstrated that various aspects of the immune response are suppressed by aflatoxin (reviewed by Lutwick, 1979).

A second hypothesis proposed to link HBV infections and aflatoxin in the genesis of PHC is that persons with chronic HBV infection use different hepatic pathways to metabolize aflatoxin compared with people not infected with HBV. This is based on known

variations in susceptibility to aflatoxin shown in animals (Table 1.1) which appear to depend on differences in their metabolic handling of the toxin.

Studies demonstrating a correlation between HBV infection and the incidence of PHC do not demonstrate a cause and effect relationship unless there is evidence that the carrier state of HBsAg preceded the development of liver cancer. An indication of this was provided by Beasley *et al.* (1981). Between 1975-1978, 22,707 male Chinese in Taiwan were enrolled in a cohort study to determine prospectively the incidence and the relative risk of PHC among HBsAg carriers and to determine whether the HBsAg - carrier state is antecedent to the development of PHC. On recruitment 15.2 per cent of the men were discovered to be carriers of surface antigen. At the end of 1980 a pronounced excess of deaths from PHC and cirrhosis was observed in those who were HBsAg-positive. Forty out of 41 men who died from PHC and 17 of 19 men who died from cirrhosis were HBsAg-positive. PHC and cirrhosis together accounted for 57 of 105 deaths among HBsAg-positive subjects (54.3 per cent) compared with 3 of 202 deaths among subjects negative for HBsAg (1.5 per cent). The relative risk of developing PHC between the two groups was 223.

Although a correlation between HBV and PHC has been reported in many studies there are also a number of conflicting reports. A study carried out in Kenya (Bagshawe *et al.*, 1975) revealed no significant difference in the prevalence of HBsAg between two distinct areas known to have differing liver cancer rates and where previous work (Peers and Linsell, 1973) had shown a significant correlation of PHC incidence and dietary aflatoxins.

Skinhoj *et al.* (1978) examined the occurrence of cirrhosis and liver cancer in the Eskimo population of Greenland where hepatitis B is hyperendemic. Neither cirrhosis nor PHC were found to be a more prevalent cause of death in this population than in Northern Europe where hepatitis B is at least ten-fold less prevalent, suggesting hepatitis B infection *per se* does not contribute significantly to the development of liver cancer at least in the Eskimo population of Greenland.

After examining available epidemiological data on the HBV-PHC hypothesis Van Rensburg (1977) concluded that populations with both high PHC and hepatitis-B antigenemia rates show no correlation between the extent of elevation of these two parameters, unlike populations with both high liver cancer rates and aflatoxin intakes (Table 1.4 and Figure 1.2). Furthermore he pointed out that PHC is virtually unknown in Whites living amongst Blacks in the high liver cancer area of Mozambique and believes this striking difference to be more likely due to the completely different dietary pattern than exposure to a virus.

The next three lines of evidence for the implication of HBV in the pathogenesis of liver cancer come from the field of molecular biology. The most striking advance has been the finding of integrated HBV-DNA in patients with chronic hepatitis and hepatocellular carcinoma. Lutwick and Robinson (1977) reported the presence of hepatitis B virus DNA in primary hepatocellular carcinoma tissue. Then with the widespread use of recombinant DNA technologies, the integration of viral DNA sequences into the host genome was demonstrated. Shafitz and Kew (1981), by molecular hybridization using ^{32}P - labelled recombinant,

cloned and purified HBV-DNA, found HBV-DNA sequences covalently integrated into the host genome in carcinomateous tissue of PHC patients known to be carriers of HBsAg. HBV-DNA was not found in DNA extracts from tumours of patients who were not carriers of the surface antigen. Integrated HBV-DNA has also been found in non-tumour tissue of PHC patients (Shafritz *et al.*, 1981), suggesting integration precedes the development of neoplasia (Zucterman, 1982). Many recent studies have detailed the integration patterns of HBV-DNA into host cell DNA and have been well reviewed by Zuckerman, 1982; WHO, 1983; Mason *et al.*, 1984.

One further line of evidence for the HBV hypothesis comes from experiments using animals. Several reports of experiments with mice (*e.g.* Desmyter *et al.*, 1978; Bassendine *et al.*, 1980) have demonstrated the transplantability of a HBsAg-producing cell line from a human hepatocellular carcinoma and the subsequent development of liver tumours. In contrast, chimpanzees infected with HBV have been shown to develop chronic hepatitis but none have developed liver cancer (cited in WHO Technical Report, 1983). A study with marmosets again demonstrated that viral hepatitis did not induce hepatic tumours, although when given in combination with aflatoxin B_1, it was shown to enhance the development of liver disease (Lin *et al.*, 1974).

The detection of a high incidence of liver cancer occurring "naturally" in certain animal species led researchers to the discovery of hepatitis B-like animal viruses. These viruses share similar morphological and biological properties and genomic organization with the human hepatitis B virus and have been identified in eastern woodchucks, prairie-dogs, ground-squirrels, and certain ducks (reviewed by Summers and Mason, 1982).

Hepatitis B infection has now been shown to be preventable by a hepatitis B vaccine (Szmuness *et al.*, 1980). Presently trials of this vaccine are being carried out in Katete in the Eastern Province of Zambia (Bayley, personal communication). In the near future it should be possible, by immunization early in life, to prevent the establishment of the HBV carrier state in high prevalence regions of the world. If this vaccine is accompanied by a marked fall in the incidence of PHC this would provide the final link in the chain of evidence implicating HBV as an etiologic agent in PHC.

9.3. Genetic Factors

Reports of a familial occurrence of liver cell carcinoma have been documented. One study in Chile (Velasco *et al.*, 1971) reported a family of eight in which one member, a male aged 21 years, had liver cancer and two of his brothers had died of liver cancer at ages of 14 and 17 years. It was shown that four of the family members were carriers of hepatitis antigen, the propositus, a brother, the mother and a maternal aunt.

Another study carried out in Japan (Ohbayashi *et al.*, 1972), described three family cases. Of 54 members, 15 had chronic liver disease or liver cancer. Again, a high proportion were positive for hepatitis antigen, especially the siblings of affected members.

Familial clustering of hepatitis B antigenemia without any liver disease has also been reported and therefore there must be additional factors, genetic or otherwise, present for carcinoma to develop (Anthony, 1977).

9.4. Cirrhosis

Cirrhosis, a degenerative condition in which the liver becomes hard and fibrotic, results from long-continued loss of liver cells accompanied by compensatory liver cell hyperplasia and nodule formation and progressive replacement by fibrous tissue. Cirrhosis due to any cause predisposes to the condition of PHC. In Britain, 10-15 per cent of patients with cirrhosis from various causes, including chronic hepatitis and alcohol, progress to liver cancer (MacSween and Scott, 1973). In patients with PHC, underlying cirrhosis is found in more than 70 per cent (Lefkowitch, 1981) and this figure may in fact be as high as 90 per cent (Mason *et al.*, 1984). In Africa, the precise frequency of an underlying cirrhosis in hepatoma cases is difficult to assess because of lack of *post mortem* data and the fact that livers of such patients are often largely replaced by the tumour (Bagshawe *et al.*, 1971).

These observations have led to the postulation that some etiologic agents implicated in PHC act indirectly by producing cirrhosis. For instance, it has been suggested that persistent infection with HBV leads to cirrhosis and that carcinoma then arises from regenerative nodules by mechanisms in which the virus is not involved. However, Zuckerman (1977) has pointed out that liver cancer associated with persistent hepatitis B infection is seen in about 25 per cent of patients in the absence of fibrosis. It would appear more likely, from the evidence given, that both HBV and aflatoxin are intrinsically carcinogenic.

9.5. Alcohol

In Western countries alcoholic cirrhosis is the most common antecedent of liver cell carcinoma (Anthony, 1977). However, it is generally believed that the development of cancer in this case is more likely the result of the increased rate of cell turnover inherent in cirrhosis and that alcohol by itself, without cirrhosis, does not increase the risk of liver cancer.

In Africa, alcohol consumption, although prevalent, is not believed to be a major factor in liver cancer. A study in Uganda (Alpert *et al.*, 1969) showed the rate and type of alcohol ingestion to be similar in liver cancer patients and controls and this was similarly reported in Mozambique (Prates and Torres, 1965) and South Africa (Higginson and Oettle, 1960). In addition, Oettle (1965) has pointed out that fatty change of the liver, a characteristic feature of alcoholic liver disease, is strikingly absent in the Bantu population of South Africa who are known to have a high incidence of PHC.

9.6. Malnutrition

The view that malnutrition is a factor in liver cancer is based on the high incidence of both conditions in the same populations and on experimental data showing the importance of protein and other nutrient deficiencies in the development of hepatomas in rats (Oettle, 1965). It is now believed that the association of liver cancer with malnutrition is not of causal significance but is due to the fact that both are promoted by many similar environmental and socioeconomic circumstances. Malnutrition may play a contributory role in liver cancer by damaging the liver and thus aggravating the effects of aflatoxin and other hepatotoxic agents.

9.7. Parasites

The incidence of bile-duct carcinoma in the East is believed to be related to infestation with *Clonorchis sinensis* and other liver flukes (Gibson, 1971). This led to suspicion that bilharziasis might be responsible for liver cancer in Africa. This was hypothesized before aflatoxins and hepatitis virus had been discovered and this theory, although not disproven, has now been largely discarded.

9.8. Iron Overload

Haemochromatosis has been considered by some to be a predisposing factor in liver cancer. Much of this evidence comes from studies of the Bantu population of South Africa who have a high incidence of PHC and who, by brewing in iron pots, have a very high iron intake. However, it has been observed that liver cancer only arises in people with haemochromatosis after development of cirrhosis. Thus, cancer is likely the outcome of cirrhosis, not the condition of iron overload.

9.9. Environmental Chemical Carcinogens

This term encompasses a vast array of chemicals, both naturally occurring and synthetic. Naturally occurring substances, other than aflatoxins, which are thought to be liver carcinogens include the additional mycotoxins, cyclochlorotine, luteoskyrin and sterigmatocystin, and a number of plant substances, namely pyrollizidine alkaloids found in herbal teas and medicines, cycasin from the cycad tree, safrole, and tannic acid, found in coffee and tea.

Synthetic chemicals implicated as liver carcinogens include chlorinated hydrocarbons, such as organochlorine and polychlorinated biphenyl pesticides, the solvents carbon tetrachloride and chloroform, and nitrosamines, possibly formed in the gastrointestinal tract from the common food additive, nitrite.

10. Aflatoxin Consumption in Zambia

Maize is the most important staple food in Zambia, supplemented by a "relish" usually of green leaves, sometimes with onion or tomato, cooked in oil. Groundnuts are commonly available and are eaten as a snack or cooked into a paste. Maize and groundnuts are the two main foods commonly reported to be contaminated with aflatoxins and the importance of them in the Zambian diet, combined with its tropical climate and the fact that the rural population grow and store their own food, make Zambia a country of high potential risk to aflatoxin exposure.

Aflatoxin consumption has been demonstrated in Zambia. Analysis of 200 plate food samples, collected from five villages in Eastern province during the late dry seasons of 1975 and 1977, revealed six contaminated samples with aflatoxin levels ranging from 0.6-43.9 μg/kg (Lovelace and Salter, 1979). It is interesting that four of the six contaminated samples were green vegetables (cooked and dried).

Other studies, in Kenya (Peers and Linsell, 1973) and Nigeria (Okonkwo and Obionu, 1981), have shown seasonal variations in aflatoxin levels of foods: higher levels are generally found in food samples collected in the rainy season. This has been attributed to two factors.

Firstly, a warm, humid environment favours proliferation of *A. flavus* and secondly, the rainy season is generally the planting period and foods sold in the markets at that time typically come from the previous harvest and have therefore been stored for a prolonged period (Okonkwo and Obionu, 1981).

More recently, in Zambia, food samples were collected during the rainy season (Njapau, personal communication). Collection began in February 1983 and to date 211 samples have been analyzed from or near St. Francis' Hospital, Katete and 35 samples from Eastern Co-Operative Union, Chipata, both in the Eastern Province. The results are given in Table 1.9(a) and (b). Average levels of contamination ranged from 10-15 µg/kg, except for samples of germinated maize which were more heavily contaminated with levels up to 150 µg/kg. This level is very high. Moreover, the rate of contamination was 73 per cent. This is potentially serious as germinated maize, *chimela*, is used to prepare sweet beer, *thobwa*, which is consumed by children as well as adults.

11. The Purpose of the Present Study

Contamination of foods with aflatoxin has been shown to be a problem in various African countries. In Zambia aflatoxin has been identified in plate foods (Lovelace and Salter, 1979) and further confirmation of aflatoxin consumption was obtained from a study showing the presence of aflatoxin in human urine (Lovelace *et al.*, 1983). In this study urine samples were obtained from patients with and without liver disease who had been admitted to the hospital in Lusaka, in an attempt to examine the possible effect of liver pathology on the type and amount of aflatoxin metabolite(s) excreted. However, no significant differences between liver disease and control patients were demonstrated.

Table 1.9(a)

FOOD SAMPLES OBTAINED AT OR NEAR ST. FRANCIS' HOSPITAL, KATETE

COMMODITY	NO. OF SAMPLES	NO. CONTAMINATED
Soaked maize (phale)	27	0
Shelled dry maize	31	8 (26 Percent)
Germinated maize (chimela)	11	8 (73 Percent)
Institutional nsima (maize)	43	3 (7 Percent)
Institutional beans	34	0
Village meals	65	0
Total	211	19 (9 Percent)

Table 1.9(b)

MAIZE PRODUCTS OBTAINED FROM EASTERN CO-OPERATIVE UNION, CHIPATA

PRODUCT	NO. OF SAMPLES	NO. CONTAMINATED
Breakfast meal	14	6 (43 Percent)
Roller meal	11	3 (27 Percent)
Maize bran	10	3 (30 Percent)
Total	35	12 (34 Percent)

(Njapau, personal communicatio

It was there decided to carry out a further study to look at this in more depth.

Urine samples, from patients with and without liver disease, would be collected for one year and analyzed for both free and conjugated aflatoxin metabolites. Studies have shown that conjugation reactions play an important role in aflatoxin excretion in animals and the amount and type of aflatoxin conjugates in humans is not known. First of all, a method for enzymatic hydrolysis of urinary aflatoxin conjugates would be developed using the rat as the experimental animal. Analysis of both free and conjugated metabolites, if detected, would provide information on the detoxification processes involved in humans as well as giving a better, more sensitive estimate of the amount of aflatoxin being consumed by people in Zambia. By examining patients with and without liver disease the possible effect of liver pathology on the metabolism and excretion of aflatoxin would be examined further. The use of a year long survey would provide information on the seasonal variation of aflatoxin contamination of foods.

Aflatoxin contamination has been demonstrated to show variation between urban and rural areas. Thus additional urinary samples would be analyzed from a rural area of Zambia and these results compared with those of Lusaka.

The method employed for the previous study carried out in Lusaka was acceptable but since that time newer extraction methods have been developed that have lower detection limits and require less time and solvents. Therefore various methods would be performed and one of these selected and standardized for urinary analyses.

Liver tissues, taken at autopsy from subjects with and without liver disease, would also be examined where possible, as this would give direct evidence of the presence of aflatoxin at the target organ.

Concurrent with this work, serum and liver samples would be analyzed for HBV so that the role of both etiological factors in PHC could be assessed.

CHAPTER TWO

METHODOLOGY

1. Reagents

Solvents were analar grade where possible, or laboratory grade in which case distillation was carried out before use. Distilled chloroform had 0.75 per cent ethanol added as stabilizer. Solvents and chemicals were obtained from BDH (Poole, Dorset, England) or Merck (Darmstadt, Germany). Chromatographic materials were supplied by Merck and aflatoxin standards were obtained from Sigma Chemical Company (Poole, Dorset, England) and Makor Chemicals Limited. (Jerusalem, Israel). B-Glucuronidase and sulphatase (type H-2) and assay chemicals were obtained from Sigma Chemical Company.

2. Sampling

2.1. Urine Samples from University Teaching Hospital, Lusaka

Sample collection commenced in September 1983 and continued for one year. Patients with liver pathology (hepatitis, cirrhosis, PHC) were selected soon after admission to the University Teaching Hospital (U.T.H.) and a urine sample was collected. A second sample was collected after 24 hours. Control patients, matched with liver patients by sex and age where possible, and showing no liver pathology, were selected soon after admission and urine samples were taken as for patients with liver pathology. Each sample was accompanied by a data sheet (Figure 2.1). A 50 ml portion was extracted immediately or frozen at -20^{o}C for approximately one week before extraction. A further portion was stored at -20^{o}C for analysis of conjugated metabolites.

AFLATOXIN METABOLITE PROJECT

Name ..... Project No: .....

Age ..... Sex M F Ward .....

Provisional Diagnosis .....

Date of Admission .....

Urine 1st sample ..... 2nd sample .....

3rd sample .....

Blood 10 ml taken date .....

Examination	Liver	Yes	No ..... cm RCM (Right Costal Margin)
	Spleen	Yes	No
	Ascites	Yes	No
	Jaundice	Yes	No
	Oedema	Yes	No
Diagnosis confirmed	Yes	No	
	Histology	No:	S/ /
	Histology Report		
	.....		

COMMENT:

Figure 2.1 Data Sheet for Urine Sample Collection

2.2. Serum Samples from University Teaching Hospital

A sample of blood was collected from both control and liver disease patients at the time of the first urine collection. Serum was prepared and immediately frozen at -20^{o}C to await analysis for HBsAg and anti-HBs.

2.3. Urine Samples from St. Francis' Hospital, Katete

Urine samples were collected over a four day period in April, 1984 from all newly admitted patients to St. Francis' Hospital in Katete. The age, sex and diagnosis of each patient was recorded. Samples were immediately frozen and analyzed within 14 days for free aflatoxin metabolites. Where possible a further portion was stored frozen for analysis of conjugated metabolites.

2.4. Liver Samples from University Teaching Hospital

Samples were collected during December 1983 to November 1984. Specimens of liver tissue were taken at autopsy from subjects with and without liver pathology. Each sample was accompanied by a data sheet (Figure 2.2). Samples were frozen immediately until aflatoxin analysis was performed. A further portion was stored for analysis for HBsAg.

3. Experimental Animals

Male albino rats weighing 200-250 grams were used for all experiments. They were caged individually for the duration of the experiment.

AFLATOXIN METABOLITES IN THE LIVER

PROJECT NO. AFL/..../....

NAME ..... AGE ..... SEX .....

WARD ..... FIRM/UNIT ..... DATE OF ADMISSION .....

DATE OF DEATH .....

DATE OF POSTMORTEM .....

CLINICAL DIAGNOSIS .....

RELEVANT LABORATORY RESULTS - Hb

- ESR

- LFT

POSTMORTEM DIAGNOSIS - PM NO.....

POSTMORTEM HISTOLOGY NO.....

AFLATOXIN

Figure 2.2. Data Sheet for Liver Sample Collection

4. Preparation of Aflatoxin Standards (AOAC Official Method)

4.1. Preparation of Stock Solutions

Aflatoxin standards were obtained as either crystals or solutions. They were diluted with chloroform to an approximate concentration of 8-10 µg/ml, using the label statement of aflatoxin weight or concentration as a guide. The vial was then agitated vigorously for one minute using a Vortex mixer (Gallenkamp Spin Mix).

4.2 Calibration of Spectrophotometer

A spectrophotometer (Shimadzu UV-120-01) with 1 cm quartz - face cells was calibrated as follows:

Three standard potassium dichromate solutions were prepared, 0.25 mM, 0.125 mM and 0.0625 mM, in 9 mM H_2SO_4. The absorbance, A, of each solution was determined at maximum absorption near 350 nm, against H_2SO_4, 9 mM, as a solvent blank. The molar absorptivity coefficient, ε , was determined at each concentration:

$$\varepsilon = \frac{A}{bXc} \text{ , where}$$

b = path length in m

c = concentration in mmol/l

The three ε values were averaged and the correction factor, CF, for the instrument and cells was determined by substituting in equation: $CF = \frac{3160}{\varepsilon}$, where 3160 is the literature value for ε of

$K_2Cr_2O_7$ solutions. If CF was <0.95 or >1.05 the instrument and technique were checked and the cause was eliminated before continuing with the standardization procedure.

4.3 Determination of Aflatoxin Concentrations

The concentrations of aflatoxin stock solutions were determined by measuring A at the wavelength of maximum absorption close to 350 nm and using the following equation:

$$\text{g aflatoxin/ml} = \frac{A \times MW \times 1000 \times CF}{\varepsilon}, \text{ where}$$

MW = molecular weight

CF = correction factor obtained in section 4.2

ε = molar absorptivity of aflatoxin

4.4 Preparation of Standard Solutions

A portion of the stock solution from 4.3 was diluted away from light, with chloroform, to obtain a standard solution with a concentration suitable for thin layer chromatography (TLC) analysis (usually between 0.1-0.5 μg aflatoxin/ml). The vial was agitated vigorously for one minute using a Vortex mixer. It was then wrapped tightly in aluminum foil and stored at -20°C. Before use solutions were brought to room temperature to avoid incorporation of water by condensation. The concentration of these standard solutions was considered to be stable for two weeks. Thereafter, before any quantitative analyses were carried out they would be restandardized. To facilitate the spotting procedure during TLC analysis a portion

of these solutions was used to make up one solution containing a mixture of aflatoxins, usually AFB_1, AFM_1, AFL and AFP_1, for qualitative analysis of urine.

5. Extraction of Free Aflatoxin Metabolites

5.1. Extraction from Urine

Few procedures exist in the literature for the extraction of aflatoxins from urine. However, numerous methods have been developed for various foodstuffs and some of these, particularly those for milk, are suitable for aflatoxin extraction from urine. Five methods were investigated. Each method was performed using urine samples spiked with aflatoxins B_1 and M_1 at levels ranging from 0.05 to 1.00 ppb. The same aflatoxin standard solutions were used for both spiking and TLC determination. The urine was from healthy individuals and from jaundiced patients admitted to U.T.H. The final extract obtained from urine of jaundiced patients often contains more background interferences on the TLC plate, making identification of aflatoxin spots more difficult.

Van Egmond *et al.* (1981) have developed a method for milk which is suitable for urine analysis. The extraction column consists of a polypropylene tube (15 cm length, 4 cm width), half filled with inert hydrophilic matrix (Chem Tube No. 2050, Analytichem International, Inc., Harbor City, CA 90710, U.S.A.). A 50 ml urine sample was measured, poured onto the column, and allowed to be absorbed by the matrix for 10 minutes. Then the aflatoxins were eluted with three 50 ml portions of chloroform:acetone (3:1). The solvent was allowed to drain through

the column after each addition but the column was not permitted to dry between extractions. The eluates were collected in a 250 ml round bottom flask and evaporated to near dryness on a rotary evaporator under vacuum. The residue was then cleaned up.

The clean-up step employs a silica gel mini-column. (15 cm x 0.7 cm id, fitted with a sintered glass filter and a luer nylon stopcock-Econo-columns, Bio-Rad Laboratories, Richmond, CA, U.S.A.) (Figure 2.3). A silica gel (Silica Gel 60, Merck, mesh 0.05-0.20 mm) slurry (1.5 g in chloroform) was prepared. The slurry was poured onto the column and stirred with a glass rod until all air bubbles were removed. The column was then capped with 1 g anhydrous sodium sulphate. The urine residue was transferred quantitatively to the column with 10 ml chloroform (Figure 2.4). This was allowed to drain through the column and the eluate discarded. n-Hexane (3 ml) was passed through the column followed by 12 ml acetonitrile:ether:hexane (2:3:5). Both eluates were discarded. During preliminary analyses, using samples spiked with aflatoxins, these eluates were collected and shown to be free of aflatoxin. Aflatoxins were then eluted from the column with 7.5 ml chloroform:acetone (3:1) and the eluate collected in a 10 ml teflon-lined screw-cap vial. This was evaporated under a stream of nitrogen in a heating block at approximately 50^{o}C and redissolved in 100 µl chloroform for TLC analysis. During preliminary analyses, using samples spiked with aflatoxins, a further 7.5 ml of solvent was drained through the column to confirm that all aflatoxin had been removed during the first elution.

Figure 2.3 Silica Gel Mini-Columns

Figure 2.4 Transfer of Urine Extract to Mini-Columns

Average recoveries for AFB_1 and AFM_1 were 65 and 70 per cent respectively using this method. The extracts were found to be clean, with low background fluorescence.

Hsieh *et al.* (1981) have developed two methods for milk which can be applied to urine samples. These methods employ the same extraction column described by Van Egmond *et al.* but use a chloroform: acetone ratio of 9:1 instead of 3:1 for elution. In addition, the eluate is passed through 10 g anhydrous sodium sulphate, held in Whatman No. 1 filter paper, before being collected in a round bottom flask.

The first of these methods also uses a silica gel mini-column for the clean-up step. The silica gel was first oven activated at $105^{o}C$ for one hour, then deactivated by adding 3 per cent water and allowed to equilibrate overnight in a tightly sealed container at room temperature as recommended by the authors. The urine residue was transferred quantitatively to the column with anhydrous diethyl ether (5 ml + 10 ml). The solvent was allowed to drain through the column and the eluates were discarded. Aflatoxins were eluted with two 10 ml portions chloroform:acetone (9:1) and collected in a 50 ml round bottom flask. The extract was then evaporated to dryness under vacuum and quantitatively transferred with chloroform to a small vial. This was evaporated to dryness under nitrogen in a heating block and redissolved in 100 µl chloroform for TLC analysis.

This method gave recoveries similar to the Van Egmond method; however, the background fluorescence was higher. This is a significant disadvantage because urine extracts from patients with liver pathology generally show considerable interfering fluorescence.

In the second method by Hsieh *et al.* (1981) the silica gel mini-column is replaced by a Sep-Pak silica cartridge (No. 51900, Waters Assoc., Inc., Milford MA 01757, U.S.A.). The long end of the Sep-Pak is attached to the Luer tip of a small (5-10 ml) syringe. The urine residue was dissolved in 2 ml chloroform, transferred to the syringe barrel, and loaded onto the Sep-Pak slowly. This was repeated and the eluates were discarded. 2 ml chlorofom:acetone (4:1) were added to the flask, passed through the Sep-Pak slowly and collected in a small vial. This step was repeated twice and the combined eluates were evaporated to dryness under nitrogen and redissolved in 100 µl chloroform for TLC analysis.

This method was faster than the methods using a silica gel column and gave recoveries of 70-75 per cent for both AFB_1 and AFM_1. The background fluorescence on the TLC plate was low. It also used small volumes of solvent which is of importance considering all solvents are imported into Zambia.

The fourth method, reported by Stubblefield (1979), involves extraction of urine with chloroform in a separatory funnel. 50 ml urine, 10 ml saturated sodium chloride solution and 120 ml chloroform ($35^{o}C$) were measured and shaken in a 250 ml separatory funnel for 60 seconds. The chloroform layer was drained into a 125 ml Erlenmeyer flask and 10 g anhydrous sodium sulphate were added. This solution was stirred occasionally for three minutes before being filtered into a graduated cylinder to measure the filtrate volume. Then a silica gel (2 g) mini-column was set up as described previously and the sample was added in portions and drained through the column. The column was washed with 25 ml glacial acetic acid:toluene (1:9),

followed by 25 ml hexane, and lastly with 25 ml acetonitrile:ether: hexane (2:3:5). Aflatoxins were eluted with 40 ml chloroform:acetone (4:1) into a round bottom flask. The extract was evaporated to near dryness and transferred quantitatively with chloroform to a vial. This was then evaporated to dryness and redissolved in 100 µl chloroform for TLC analysis.

The recoveries of this method were found to be lower, approximately 50 per cent, and the background fluorescence on the TLC plate was high. In addition, when compared to the first three methods, more steps, and thus time, were involved. This method also required larger volumes of solvent.

The last method to be investigated was one which had been used successfully for a previous study of aflatoxins in urine samples in Zambia (Lovelace *et al.*, 1982). It was based on the method described by Messripour and Nesheim (1977). Celite 545 filter aid (10 g) was added to 100 ml urine. The mixture was stirred continuously for 10 minutes with a magnetic stirrer while 200 ml acetone were added slowly. Then the mixture was filtered into a 600 ml beaker. The filtrate was stirred and 100 ml distilled water were added, followed by 10 ml 20 per cent lead acetate solution. The mixture was allowed to stand for 5-10 minutes, until the precipitate coagulated, and then 5 ml saturated sodium chloride were added slowly, with stirring, followed by 5 g filter aid. This mixture was filtered into a 600 ml beaker and a freshly prepared ferric hydroxide slurry (30 ml 6.7 per cent ferric chloride and 170 ml 0.2 M sodium hydroxide) was added slowly, with stirring, followed by 5 g filter aid. The mixture was

stirred for three minutes, then transferred to two 500 ml separatory funnels and 50 ml 0.1 per cent sulphuric acid was added to each. The filtrate in each flask was extracted with two portions of chloroform (15 ml + 10 ml) and the two extracts combined and washed by shaking with 100 ml 5 per cent sodium chloride solution. The washed extract was evaporated to near dryness on a rotary evaporator under vacuum and transferred quantitatively with chloroform to a vial. This was evaporated to dryness under nitrogen and redissolved in 0.6 ml of chloroform for TLC analysis.

This method had average recoveries of 55 and 60 per cent for AFB_1 and AFM_1 respectively. The biggest disadvantage of this method was that it required more time than the other methods, making it unsuitable for a survey. In addition it required larger volumes of solvent than the first three methods described.

Thus after consideration the method of Hsieh *et al.* (1981), using Sep-Pak silica cartridges, was selected for urine analysis for the one year survey. Further preliminary work with spiked samples showed that the lowest detectable concentrations of aflatoxins B_1, M_1, and AFL were 2.5, 3.0 and 3.0 ng/50 ml urine respectively, using this method.

As there was a limited number of Sep-Pak cartridges available the method of Van Egmond *et al.* (1981) was chosen to replace the method of Hsieh *et al.* (1981) during the latter part of the survey. It was also used for the St. Francis' Hospital, Katete samples.

5.2 Extraction from Liver

The method used for the extraction of free metabolites from liver was as reported by Stubblefield and Shotwell (1981). The volumes given are for a 10 g sample although sample weights varied from 5 to 25 g and volumes were adjusted accordingly. A sample was blended to form a paste. The paste was transferred to a wide-mouthed, stoppered flask, weighed accurately and 20 per cent citric acid solution (1 ml) was added and mixed in thoroughly with a glass rod. After five minutes 2 g of diatomaceous earth (Celite Filter aid) was added, followed by 20 ml dichloromethane. The flask was shaken vigorously for 30 minutes and the mixture subsequently filtered into a 50 ml flask containing 1 g anhydrous sodium sulphate. The filter paper top was closed and the residue compressed against the funnel to expel a maximum filtrate. The flask was gently swirled intermittently for two minutes and then refiltered into a 25 ml graduated cylinder and the filtrate volume recorded. This was then evaporated to near dryness in a round bottom flask, under vacuum.

The clean-up step procedure employed a silica gel mini-column as described in the methods for urine (page 73). The silica gel was pre-treated by first stirring one hour in methanol, filtering, and stirring one hour in chloroform. After filtration it was air-dried in a shallow pan overnight, oven activated at 105^{o}C for one hour, then mixed with 1 per cent water and allowed to equilibrate overnight in a tightly sealed container at room temperature. The column was set up by making a silica-dichloromethane slurry, pouring it into the column and stirring until all air bubbles were removed. The extract was quantitatively transferred to the column with 7 ml dichloromethane (3 ml + 2 ml + 2 ml). The solvent

was allowed to drain through the column and the eluates discarded. The column was washed with 5 ml glacial acetic acid:toluene (1:9), 5 ml hexane and 5 ml acetonitrile:ether:hexane (1:3:6) and the washes discarded. Aflatoxins were eluted with two 10 ml volumes chloroform: acetone (4:1) and the eluates evaporated to near dryness under vacuum. The extract was then quantitatively transferred to a vial with chloroform, evaporated to dryness under nitrogen, and dissolved in 50 µl chloroform for TLC analysis.

This method was carried out without difficulty using rat and human liver samples and the final extract was clean, with minimal interference on the TLC plate. Average recoveries for aflatoxins B_1 and M_1, at spiking levels ranging from 0.2-1.2 ng/g, were found to be 65 and 75 per cent respectively. The lowest detectable concentrations of AFB_1 and AFM_1 were 0.20 ng/g and 0.25 ng/g respectively. These values are somewhat higher than those reported by other investigators. In a collaborative study involving 13 laboratories (Stubblefield *et al.*, 1982) some reported detection limits of 0.03 ng AFB_1 and AFM_1/g. However the limit of detection value depends, to some extent, on sample size. The larger the sample, the more aflatoxin in the final extract. In this collaborative study 100 g of liver were used compared with only approximately 10 g in the present study.

6. Extraction of Conjugated Metabolites from Urine

The method used for analysis of conjugated metabolites in urine was based on the procedure described by Wong and Hsieh (1980). In order to standardize this method preliminary experiments using rats were carried out.

6.1 Administration of AFB_1 and Urine Collection

AFB_1 was administered orally to rats by absorbing a chloroform solution containing 150 µg AFB_1 (1 µg/µl) onto a food pellet and removing all other pellets until the contaminated one had been completely consumed. This dose is approximately 3 per cent of an LD_{50} dose.

The rats were caged individually and clean trays, with wire mesh above them, were placed under the cages. Urine was collected 24 and 48 hours after the aflatoxin had been consumed. This was done by washing the trays thoroughly with water (20 ml) and chloroform:acetone (4:1) (20 ml), with the aid of a Pasteur pipette. The combined rinses were collected in a 100 ml glass-stoppered flask and stored at -20°C. It was not possible, due to the design of the cages, to collect urine free of faecal contamination. However, as the purpose of these experiments was to perfect a method for the recovery of conjugated metabolites from human urine this was not considered important.

6.2 Extraction of Water Soluble Conjugates and Their Enzymatic Hydrolysis

After removal of particulate matter by vacuum filtration the samples were extracted five times with an equal volume of chloroform in a separatory funnel. With some samples severe emulsions formed and phase separation could only be achieved by centrifugation. The residual aqueous phases were collected in a 250 ml Erlenmeyer flask and recycled several times through an Amberlite XAD-4 (Rohm and Hass, Philadelphia,

PA., U.S.A.) column to recover aflatoxin conjugates. The urinary conjugates were eluted from the column with 25 ml acetone followed by 25 ml methanol and collected in a round bottom flask. The solvents from the eluate were evaporated under vacuum and the residue was quantitatively transferred to a small vial with acetone followed by methanol. The solvents were again removed, under a stream of nitrogen in a heating block, and the remaining extract was suspended in 2 ml of a 0.2 M acetate buffer (pH 5.0). This extract, rather than being completely dry as reported by Wong and Hsieh (1980), was found to contain approximately 2 ml water from the resin column which could not be evaporated at the low temperature required when working with aflatoxins. Thus, instead of suspending a dry extract in 2 ml of a 0.2 M acetate buffer, a 2 M acetate buffer was prepared and 0.2 ml was added to the 2 ml water already present in the extract. The conjugates were then subjected to enzymatic hydrolysis with 200 units of sulphatase and 10,000 units of β-glucuronidase for 48 hours in a shaking water bath at 37°C. The activity of the enzymes was determined before use as recommended by Sigma.

6.3 Analysis of Hydrolyzed Extracts

The hydrolysate from 6.2 was extracted five times with an equal volume of chloroform and the extracts combined, concentrated and analyzed for aflatoxin metabolites using TLC. However it became apparent that this extract was too dirty to proceed directly to TLC analysis and subsequently a clean-up step was introduced. The

concentrated extract was passed through a silica gel mini-column as described previously by Van Egmond *et al.* (1981) (Section 5.1) and thereafter TLC analysis was successful. Using this method on rat urine conjugates of AFM_1 and AFP_1 were recovered.

6.4 Human Studies

Following these experiments human samples were analyzed by the same method. The sample size was 50 ml. The first step, removal of particulate matter, was not necessary and fewer emulsions were encountered than with rat samples.

7. Thin Layer Chromatography Analysis

7.1 Separation

Using spiked urine and liver samples numerous one-and two-dimensional solvent systems were tested. It was found that one-dimensional TLC rarely gave satisfactory separation of extracts and therefore two-dimensional TLC was used for all samples. Solvent ratios were varied until the best developing mixture was chosen.

The two-dimensional method of Schuller *et al.* (1973) as described by Van Egmond *et al.* (1978) was used with slight modifications. Pre-coated 10 x 10 cm TLC plates (Merck, Darmstadt, Germany, 0.25 mm), self-cut from 20 x 20 cm plates, were used. Two straight lines were scored at right angles 1.5 cm from each edge to limit migration of developing solvent fronts. Extract and standard spots were applied on TLC plates using microsyringes (Hamilton, Bonaduz, Switzerland) as

indicated in Figure 2.5. The following aliquots were spotted: A, 20 µl sample extract; B and C, 25 µl standard solution containing 3 ng of AFM_1; E and C, 25 µl standard solution containing 3 ng AFB_1. Additional standards (AFP_1, AFL) were sometimes applied on top of B and C. Samples and standards were applied as quickly as possible in subdued light to minimize decomposition and care was taken to ensure spots were uniformly sized and $\leq$0.5 cm in diameter.

The plate was developed in the first direction in a saturated tank containing ethylether:methanol:water (92:6.5:1.5). After development the plate was allowed to dry for 10-15 minutes in the dark, then developed in the second direction with chloroform:acetone: methanol (90:10:2) in an unsaturated tank. All plates were developed in the dark by placing the tank in a cupboard or by covering it with thick cardboard, as exposure of aflatoxins on adsorbent surface to ultraviolet (UV) light may lead to decomposition, especially in the presence of solvents (Schuller and Van Egmond, 1981).

As an additional check on the location and appearance of aflatoxin spots from the sample a second plate was prepared. At A (Figure 2.5) 25 µl of AFB_1 and AFM_1 standard solutions were spotted, together with 20 µl of sample extract on top of the standard spot. This plate was developed as described above. These two plates were always placed in the same tank, facing each other, to obtain developing conditions as equal as possible.

The plates were examined under UV light (365 nm). If aflatoxins M_1 and B_1 were present in the extract blue fluorescing spots were visible at A^1 and D^1 respectively (Figure 2.5). The spot pattern of this plate was compared to the pattern of the second plate with the sample spot containing the internal standards.

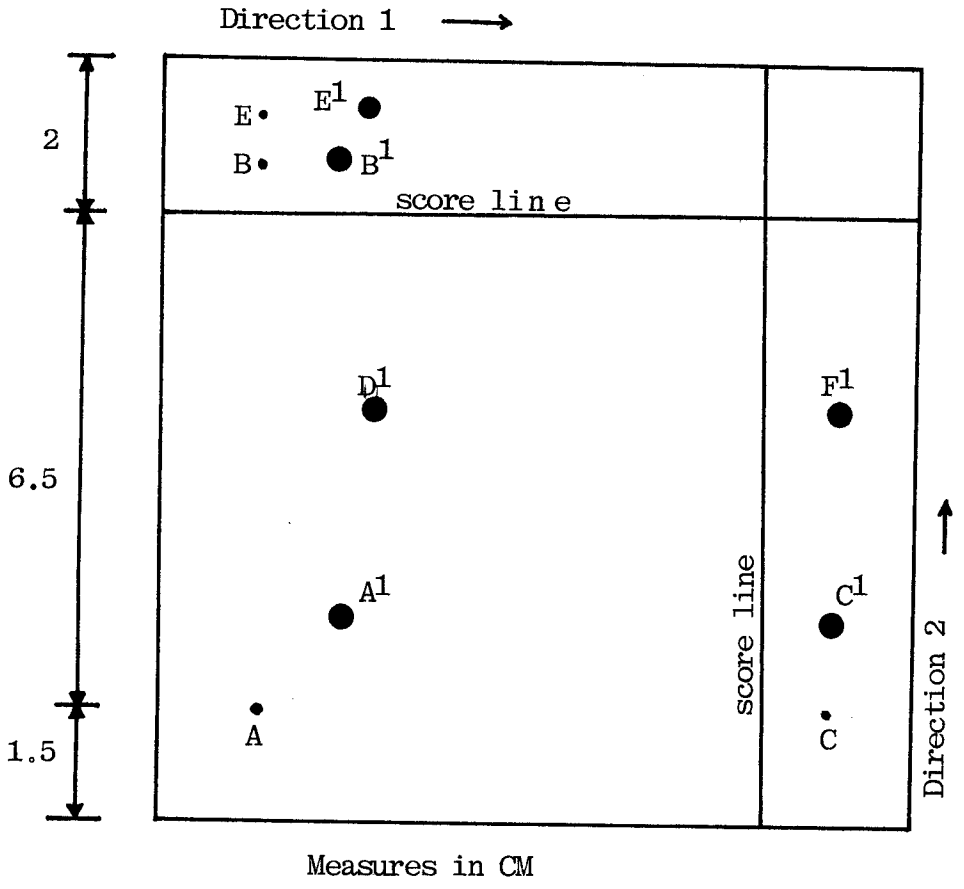


Figure 2.5 Schematic representation of thin layer chromatogram

Direction 1: ethyl ether: methanol: water (92:6.5:1.5, lined tank)

Direction 2: chloroform: acetone: methanol (90:10:2, unlined tank)

A^1 = AFM_1 spot from sample

B^1 and C^1 = locations of standard AFM_1 spots

D^1 = AFB_1 spot from sample

E^1 and F^1 = locations of standard AFB_1 spots

In order for a sample to be suspected as positive the superimposed standard and the presumed aflatoxin spot from the sample had to coincide and the spot from the sample containing the internal standard had to be more intense than either sample or standards alone. Figure 2.6 shows a chromatoplate on which an extract of urine containing an internal standard (AFB_1, AFM_1 and AFP_1) was subjected to two-dimensional TLC.

7.2 Confirmation

Once a sample was found that was believed to be positive the identity of the aflatoxin(s) was confirmed with the method of van Egmond et al., (1978). This step is essential, as numerous substances can resemble aflatoxins on the TLC plate. The locations of AFM_1 and AFB_1 spots from sample (A^1 and D^1) and standards (B^1 and E^1) (Figure 2.7) were marked with a pencil carefully. These spots were then treated with 1-2 µl trifluoroacetic acid (TFA) using a microsyringe. The plate was kept in the dark 3-5 minutes at room temperature and then placed on a hot glass plate (20 x 20 cm) in the oven at 75°C. The marked areas (A^1, B^1, D^1 and E^1) were covered with a hot glass plate and heated for five minutes. The plate was allowed to cool for one minute on a cold surface and was then developed in the dark in the first direction with a mixture of chloroform:methanol: acetic acid (90:10:2) in an unsaturated tank.

The plate was examined under UV light (365 nm). Aflatoxin M_1 standard from B^1 shows a blue fluorescent spot of the reaction product of M_1 with TFA (B^{11}) and a weak yellowish fluorescent spot of unreacted M_1 at a higher R_F(B^{111}). The M_1 derivative from the

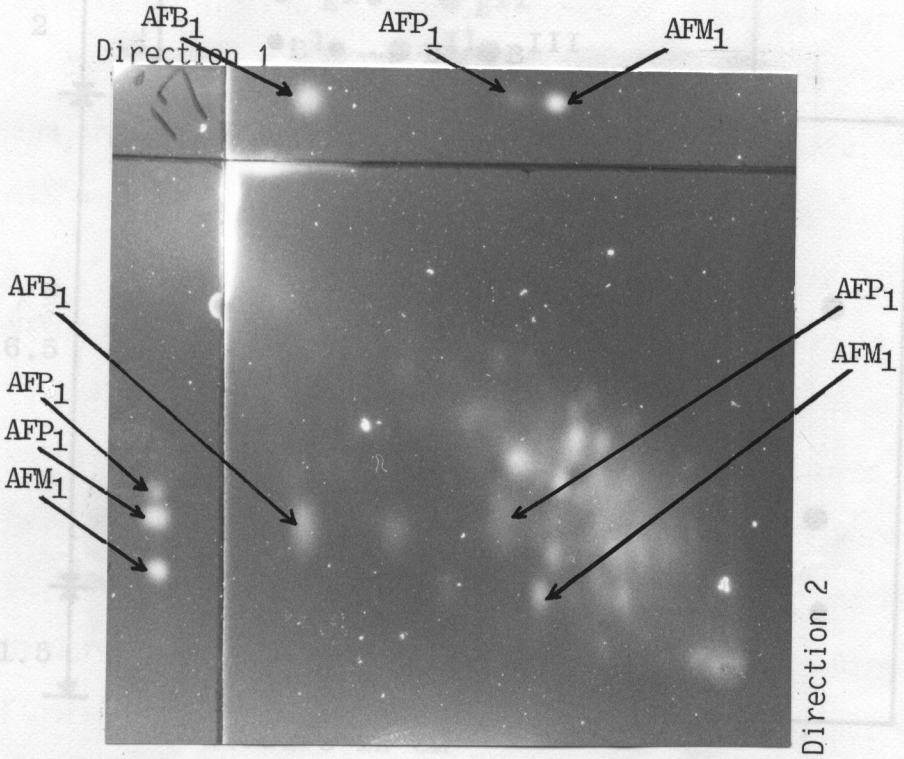


Figure 2.6 Two-dimensional TLC separation of urine extract containing internal standards AFB_1, AFM_1 and AFP_1

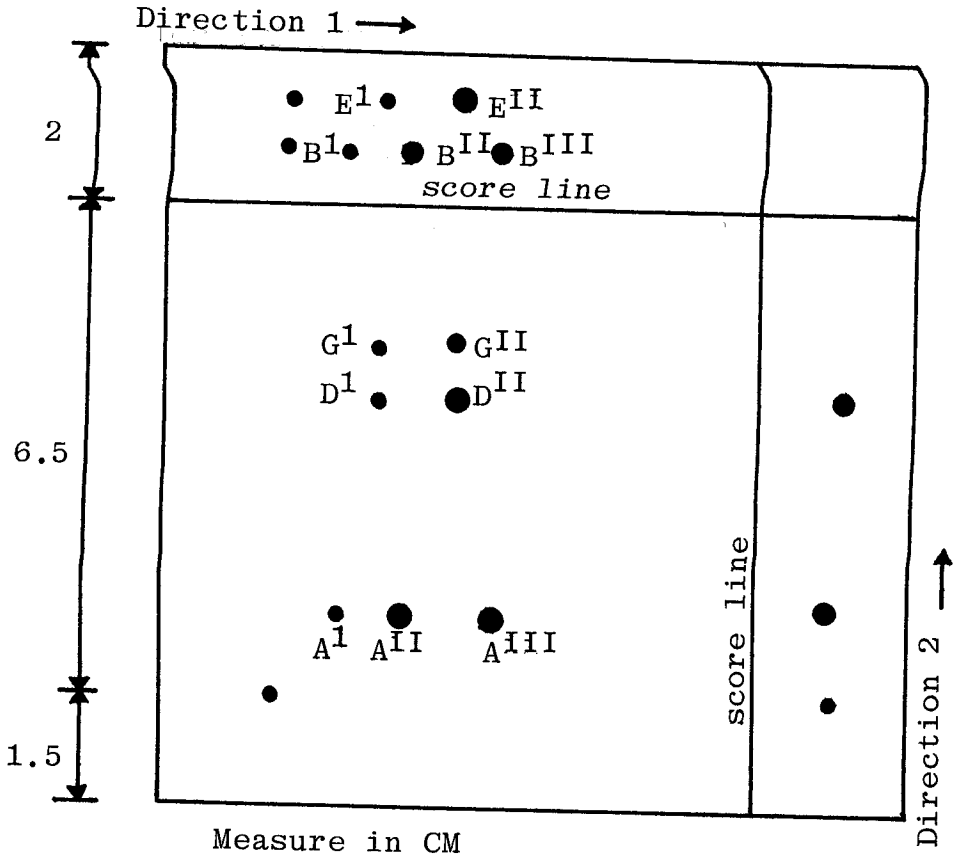


Figure 2.7 Schematic represenation of thin layer chromatogram after reaction with TFA and third development in Direction 1 in chloroform: methanol: acetic acid (90:10:2, unlined tank)

A^1, B^1, D^1 and E^1 = locations were TFA has been superimposed, corresponding to A^1, B^1, D^1 and E^1 of Figure 2.5

G^1 = location for spotting AFB_1 standard after 2-dimensional TLC, and for superimposing TFA

A^{II} = reaction product of AFM_1

B^{II} = reaction product of AFM_1 standard

D^{II} = reaction product of AFB_1 from sample

E^{II} and G^{II} - reaction products of AFB_1 standard

A^{III} = residual AFM_1 from sample

B^{III} = residual AFM_1 from standard

extract (A^{11}) has moved the same distance as the standard M_1 derivative (B^{11}). The identity of AFM_1 in the extract is confirmed when the R_F values of the M_1 derivatives from sample and standard match. The B_1 derivatives from the sample and extract are blue fluorescent spots and are located at D^{11} and E^{11} respectively. The AFB_1 derivative is its hemiacetal, AFB_{2a} (Schuller and Van Egmond, 1981).

Figure 2.8(a) shows a chromatoplate on which an extract of urine was subjected to two-dimensional TLC. The sample was found to be positive for AFM_1. Figure 2.8(b) demonstrates the same urine extract, after the confirmatory test with TFA. Figures 2.9 and 2.10 show chromatoplates on which extracts of urine were subjected to two-dimensional TLC and then confirmed for AFM_1 and AFB_1 respectively by derivatization with TFA and third development in Direction 1. From Figures 2.8(b) and 2.9 it can be seen that AFM_1 has not reacted completely with TFA.

Occasionally an additional test was applied to sample chromatograms. Sulphuric acid (25 per cent) was sprayed onto a plate after the second development. This changes the fluoresence of aflatoxin from blue to yellow. This test only confirms the absence of aflatoxins; *i.e.* spots which do not turn yellow are positively not aflatoxin, whereas many substances other than aflatoxin may give a yellow spot with sulphuric acid. This simple test was carried out on samples that had a blue fluorescent spot at or near standard aflatoxin locations but which, from past experience of examining many chromatograms, are thought to be most likely negative. As this occurred fairly often, solvents were minimized by confirming a negative result before proceeding with the derivatization procedure.

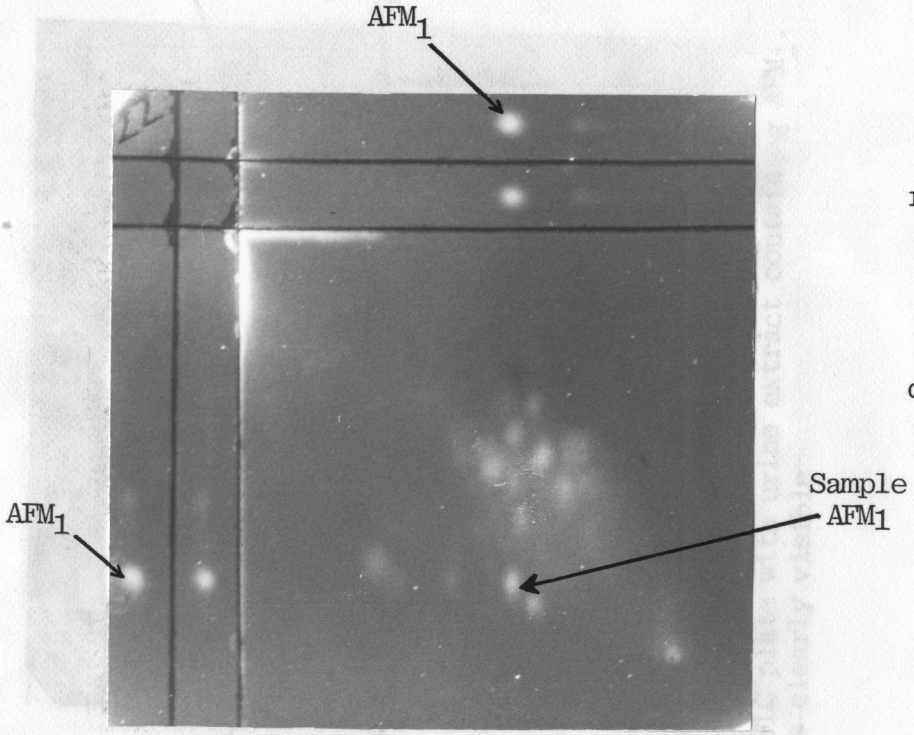


Figure 2.8(a) Two-dimensional TLC separation of urine extract. Sample was suspected to contain AFM_1

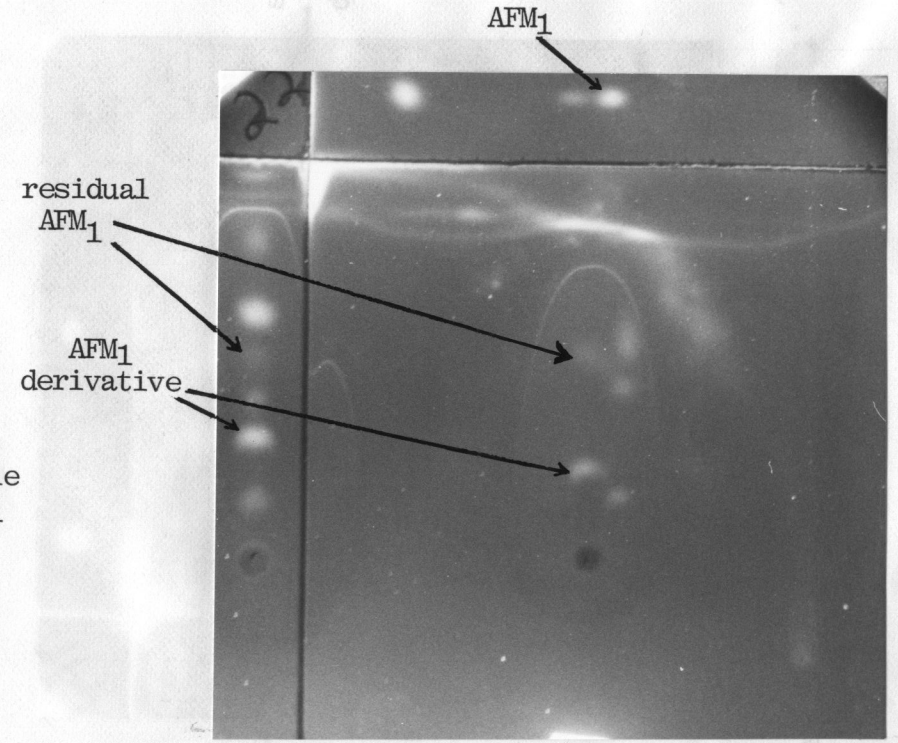


Figure 2.8(b) TLC plate with urine extract of (a) after treatment with TFA and development. The AFM_1 derivative is clearly visible, confirming the presence of AFM_1. Residual AFM_1 is also visible.

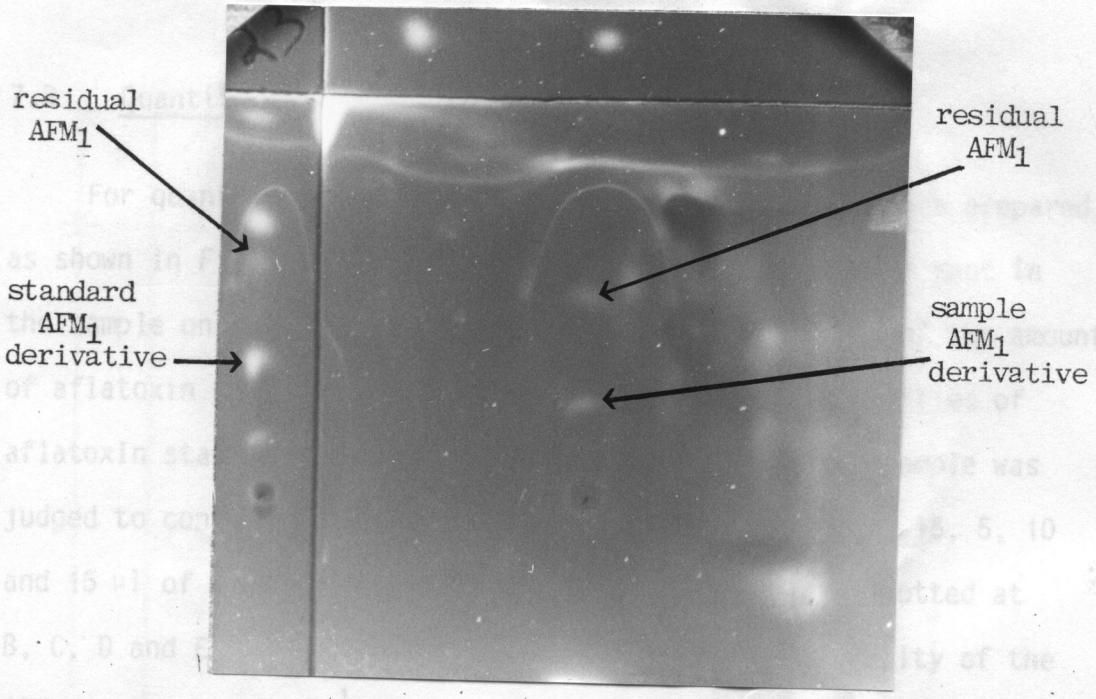


Figure 2.9 TLC plate with urine extract containing AFM_1. The TFA derivative is clearly visible

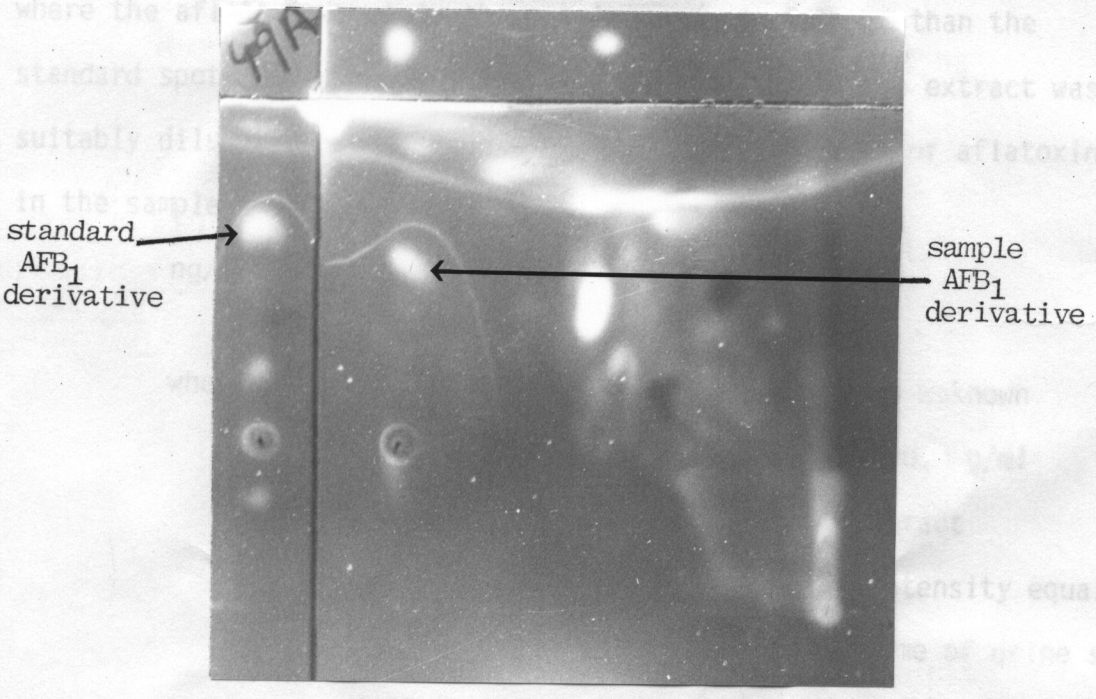


Figure 2.10 TLC plate with urine extract containing AFB_1. The TFA derivative is clearly visible.

7.3 Quantification

For quantification of aflatoxin a second TLC plate was prepared as shown in Figure 2.11. By examination of the aflatoxin spot in the sample on the first plate an approximation was made of the amount of aflatoxin present and from this three different quantities of aflatoxin standard were spotted. For instance, if the sample was judged to contain roughly 1 ng AFM_1 in 20 µl of extract, 15, 5, 10 and 15 µl of a 0.1 ng/µl AFM_1 standard solution were spotted at B, C, D and E respectively. Then the fluorescent intensity of the AFM_1 in the sample (A^1) was compared with those of the standard spots (C^1, D^1 and E^1) and it was determined which of these matched the sample spot. If necessary an interpolation was made or, in the case where the aflatoxin spot in the sample was more intense than the standard spot with the greatest amount of aflatoxin, the extract was suitably diluted. The calculation of the concentration of aflatoxin in the sample was made using the following formula:

$$\text{ng/g or ng/ml} = \frac{S \times Y \times V}{X \times W}$$

where: S = µl of aflatoxin standard equal to unknown

Y = concentration of aflatoxin standard, µg/ml

V = µl of final dilution of sample extract

X = µl sample extract giving a spot intensity equal to S

W = weight of liver sample (g) or volume of urine sample (ml) represented by final extract for TLC

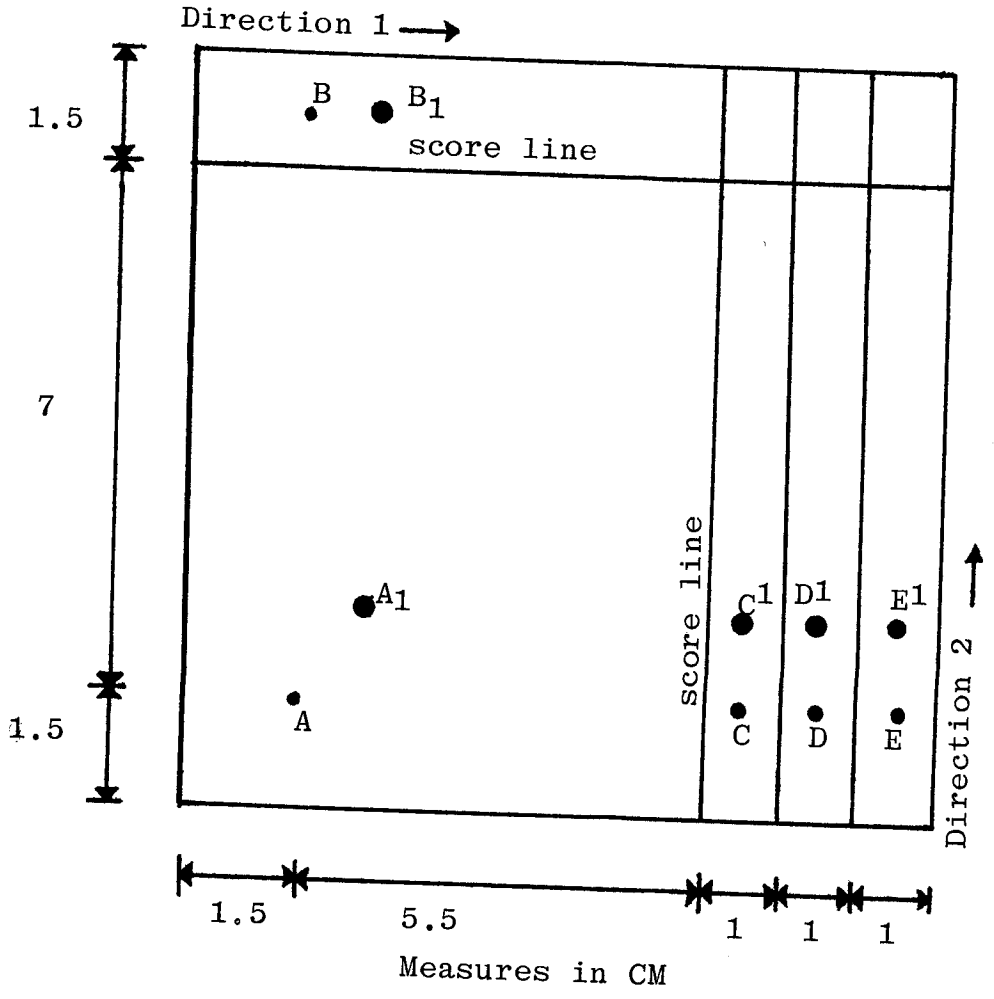


Figure 2.11 Schematic representation of thin layer chromatogram used for quantification of aflatoxin in sample

In the method used for liver (Stubblefield and Shotwell, 1981) and in the method of Stubblefield (1979) for urine the filtrate volume is taken into consideration by multiplying W by : filtrate volume (ml)/extraction solvent volume (ml) *i.e.* 20 ml dichloromethane for a 10 g liver sample gives approximately 12 ml filtrate.

8. Analysis of Samples for Hepatitis B Virus Markers

8.1. Serum Samples

Serum samples were analyzed for HBsAg and anti-HBs by radio-immunoassay (RIA) by the Bureau of Biologies, National Institute of Health, Maryland, U.S.A.

8.2 Liver Samples

Liver samples were analyzed for HBsAg using Shikata's method of orcein staining performed by Dr. N.K. Nkanza, Department of Pathology, U.T.H.

9. Statistical Procedures

The significance of difference between two values was calculated using Fisher's Exact Test except where otherwise indicated. The *Chi*-square test was not used as frequency values were low. A result was considered to be statistically significant where probability P was ≤ 0.05.

CHAPTER THREE

RESULTS

1. Free Aflatoxin Metabolites in Urine Samples Collected from University Teaching Hospital, Lusaka, September 1983 - September 1984

Urine samples from 136 patients at U.T.H. were analysed, 67 were patients showing liver pathology and 69 were control patients with clinically normal liver function. The results are presented in Table 3.1 and tabulated according to age, sex and season of the year in Table 3.2.

The presence of urinary aflatoxin was confirmed for five patients of whom three were patients with liver disease and two were controls. Aflatoxins B_1 and M_1 and AFL were detected. AFP_1 was also looked for but was not observed. In these cases aflatoxin was observed in the initial or 24-hour or both urine collections. In a previous study by Lovelace *et al.* (1983) a patient's urine was only considered positive when both samples were found to contain aflatoxin. However, Wong and Hsieh (1980) have shown that, in monkeys, urinary levels drop sharply within 24 hours. Thus it was decided that only one urine collection need contain aflatoxin for a patient's urine to be considered positive.

The incidence of aflatoxin excretion was 3.7 per cent and the range of urinary aflatoxin was 4.0-20.0 ng/100 ml, with a mean concentration of 9.1 ng/100 ml. The difference in incidences of aflatoxin excretion for the two groups was not significant ($P < 0.05$). It can be seen that the mean urinary aflatoxin concentration of control patients was more than twice that of liver disease patients. However the number of positive samples was considered too small for a test of significance to be carried out.

Table 3.1 Aflatoxin Analysis of Urine Samples Collected from University Teaching Hospital, Lusaka, September 1983 to September 1984

	Total	Liver Patients	Control Patients
Number of Samples	136	67	69
Number of Positive Samples	5	3	2
Incidence of Positive Samples (percent)	3.7	4.5	2.9
Range of Aflatoxin Concentration (ng/100ml)	4.0-20.0 (mean=9.1)	4.0-6.6 (mean=5.6)	5.0-20.0 (mean=13.9)
Aflatoxin metabolite(s) observed		AFB_1 (1) AFM_1 (1) AFL (1)	AFB_1

Table 3.2 Classification of Patients used for Aflatoxin Analysis of Urine Samples collected from University Teaching Hospital, Lusaka.

(a) Age Range (in years)

	0-19	20-29	30-39	40-49	50+
Controls	7	30	18	7	7
Patients	10	32	8	8	9
Total	17	62	26	15	16
Number of Positive Samples	0	3	0	0	2
Incidence of Positive Samples (Percent)	0	4.8	0	0	12.5

(b) Sex

	Male	Female
Controls	50	19
Patients	53	14
Total	103	33
Number of Positive Samples	5	0
Incidence of Positive Samples (percent)	4.9	0

Table 3.2 cont'd

(c) Season

	Rainy Season (Nov. - Mar.)	Dry Season (Apr. - Oct.)
Controls	39	28
Patients	37	32
Total	76	60
Number of Positive Samples	5	0
Incidence of Positive Samples (percent)	6.6	0

From Table 3.2 it can be seen that aflatoxin excretion was observed only in males, in the age groups 20-29 and 50+ years, although this finding was not significant at $P < 0.05$. All samples containing aflatoxin were collected during the rainy season showing that the likelihood of consuming aflatoxin was significantly higher in the rainy season ($P < 0.05$).

2. Free Aflatoxin Metabolites in urine Samples Collected from St. Francis' Hospital, Katete, April 1984

Urine samples from 49 patients at St. Francis' Hospital were analysed, five were patients showing liver pathology and 21 were control patients with clinically normal liver function. A further 23 samples were collected from children under seven years of age who suffered mainly from protein energy malnutrition. As these patients could not easily be classified as control or liver disease patients they were excluded. (There was one positive sample in this group, collected from a child with marasmic kwashiorkor, 22 ng AFM_1/100 ml urine).

The results are presented in Table 3.3 and tabulated according to age and sex in Table 3.4. The presence of AFM_1 was confirmed for four patients, all of whom were controls. Aflatoxins B_1, P_1 and AFL were also looked for but were not observed. The incidence of AFM_1 excretion was 15.4 per cent and the range of urinary AFM_1 was 8.0 - 25.0 ng/100 ml, with a mean concentration of 16.5 ng/100 ml.

Table 3.3 Aflatoxin Analysis of Urine Samples Collected From St. Francis' Hospital, Katete, April 1984

	Total	Liver Patients	Control Patients
Number of Samples	26	5	21
Number of Positive Samples	4	0	4
Incidence of Positive Samples (Percent)	15.4	0	19.0
Range of aflatoxin concentration (ng/100ml)	8.0-25.0 (mean=16.5)	-	8.0-25-0 (mean=16.5)
Aflatoxin metabolite(s) observed	AFM_1	-	AFM_1

Table 3.4 Classification of Patients used for Aflatoxin Analysis of Urine Samples collected from St. Francis' Hospital, Katete, April 1984.

(a) Age Range (in years)

	7.-19	20-29	30-39	40-49	50+
Number of samples	11	8	0	2	5
Number of Positive samples	2	1	0	0	1
Incidence of Positive Samples (percent)	18.2	12.5	0	0	20.0

(b) Sex

	Male	Female
Number of Samples	11	10
Number of Positive Samples	1	3
Incidence of Positive Samples (Percent)	9.1	30.0

No significant findings were made with respect to age or sex and the incidence of urinary aflatoxin excretion. However, it was interesting to note that two of the AFM_1-positive samples were from young women who suffered from incomplete and threatened abortions.

3. Conjugated Aflatoxin Metabolites in Urine Samples Collected from University Teaching Hospital, Lusaka and St. Francis' Hospital, Katete

Glucuronide and sulphate conjugates of AFM_1, AFP_1 and AFL were looked for in urine specimens from 48 of the patients from U.T.H., including all samples previously shown to be positive for free aflatoxin metabolites, and eight of the patients from St. Francis' Hospital, including one sample known to contain a high level of AFM_1. No conjugates were detected in any of these samples.

4. Comparison of Urban and Rural Urine Samples

The incidence of urinary aflatoxin excretion was 3.7 per cent in samples collected from U.T.H. and 15.4 per cent in samples collected from St. Francis' Hospital. Using the Test of Proportions this difference was found to be highly significant ($P < 0.01$). The mean urinary aflatoxin concentrations of U.T.H. and St. Francis' Hospital samples differed by almost two-fold, the mean concentrations being 9.1 and 16.5 ng/100 ml at U.T.H. and St. Francis' Hospital, respectively.

5. Free Aflatoxin Metabolites in Liver Autopsy Samples Collected from University Teaching Hospital, Lusaka, December 1983 - November 1984

Liver samples were taken at autopsy from 34 subjects and analysed for aflatoxins B_1, G_1, M_1, P_1 and AFL. The results are presented in Table 3.5 and tabulated according to age, sex and season in Table 3.6. The presence of AFB_1 was confirmed in five samples (14.7 per cent), of which four were from control subjects and one was from a male who had suffered from PHC. Only AFB_1 was detected, at concentrations ranging from 0.3-0.86 ng/g in control cases, and at a level of 9.5 ng/g in the PHC case. Thus the sample from the hepatocellular carcinoma subject contained over 10 times more aflatoxin than the highest level observed in controls.

From Table 3.6 it can be seen that aflatoxin was observed in males more than females and this difference was significant ($P < 0.05$). With respect to age the 50+ years group had the highest percentage of positive samples. As in the case of urine samples collected from U.T.H., all liver samples containing aflatoxin were collected during the rainy season, although this finding was not significant ($P > 0.05$).

6. Hepatitis B Virus Markers in Samples Collected from University Teaching Hospital

Table 3.5 Aflatoxin Analysis of Liver Autopsy Samples Collected from University Teaching Hospital, Lusaka

	Total	Liver Cancer Subjects	Control Subjects
Number of Samples	34	1	33
Number of Positive Samples	5	1	4
Incidence of Positive Samples (Percent)	14.7	100	12.1
Range of aflatoxin concentration (ng/g)	0.3-9.5 (mean=2.3)	9.5	0.3-0.86 (mean=0.54)
Aflatoxin metabolite(s) observed		AFB_1	AFB_1

Table 3.6 Classification of Patients Used for Aflatoxin Analysis of Liver Autopsy Samples, University Teaching Hospital, Lusaka.

(a) Age Range (in years)

	1-19	20-29	30-39	40-49	50+
Controls	7	8	7	5	6
Patients	0	0	1	0	0
Total	7	8	8	5	6
Number of Positive Samples	0	2	1	0	2
Incidence of Positive Samples (Percent)	0	25.0	12.5	0	33.3

(b) Sex

	Male	Female
Controls	20	13
Patients	1	0
Total	21	13
Number of Positive Samples	4	1
Incidence of Positive Samples (Percent)	19.0	7.7

Table 3.6 cont'd

(c) Season

	Rainy Season (Nov.-Mar.)	Dry Season (Apr.-Oct.)
Controls	30	3
Patients	1	0
Total	31	3
Number of Positive Samples	5	0
Incidence of Positive Samples (Percent)	16.1	0

6.1 Serum Samples

Serum samples from 65 patients at U.T.H. were analysed for HBsAg and anti- HBs, 31 were patients showing liver pathology, mostly hepatitis, and 34 were control patients with clinically normal liver function. The results are presented in Table 3.7. The presence of HBsAg was detected in 32 per cent of serum samples obtained from control patients and in 74 per cent of samples obtained from liver disease patients. Anti- HBs was detected in 71 per cent of control cases and in 45 per cent of liver disease cases. These percentage values are age and sex dependent and therefore the data for control patients is tabulated according to age and sex in Table 3.8.

6.2. Liver Samples

Fourteen liver samples were collected and analysed for HBsAG and 11 were found to be postive (79 per cent).

Table 3.7 Hepatitis B surface antigen (HBsAg) and antibody to hepatitis B surface antigen (anti-HBs) Analysis of Serum Samples collected from University Teaching Hospital, Lusaka, September 1983 to September 1984

	Number of Samples	HBsAg Positive (Percent)	anti-HBs Positive (Percent)
Controls	34	11 (32)	24 (71)
Patients	31	23 (74)	14 (45)

Table 3.8 Classification of Control Patients used for HBsAg and anti-HBs Analysis of Serum Samples collected from University Teaching Hospital

(a) Age range (in years)

Age	Number of Samples	HBsAg Positive (Percent)	anti-HBs Positive (Percent)
15–19	4	1 (25)	3 (75)
20–29	16	5 (31)	11 (69)
30–39	8	1 (13)	5 (63)
40–49	3	2 (67)	3 (100)
50+	2	2 (100)	2 (100)

(b) Sex

Sex	Number of Samples	HBsAg Positive (Percent)	anti-HBs Positive (Percent)
Male	24	10 (42)	17 (71)
Female	10	1 (10)	7 (70)

CHAPTER FOUR

DISCUSSION

Data relating rates of PHC incidence, HBV status and aflatoxin exposure of specific rural and urban populations in Zambia has not been collected and would be valuable in investigating the relative roles of both etiological factors in PHC.

Comparison of this study with urinary studies carried out in other parts of Africa may show that aflatoxin contamination of foods in Zambia is not as serious as elsewhere. For instance, in Sudan, 25 per cent of urine samples were found to contain aflatoxin, at concentrations greater than those in the present study (Hendrickse *et al.*, (1982). However, the results of the recent food study, with rates of aflatoxin contamination as high as 73 per cent in certain maize products (Table 1.9a) indicates that aflatoxin still poses a serious health hazard in certain areas of Zambia.

The incidences of aflatoxin excretion in liver disease and control patients at U.T.H. were similar although the mean urinary aflatoxin concentration of control patients was more than twice that of liver disease patients. At St. Francis' Hospital, only urine samples collected from control patients were found to contain aflatoxin metabolites. The more frequent detection and higher mean concentrations of aflatoxin in the urine of control patients overall may suggest that patients with liver disease have an impaired ability to metabolize and excrete these substances. The three liver disease patients excreting aflatoxin metabolites suffered from cirrhosis, hepatitis and tuberculosis with liver involvement. The liver function of these patients would be impaired and thus it is possible that higher levels of aflatoxins are being retained in their tissues. It should also be noted that urine

samples from five patients with PHC were included in this survey and none were found to contain aflatoxin metabolites. This may simply be because these people had not consumed any aflatoxin contaminated food; alternatively it may indicate a decreased ability to metabolize and excrete aflatoxin.

The detection of AFB_1 in the urine of control patients at U.T.H. was an unexpected observation. At St. Francis' Hospital AFM_1 was the only urinary metabolite observed, a finding consistent with the work of Campbell et al. (1983) and Lovelace et al. (1983). Other workers have detected AFB_1 in human urine but apparently only in patients with impaired liver function. Amla et al. (1970) observed AFB_1 in urine of children suffering from Indian Childhood Cirrhosis and Hendrickse (1984) reported urinary AFB_1 in African children with protein-energy malnutrition (PEM). The observation of AFB_1 in the urine of one patient with liver disease in the present study is therefore consistent with these findings.

The detection of AFL in urine collected from a man suffering from cirrhosis and not in controls may indicate some difference in the metabolism of aflatoxin in the two categories of patients. This may be a consequence of the disease with serious potential. Aflatoxicol has been reported to be the most potent mutagenic and carcinogenic metabolite of AFB_1 (Table 1.6) and in vivo formation of AFL has been shown to parallel species susceptibility to aflatoxin carcinogenesis (Wong and Hsieh, 1980).

The failure to detect glucuronide and sulphate conjugates of AFM_1 or AFP_1 in urine, even in patients who had been excreting up to 20 ng of free aflatoxin /100 ml urine, was an unexpected finding. Conjugation

of toxic metabolites is known to be an integral part of cellular defense against environmental carcinogens (Autrup, 1982) and urinary aflatoxin conjugates have been observed in a number of species, including monkeys (Dalezios *et al.*, 1973; Wong and Hsieh, 1980). This finding is however, in agreement with the reports of Merrill and Campbell (1974a) who were unable to detect glucuronide or sulphate conjugates of AFP_1 in urine obtained from humans ingesting 9.6-40.3 µg AFB_1/48 hours. The same workers (1974b) also observed, in a study of the *in vitro* metabolism of AFB_1 by human hepatic tissue, that incubation with glucuronidase and sulphatase did not yield any additional free aflatoxin metabolites.

The results of the present study suggest that glucuronide and sulphate conjugation reactions do not represent a major aflatoxin excretory route in humans. In fact, the percentage of ingested aflatoxin recovered as urinary glucuronide and sulphate conjugates in animal studies is often low. For instance, Dalezios *et al.* (1973) estimated that, in monkeys, only 3.3 per cent of an AFB_1 dose was excreted as AFP_1 glucuronide and 1.2 per cent as sulphate conjugate and conjugates of AFM_1 were not detected. Thus in the present study glucuronide and sulphate conjugates of AFM_1 and AFP_1 may have been present in the urine of liver disease and/or control patients but at concentrations below detectable levels.

One of the purposes of this study was to investigate whether a relationship exists between liver pathology and the type of aflatoxin metabolite(s) excreted. Unfortunately, interpretation of the findings in this respect is very difficult as there is no consistent relationship

between liver pathology and the type of aflatoxin metabolite(s) excreted. The need for caution in interpreting data obtained from human metabolism studies was demonstrated by Hendrickse (1984) who carried out serial simultaneous estimations of aflatoxins in serum, urine and faeces. Specimens were collected at six hour intervals from 12 children. The results showed rapid changes in aflatoxin metabolism, reflected by qualitative and quantitative changes in all three types of samples. For example, urine samples collected from one child at 0, 6 and 12 hours contained AFL_1 AFB_1 and AFM_1 respectively.

The results of the present study lead to further questions about the metabolism and excretion of aflatoxins in humans. If free urinary aflatoxin metabolites represent only about five per cent of ingested aflatoxin as quoted by most workers, and conjugated urinary metabolites also make up a minor excretory route, one is left with a very high level of "unaccounted for" aflatoxin. This finding is, in fact, frequently encountered in aflatoxin metabolism studies. The identification of a bound aflatoxin-albumin complex in the systemic blood of some species (Luthy *et al.*, 1980; Nassar *et al.*, 1982) has been considered as one explanation for the low excretion rates observed and needs further investigation in humans. Reports showing the faeces to constitute a major route of excretion in some animals (Dalezios *et al.*, 1973; Luthy *et al.*, 1980) and the observation of aflatoxin metabolites in human faeces at concentrations as high as 123 ng/g (Shank *et al.*, 1971) point to the need for additional study of this excretory route in humans. The findings of Luthy *et al.* (1980) must be taken into consideration. These workers showed that only a small percentage of faecal aflatoxins in pigs was extractable with methylene chloride, suggesting that most

of the aflatoxin was present as a conjugate or strongly bound to faecal components.

The results of the liver analyses show that aflatoxin exists in the liver in its unmetabolized AFB_1 form. This finding is in agreement with other studies (Phillips *et al.*, 1976; Onyemelukwe *et al.*, 1980; Stora *et al.*, 1981) which also observed only AFB_1. More recently, however, AFL and AFM_1 were also detected in human liver (Lumplugh and Hendrickse, 1982). The concentrations of AFB_1 detected in the present study are comparable with those of Onyemelukwe *et al.*, 1980 and Stora *et al.*, 1981 although much higher levels have been reported: Shank *et al.* (1971) detected 93 ng/g in the livers of EFDV subjects and Phillips *et al.* (1976) found 520 ng/g in the liver of a man who had suffered from cancer of the liver and rectum. In addition, it has been observed in pigs dosed with AFB_1, that less than 0.2 per cent of aflatoxin present in the liver was soluble in methylene chloride (Luthy *et al.*, 1980), suggesting that the actual concentrations of aflatoxin in human liver could be much greater than those reported in the present study.

The finding that the liver of a PHC subject contained over ten times more AFB_1 than the highest level observed in controls may be significant. The presence of AFB_1 in the livers of control subjects probably reflects recent exposure but may be indicative of accumulation in the PHC case. This subject would have been extremely anorexic for a number of weeks prior to his death and exposure to aflatoxin would have been unlikely during this time. This observation is consistent with the work of Onyemelukwe *et. al.* (1980) in Nigeria who detected AFB_1 in hepatic tissue of liver cancer subjects but not in controls. Stora *et al.* (1981) also reported AFB_1 in liver tissue of PHC subjects

but no controls were included, making it impossible to compare the two categories. It was unfortunate in the present study that no significant comparisons could be made between liver disease and control subjects due to the limited number of liver samples from PHC subjects. Whether the accumulation of AFB_1 in liver tissue is causally related to PHC or is a consequence of the disease cannot be determined. Studies that demonstrate the integration of AFB_1 into DNA of tumour tissue are required for more conclusive evidence of the role of aflatoxin in PHC.

Aflatoxin was detected significantly more often in livers obtained from males than females ($P < 0.05$) and, at U.T.H., only urine samples collected from males were positive for aflatoxin, although this finding was not significant at $P < 0.05$. Males generally eat more food and drink more alcoholic beverages than females and therefore would have greater exposure to aflatoxins. This is believed to explain, in part, the male preponderance of PHC (Harris and Sun, 1984) and the present study supports this idea. However, a number of studies using rats have demonstrated that males, when concurrently subjected to the same dosing protocol as females, are more sensitive to the carcinogenic action of aflatoxin (Wogan and Newberne, 1967; Butler and Barnes, 1968; Prince and Campbell, 1982). Moreover, it has been observed that testosterone administered to females and castrated males abolishes this sex difference (Gurtoo and Motycka, 1976). Mainigi and Campbell (1981) have suggested that this female resistance could be related to their lower capacity to transport AFB_1 into or within the liver cell to the ultimate receptor site(s) and to metabolize AFB_1 to its 2,3-epoxide.

The mean age of presentation of PHC is 42 years in Zambia, however no pattern was observed between aflatoxin and age in the present study. Generally, excretion of aflatoxin metabolites was seen more frequently in younger patients, aged 16-25 years, and in older patients, over the age of 50 years. The fact that, in areas of PHC prevalence, the age peak is in approximately the fourth decade of life is difficult to understand but it is believed that the aflatoxin hypothesis, rather than the HBV hypothesis, more logically explains this observation, either through increased exposure to the carcinogen earlier in life or differences in age susceptibility to a standard dose (Van Rensburg, 1977; Harris and Sun, 1984).

The finding that the likelihood of consuming aflatoxin was significantly higher in the rainy season was expected and is in agreement with previous work carried out in Zambia (Lovelace *et al.*, 1983) and elsewhere. In Nigeria, where aflatoxin levels in food were found to be highest during the rainy season, a seasonal variation in the rate of PHC has also been observed (Okonkwo and Obionu, 1981). Although these authors do not attribute this variation to seasonal fluctuations in the aflatoxin levels of foods, they do point out a study showing the lack of a seasonal pattern of hepatitis in Nigeria (Okum, 1977). Evidence of a seasonal fluctuation in the rate of PHC diagnosis has also been observed in Mozambique (Purves, 1973; Van Rensburg, 1977). This implies a seasonal variation of exposure to the carcinogen involved and these authors feel the carcinogen is likely dietary. Therefore, the finding in the present study that aflatoxin exposure shows a seasonal fluctuation may be significant in the etiology of PHC.

The HBV carrier state of the Zambian population included in this study was observed to be exceedingly high: 32 per cent of control patients were shown to have HBsAg in their serum. Zuckerman (1982) has stated that surveys conducted in many parts of the world have shown that the prevalence of HBsAg in the serum of healthy persons varies from 0.1 - 20 per cent. The HBsAg rate of controls in the present study is higher than HBsAg values given for numerous other countries (Table 1.8) and is also considerably higher than the rate of 14 per cent reported in a previous study carried out in Lusaka (Zumla and Voller, 1982). The prevalence of anti-HBs in the serum of control patients in this study was also greater than values reported by workers elsewhere (Table 1.8) and was again higher than the previously reported rate of 54 per cent in Lusaka (Zumla and Voller, (1982). In addition, in the present study there were 16 persons in whom HBsAg and anti-HBs co-existed, perhaps indicating double infection with HBV of different subtypes. The finding that 78 per cent of liver samples collected at autopsy from controls with no signs of liver pathology had HBsAg again indicates the extraordinary prevalence of HBV in the Zambian population.

The HBV carrier state is known to be more common in males (Zuckerman, 1982) and the findings of this study are in agreement with this. Forty-two per cent of male control patients were positive for HBsAg compared with only 10 per cent of female controls (Table 3.8b). In addition, during the year long survey, more cases of hepatitis were seen in males than females: 37 males compared with 12 females. This is believed by some workers to explain, in part, the male preponderance of PHC and the present study supports this idea.

In countries where infection with HBV is common, the highest prevalence of HBsAg is observed in children 4-8 years old, with declining rates among older age groups. In the present study this was not observed (Table 3.8a), although this observation was based on a limited number of patients and may not be valid.

It has been well documented that chronic HBV infection is significantly more prevalent in PHC patients compared with controls (Section 1.9.2.). In this study it was not possible to make this comparison due to the limited number of PHC cases examined. However this correlation has been reported previously in Zambia. Zumla and Voller (1982) found HBsAg in 60 per cent of patients with PHC compared to 14 per cent of healthy controls, demonstrating a highly significant association between HBsAg and PHC ($P < 0.01$).

It is generally believed that both HBV and aflatoxin influence PHC rates. One hypothesis formulated to explain how these two factors interact is that aflatoxin exposure occurs first and, by causing immunosuppression, determines the community carrier rate for chronic HBV infections (Lutwick, 1979). Alternatively aflatoxin exposure, after HBV infection is established, may precipitate malignant change. In the present study it was demonstrated that the Zambian population is exposed relatively frequently to aflatoxin in their diet and it was clearly observed that the HBV carrier rate for this community is very high. However no clear relationship between the presence of HBV markers, aflatoxin metabolites excreted and PHC could be observed. It should be noted though, that four of the five patients shown to be excreting urinary aflatoxin were also tested for serological HBV markers and that

all of these patients had evidence of HBV infection (HBsAg, anti-HBs or both). Ideally a longitudinal, prospective study should now be carried out to study the possible interactions of these two etiological factors more closely.

REFERENCES

Adamson, R.H., Correa, P. and Dalgard, D.W. 1973. 'Occurrence of a primary liver carcinoma in a rhesus monkey fed aflatoxin B_1,' J. Natl. Cancer Inst. 50, 549-552.

Adekunle, A.A., Hayes, J.R. and Campbell, T.C. 1977. 'Interrelationships of dietary protein level aflatoxin B_1 metabolism and hepatic microsomal epoxide hydrolase activity,' Life Sci. 21, 1785-1792.

Alpert, M.E., Hutt, M.S.R. and Davidson, C.S. 1969. 'Primary hepatoma in Uganda. A prospective clinical and epidemiologic study of forty-six patients', Am. J. Med. 46, 794-802.

Alpert, M.E., Hutt, M.S.R., Wogan, G.N. and Davidson, C.S. 1971. 'Association between aflatoxin content of food and hepatoma frequency in Uganda,' Cancer 28, 253-260.

Amla, I., Kamala, C.S., Gopalakrishna, G.S., Jayaraj, A.P., Screenivasamurthy, V. and Parpia, H.A.B. 1971. 'Cirrhosis in children from peanut meal contaminated by aflatoxin,' Am. J. Clin. Nutr. 24, 609-614.

Amla, I., Shyamala, K., Screenivasamurthy, V., Jayaraj, A.P. and Parpia, H.A.B. 1970. 'Role of aflatoxin in Indian Childhood Cirrhosis,' Indian Pediat. 7, 262-266.

Amstad, P., Levy, A., Emerit, I. and Cerutti, P. 1984. 'Evidence for membrane-mediated chromosomal damage by aflatoxin B_1 in human lymphocytes,' Carcinogenesis 5, 719-723.

Anthony, P.P. 1977. 'Cancer of the liver: pathogenesis and recent aetiological factors,' Trans. Roy. Soc. Trop. Med. Hyg. 71, 466-470.

Asplin, F.D. and Carnaghan, R.B.A. 1961. 'The toxicity of certain groundnut meals for poultry with special reference to their effect on ducklings and chickens,' Vet. Record 73, 1215-1219.

Autrup, H. 1982. 'Carcinogen metabolism in human tissues and cells,' Drug Metabolism Reviews 13, 603-646.

Autrup, H. Bradley, K., Shamsuddin, K. Wakhisi, J. and Wasunna, A. 1983. 'Detection of putative adduct with characteristics identical to 2,3-dihydro-2-(7'-guanyl)-3-hydroxyaflatoxin B_1 in human urine collected in Murang'a district, Kenya,' Carcinogenesis 4, 1193-1195.

Ayres, I.L., Lee, D.J., Wales, J.H. and Sinnhuber, R.O. 1971. 'Aflatoxin structure and hepatocarcinogenicity in rainbow trout,' J. Natl. Cancer Inst. 46, 561-564.

Bagshawe, A.F., Gacengi, D.M., Cameron, C.H., Dorman, J. and Dane, D.S. 1975. 'Hepatitis B surface antigen and liver cancer - A population based study in Kenya,' Br. J. Cancer 31, 581-584.

Bagshawe, A.F., Parker, A.M. and Jindani, A. 1971. 'Hepatitis-associated antigen in liver disease in Kenya,' Brit.Med. J. 1, 88-89.

Bassendine, M.F., Arborgh, B.A.M., Shipton, N., Monjardino, I., Aranguibel, F., Thomas, H.C. and Sherlock, S. 1980. 'Hepatitis B surface antigen and alphafoetoprotein secreting human primary liver cancer in athymic mice,' Gastroenterology 79, 528-532.

Bayley, A.C. 1979. 'The aetiology and prevention of hepatocellular carcinoma,' Proceedings of the National Seminar on Environment and Development, D.S. Johnson and W. Roder (eds.) Zambia Geographical Association, Lusaka, 243-251.

Bayley, A.C. and Cairns, J.M. 1980. 'The aetiology and prevention of hepatocellular carcinoma,' Proceedings of the Association of Surgeons of East Africa, 3, 63-66.

Beasley, R.P., Lin, C.C., Hwang, L.Y. and Chien, C.S. 1981. 'Hepatocellular carcinoma and hepatitis B virus - A prospective study of 22,707 men in Taiwan,' Lancet 2 1129-1132.

Becroft, D.M.O. 1966. 'Syndrome of encephalopathy and fatty degeneration of viscera in New Zealand children,' Brit. Med. J. 2:135-140.

Becroft, D.M.O. and Webster, D.R. 1972. 'Aflatoxins and Reye's Syndrome,' Brit. Med. J. 4, 117.

Bennett, R.A., Essigmann, J.M. and Wogan, G.N. 1981. 'Excretion of an aflatoxin-guanine adduct in the urine of aflatoxin B_1-treated rats,' Cancer Res. 41, 650-654.

Blount, W.P. 1961. 'Turkey "X" disease,' Turkeys 9, 55-58.

Blumberg, B.S. 1980. 'The hepatitis B virus,' Landmarks in American Epidemiology 95, 427-435.

Booth, S., Bosenberg, H., Garner, R., Hertzog, P. and Norpoth, K. 1981. 'The activation of aflatoxin B_1 in liver slices and in bacterial mutagenicity assays using livers from different species including man,' Carcinogenesis 2, 1063-1068.

Bourgeois, C.H., Shank, R.C., Grossman, R.A., Johnsen, D.O., Woodling, W.L. and Chandavimol, P. 1971. 'Acute aflatoxin toxicity in the macaque and its similarities to Reye's Syndrome,' Lab. Invest. 24, 206-216.

Brechot, C., Pourcell, C., Louisa, A., Rain, B. and Tiollais, P. 1980. 'Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma,' Nature 286, 533-535.

Buchi, G.H., Muller, P.M., Roebuck, B.D. and Wogan, G.N. 1974. 'Aflatoxin Q_1: A major metabolite of aflatoxin B_1 produced by human liver,' Res. Commun. Chem. Pathol. Pharmacol. 8, 585-592.

Buchi, G., Spitzner, D., Paglialunga, S. and Wogan, G.N. 1973. 'Synthesis and toxicity evaluation of aflatoxin P_1,' Life Sci. 13, 1143-1149.

Bulatao-Jayme, J., Almero, E.M., Castro, C.A., Jardeleza, T.R. and Salamat, L.A. 1982. 'A case control dietary study of primary liver cancer risk from aflatoxin exposure,' International Journal of Epidemiology 11, 112-119.

Butler, W.H. and Barnes, J.M. 1966. 'Carcinoma of the glandular stomach in rats given diets containing aflatoxin,' Nature 209, 90.

Butler, W.H. and Barnes, J.M. 1968. 'Carcinogenic action of groundnut meal containing aflatoxin in rats,' Fd. Cosmet. Toxicol. 6, 135-141.

Campbell, T.C., Caedo, J.P. Jun., Bulatao-Jayme, J., Salamat, L.A. and Engel, R.W. 1970. 'Aflatoxin M_1 in human urine,' Nature 227, 403-404.

Campbell, T.C. and Hayes, J.R. 1976. 'The role of aflatoxin metabolism in its toxic lesion - A review article,' Toxicol. Appl. Pharmacol. 35, 199-222.

Campbell, T.C. and Stoloff, L. 1974. 'Implications of mycotoxins for human health,' J. Agric. Food Chem. 22, 1006-1015.

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

Chandra, R.K. and Seth, V. 1972. 'The enigma of childhood cirrhosis in India,' Clin. Pediat. 2, 128-130.

Ch'ih, J., Lin, T. and Deulin, T. 1983. 'Activation and deactivation of aflatoxin B_1 in isolated rat hepatocytes,' Biochem. Biophys. Res. Comm. 110, 668-674.

Coker, R.D. 1979. 'Aflatoxins: Past, present and future,' Trop. Sci. 21, 143-162.

Coulter, J.B.S., Lamplugh, S.M. and Omer, M.I.A. 1984. 'Aflatoxins, in human breast milk,' Ann. Trop. Paediat. 4, 61-66.

Croy, R.G. and Wogan, G.N. 1981. 'Temporal patterns of covalent DNA adducts in rat liver after single and multiple doses of aflatoxin B_1,' Cancer Res. 41, 197-203.

Dalezios, J.I., Hsieh, D.P.H. and Wogan, G.N. 1973. 'Excretion and metabolism of orally administered aflatoxin B_1 by rhesus monkeys', Fd. Cosmet. Toxicol. 2, 605-616.

Dalezios, J.I. and Wogan, G.N. 1972. 'Metabolism of aflatoxin B_1 in rhesus monkeys,' Cancer Res. 32, 2297-2303.

Dalezios, J., Wogan, G.N. and Weinreb, S.M. 1971. 'Aflatoxin P_1: A new aflatoxin metabolite in monkeys,' Science 171, 584-585.

Dann, R.E., Mitscher, L.A. and Couri, D. 1972. 'The *in vivo* metabolism of ^{14}C-labelled aflatoxins B_1, B_2, G_1 in rats,' Res. Commun. Chem. Pathol. Pharmacol. 3, 667-675.

Degen, G.H. and Neumann, H.G. 1981. 'Differences in aflatoxin B_1 susceptibility of rat and mouse are correlated with the capability *in vitro* to inactivate AFB_1-epoxide,' Carcinogenesis 2, 299-306.

Deger, G.E. 1976. 'Aflatoxin-Human colon carcinogenesis?', Ann. Intern. Med. 85, 204.

de Iongh, H., Vles, R.O. and Van Pelt, I.G. 1964. 'Milk of mammals fed on aflatoxin-containing diet,' Nature 202, 466-467.

Deo, M.G., Dayal, Y. and Ramalingaswami, V. 1970. 'Aflatoxins and liver injury in the rhesus monkey,' J. Path. 101, 47-56.

Desmyter, J. Ray, M.B. Bradburne, A.F. and Alexander, J.J. 1978. 'Human HBsAg-positive hepatoma in nude athymic mice,' In Viral Hepatitis: A Contemporary Assessment of Etiology, Epidemiology, Pathogenesis and Prevention G.N. Vyas, S.N. Cohen and R. Schmid (eds.) Philadelphia: The Franklin Institute Press, pp. 459-463.

Dvorackova, I. 1976. 'Aflatoxin inhalation and alveolar cell carcinoma,' Brit. Med. J. 1, 691.

Dvorackova, I., Brodsky, F. and Cerman, J. 1974. 'Aflatoxin and Encephalitic syndrome with fatty degeneration of viscera,' Nutr. Rep. Int. 10, 89-102.

Dvorackova, I., Kusak, V., Vesely, D., Vesela, J. and Nesnidal, 1977. 'Aflatoxin and encephalopathy with fatty degeneration of viscera (Reye's Syndrome),' Ann. Nutr. Alim. 31, 977-990.

Dvorackova, I., Stora, C. and Ayraud, N. 1981. 'Evidence for aflatoxin B_1 in two cases of lung cancer in man,' J. Cancer Res. Oncol. 100, 221-224.

Edds, G.T., Nair, K.P.C. and Simpson, C.F. 1973. 'Effects of aflatoxin B_1 on resistance in poultry against cecal coccidiosis and Marek's disease,' Am. J. Vet. Res. 34, 819-826.

Emerole, G.O. 1981. 'Excretion of aflatoxin B_1 as a glutathione conjugate,' European Journal of Drug Metabolism and Pharmacokinetics 6, 265-268.

Emerole, G.O., Neskovic, N. and Dixon, R.L. 1979. 'The detoxification of aflatoxin B_1 with glutathione in the rat,' Xenobiotica 9, 737-743.

Essigmann, J.M., Croy, R.G., Bennet, R.A. and Wogan, G.N. 1982. 'Metabolic activation of aflatoxin B_1: Patterns of DNA adduct formation removal and excretion in relation to carcinogenesis,' Drug Metabolism Reviews 13, 581-602.

Garner, R.C., Martin, C.N., Lindsay Smith, J.R., Coles, B.F. and Tolson, M.R. 1979. 'Comparison of aflatoxin B_1 and aflatoxin G_1 binding to cellular macromolecules *in vitro, in vivo* and after peracid oxidation,' characterization of the major nucleic acid adducts, Chem. Biol. Interact. 26, 57-73.

Garner, R.C. and Wright, C.M. 1975. 'Binding of [^{14}C] aflatoxin B_1 to cellular macromolecules in the rat and hamster,' Chem. Biol. Interact. 11, 123-131.

Gibson, J.B. 1971. 'Parasites, liver disease and liver cancer. Liver Cancer,' IARC Scientific Publications No. 1 (Lyon: WHO), 42-50.

Gopalan, C., Tulpule, P.G. and Krishnamurthi, D. 1972. 'Induction of hepatic carcinoma with aflatoxin in the Rhesus Monkey,' Food Cosmet. Toxicol. 10, 519-521.

Gregory, J., Goldstein, S. and Edds, G. 1983. 'Metabolite distribution and rate of residue clearance in turkeys fed a diet containing aflatoxin B_1,' Fd. Chem. Toxic. 21, 463-467.

Groopmann, J.D., Busby, W.F. and Wogan, G.N. 1980. 'Nuclear distribution of aflatoxin B_1 and its interaction with histones in rat liver *in vivo*,' Cancer Res. 40, 4343-4351.

Gurtoo, H.L. and Dahms, R. 1974. 'On the nature of the binding of aflatoxin B_{2a} to rat hepatic microsomes,' Res. Commun. Chem. Pathol. Pharmacol. 9, 107-118.

Gurtoo, H.L. and Motycka, L.E. 1976. 'Effect of sex difference on the *in vitro* and *in vivo* metabolism of aflatoxin B_1 by the rat,' Cancer Res. 36, 4663-4671.

Halver, J.E. 1965. 'Aflatoxicosis and rainbow trout hepatoma,' In Mycotoxins in Foodstuffs, G.N. Wogan (ed.) (Massachusetts Institute of Technology Press), pp. 209-234.

Hamilton, P.B. 1977. Federation Proceedings 36, 1899.

Harris, C.C. and Sun, T. 1984. 'Multifactoral etiology of human liver cancer - Commentary,' Carcinogenesis 5, 697-701.

Hartley, R.C., Nesbitt, B.F. and O'Kelly, J. 1963. 'Toxic metabolites of *Aspergillus flavus*,' Nature 198, 1056-1058.

Heathcote, J.G. and Hibbert, J.R. 1978. Aflatoxins: Chemical and Biological Aspects. Amsterdam: Elsevier Scientific Publishing Company, p. 142.

Hendrickse, R.G. 1984. 'The influence of aflatoxins on child health in the tropics with particular reference to kwashiorkor,' Trans. Roy.Soc.Trop.Med.Hyg. 78, 427-435.

Hendrickse, R.G., Coulter, J.B.S., Lamplugh, S.M., MacFarlane, S.B.J., Williams, T.E., Omer, M.I.A. and Suliman, G.I. 1982. 'Aflatoxins and kwashiorkor: A study in Sudanese children,' Brit. Med. J. 285, 843-846.

Hendricks, J.D., Sinnhuber, R.O., Nixon, J.E., Wales, J.H., Masri, M.S. and Hsieh, D.P.H. 1980. 'Carcinogenic response of rainbow trout (*Salmo gairdneri*) to aflatoxin Q and L, synergistic effect of cyclopropenoid fatty acids,' J. Natl. Cancer. Inst. 64, 523-527.

Higginson, J. and Oettle, A.G. 1960. 'Cancer incidence in the Bantu and 'Cape coloured' races of South Africa,' J. Natl. Cancer. Inst. 24, 509-512.

Hsieh, D.P.H., Beltran, L.M. and Fukayama, M. 1981. 'Analytical procedures for aflatoxin M_1 in dairy products,' In Workshop on Mycotoxin Analytical Methods, Cairo, Egypt, September 9-16, 1981.

Hsieh, D.P.H., Salhab, A.S. Wong, J.J. and Yang, S.L. 1974. 'Toxicity of aflatoxin Q_1 as evaluated with the chicken embryo and bacterial auxotrophs,' Toxicol. Appl. Pharmacol. 30, 237-242.

Hsieh, D.P.H., Wong, Z.A., Wong, J.J., Michas, C. and Ruebner, B.G. 1977. 'Comparative metabolism of aflatoxin,' In Mycotoxins in Human and Animal Health. Park Forest South, III.: Pathotox Publishers Inc., pp. 37-50.

IARC Monographs, 1975. The Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Naturally Occurring Substances Volume 10, 51-71.

Jayaraj, A. and Richardson, A. 1981. 'Metabolic activation of aflatoxin B_1 by liver tissue from male Fischer F344 rats of various ages,' Mech. Ageing. Dev. 17, 163-171.

Kamden, L., Siest, G., Magdalou, J. 1982. 'Differential toxicity of aflatoxin B_1 in male and female rats: relationship with hepatic drug - metabolizing enzymes,' Biochem. Pharmacol. 31, 3057-3062.

Keen, P. and Martin, P. 1971. 'Is aflatoxin carcinogenic in man? The evidence in Swaziland,' Trop. Geog. Med. 23, 44-53.

Kew, M.C., Dos Santos, H.A., Sherlock, S. 1971. 'Diagnosis of primary cancer of the liver,' Brit. Med. J. 4, 408-411.

Kew, M.C., Rossouw, E., Hodkinson, J., Paterson, A., Pusheiko, G.M. and Whitcutt, J.M. 1963. 'Hepatitis B virus status of Southern African blacks with hepatocellular carcinoma: Comparison between rural and urban patients,' Hepatology 3, 65-68.

Krishnamachari, K.A.V.R., Bhat, R.V., Nagarajan, V. and Tilak, T.B.G. 1975. 'Hepatitis due to aflatoxicosis - An outbreak in Western India,' Lancet, 1061-1063.

Lamplugh, S.M. and Hendrickse, R.G. 1982. 'Aflatoxins in the livers of children with kwashiorkor,' Ann. Trop. Paediatr. 2, 101-104.

Larouze, B., Saimot, G., Lustbader, G., London, W.T., Werner, P.G., Payet, M. 1976. 'Host reponses to hepatitis B infection in patients with primary hepatic carcinoma and their families - A case controlled study in Senegal, West Africa,' Lancet 2, 534-538.

Lefkowitch, J.H. 1981. 'The epidemiology and morphology of primary malignant liver tumours,' The Surgical Clinics of North America 61, 169-180.

Lillehoj, E.B., Kwolek, W.F., Manwiller, A., Durant, J.A., Laprade, J.C., Horner, E.S., Reid, J. and Zuber, M.S. 1976. Crop. Sci. 16, 483.

Lin, J.L., Liu, C. and Svoboda, D.J. 1974. 'Long term effects of aflatoxin B_1 and viral hepatitis on marmoset liver - A preliminary report,' Lab. Invest. 30, 267-277.

Linsell, C.A. and Peers, F.G. 1977. 'Aflatoxin and liver cell cancer,' Trans. Roy. Soc. Trop. Med. Hyg. 71, 471-473.

Lovelace, C.E.A., Njapau, H., Bayley, A.C. and Kasobe, L. 1983. 'Aflatoxin metabolites in human urine,' Proceedings of the National Symposium on Mycotoxins. Cairo: Government Printers, pp. 267-270.

Lovelace, C.E.A., Njapau, H., Salter, L.F. and Bayley, A.C. 1982. 'Screening method for the detection of aflatoxin and metabolites in human urine: Aflatoxins B_1, G_1, M_1, B_{2a}, G_{2a}, aflatoxicols I and II,' J. Chromatogr. 227, 256-261.

Lovelace, C.E.A. and Salter, L.F. 1979. 'Environmental factors which may contribute to the causation of hepatocellular carcinoma in Zambia - Analysis of fungal toxins in village food samples,' Proceedings of the National Seminar on Environment and Development, D.S., Johnson and W. Roder (eds.) Zambia Geographical Association, Lusaka, 243-251.

Luthy, J., Zweifel, U. and Schlatter, Ch. 1980. 'Metabolism and tissue distribution of [^{14}C] aflatoxin B_1 in pigs,' Fd. Cosmet. Toxicol. 18, 253-256.

Lutwick, L.I. 1979. 'Relation between aflatoxin, hepatitis B virus, and hepatocellular carcinoma,' Lancet 1, 755-757.

Lutwick, L.I. and Robinson, W.S. 1977. 'DNA synthesized in the hepatitis B Dane particle DNA polymerase reaction,' J. Virol. 21, 96-104.

Lutz, W.K., Jaggi, W., Luthy, J., Sagelsdorff, P. and Schalatter, C. 1980. '*In vivo* covalent binding of aflatoxins B_1 and M_1 to liver DNA of rat, mouse and pig,' Chem. Biol. Interact. 32, 249-256.

Mac Sween, R.N.M. and Scott, A.R. 1973. 'Hepatic cirrhosis. A clinico-pathological study of 520 cases,' J. Clin. Path. 26, 936-942.

Madhavan, T.V. and Gopalan, C. 1965. 'Effect of dietary protein on aflatoxin liver injury in weanling rats,' Arch. Pathol. 80: 123-126.

Madhavan, T.V. and Gopalan, C. 1968. 'The effect of dietary protein on carcinogenesis of aflatoxin,' Arch. Pathol. 85, 133-137.

Mainigi, K.D. and Campbell, T.C. 1981. 'Effect of sex differences on subcellular distribution of aflatoxin in F-344 rats treated with various risk - modifying factors,' Toxicol. Appl. Pharmacol. 58, 236-243.

Masri, M.S., Lundin, R.E., Page, J.R. and Garcia, Mason, W.S., Halpern, M.S. and London, W.T. 1984. 'Hepatitis B virus liver disease and hepatocellular carcinoma,' Cancer Surveys 3, 25-43.

Masri, M.S., Lundin, R.E., Page, J.R. and Garcia, V.C. 1967. 'Crystalline aflatoxin M_1 from urine and milk,' Nature 215, 753-754.

Merrill, A.H. and Campbell, T.C. 1974a. 'Studies on the excretion of aflatoxin and its derivatives by humans,' Toxicol. Appl. Pharmacol. 89, 94.

Merrill, A.H. and Campbell, T.C. 1974b. 'Preliminary study of *in vitro* aflatoxin B_1 metabolism by human liver,' Toxicol. Appl. Pharmacol. 27, 210-213.

Messripour, M. and Nesheim, S. 1977. Arch. Inst. Pasteur Tunis 54, 363-371.

Muhihu, S.K. 1978. 'Grain moisture and microbial infections (including *Aspergillus*) in stored produce,' The Kenya Entomologist's Newsletter No. 8, 7-9.

Nassar, A.Y., Megalla, S.E., Abd-Fattah, H.M., Hafez, A.H. and El-Deap, T.S. 1982. 'Binding of aflatoxins B_1, G_1 and M_1 to plasma albumin,' Mycopathologia 79, 35-38.

Neal, G.E., Judah, D.J., Stirpe, F. and Patterson, D.S.P. 1981. 'The formation of 2,3-dihydroxy -2,3-dihydro-aflatoxin B_1 by the metabolism of aflatoxin B_1 by liver microsomes isolated from certain avian and mammalian species and the possible role of this metabolite in the acute toxicity of aflatoxin B_1,' Toxicol. Appl. Pharmacol. 58, 431-432.

Newberne, P.M. and Butler, W.H. 1969. 'Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: A review,' Cancer Res. 29, 236-244.

Newberne, P.M. and Rodgers, A.E. 1973. 'Rat colon carcinomas associated with aflatoxin and marginal vitamin A,' J. Natl. Cancer Inst. 50, 439-448.

Ngindu, A., Kenya, P., Ocheng, D., Omondi, T., Ngare, W., Gatei, D., Johnson, B., Ngira, J., Nandwa, H., Jansen, A., Kaviti, J. and Siongok, T. 1982. 'Outbreak of acute hepatitis caused by aflatoxin poisoning in Kenya,' Lancet 1, 1346-1348.

Niranjan, B.G., Bhat, N.K. and Avadhani, N.G. 1982. 'Preferential attack of mitochondrial DNA by aflatoxin B_1 during hepatocarcinogenesis,' Scienc 215, 73-75.

O'Brien, K., Moss, E., Judah, D. and Neal, G. 1983. 'Metabolic basis of the species difference to aflatoxin B_1-induced hepatotoxicity,' Biochem. Biophys. Res. Comm. 114, 813-821.

Oettle, A.G. 1965. 'The aetiology of primary carcinoma of the liver in Africa: A critical appraisal of previous ideas with an outline of the mycotoxin hypothesis,' S. Afr. Med. J. 39, 817-825.

Official Methods of Analysis 1980. Washington: Association of Official Analytical Chemists, Chapter 26.

Ohbayashi, A., Okochi, K. and Mayumi, M. 1972. 'Familial clustering of asymptomatic carriers of Australia antigen and patients with chronic liver disease or primary liver cancer, Gastroenterology 62, 618-625.

Okonkwo, P.O. and Obionu, C.N. 1981. 'Implications of seasonal variations in aflatoxin B_1 levels in Nigerian market foods,' Nutr. Cancer 3, 35-39.

Okum, G.E.O. 1977. 'Reported cases of infectious diseases in the East Central State of Nigeria since the end of the Civil War,' Niger. *Med. J. 7, 388-393.*

Onyemelukwe, C.G., Nirodi, C. and West, C.E. 1980. 'Aflatoxin B_1 in hepatocellular carcinoma,' Trop. Geogr. Med. 32, 237-240.

Patterson, D.S.P., Glancy, E.M. and Roberts, B.A. 1980. 'The "Carry Over" of aflatoxin M_1 into the milk of cows fed rations containing a low concentration of aflatoxin B_1,' Fd. Cosmet. Toxicol. 18, 35-37.

Patterson, D.S.P. and Roberts, B.A. 1970. 'The formation of aflatoxins B_{2a} and G_{2a} and their degradation products during the *in vitro* detoxification of aflatoxin by livers of certain avian and mammalian speices,' Food Cosmet. Toxicol. 8, 527-538.

Payet, M., Camain, R. and Pene, P. 1956. 'Le cancer primitif du foie; etude critique a propos de 240 cas,' Rev. Intern. d' Hepatol. 6, 1-86.

Peers, F.G. 1980. 'Aflatoxin in relation to the epidemiology of human liver cancer,' In Medical Mycology, Preusser (eds.), Zbl. Bakt. Suppl. 8, Gustav Fischer Verlag, Stuttgart, New York. pp. 279-289.

Peers, F.G., Gilman G.A. and Linsell, C.A. 1976. 'Dietary aflatoxins and human liver cancer,' Int. J. Cancer 17, 167-176.

Peers, F.G. and Linsell, C.A. 1973. 'Dietary aflatoxins and liver cancer - A population based study in Kenya,' Br. J. Cancer 27, 473-484.

Phillips, D.L., Yourtee, D.M. and Searles, S. 1976. 'Presence of aflatoxin B_1 in human liver in the United States,' Toxicol. Appl. Pharcacol. 36, 403-406.

Prates, M.D. and Torres, F.O. 1965. 'A cancer survey in Lourenco Marques, Portugese East Africa,' J. Natl. Cancer Inst. 35, 729-734.

Prince, A.M. 1978. 'Human HBsAg-positive hepatoma in nude athymic mice', In Viral Hepatitis: A Contemporary Assessment of Etiology, Epidemiology, Pathogenesis and Prevention. G.N. Vyas, S.N. Cohen and R. Schmid (eds.) Philadelphia: The Franklin Institute Press, P. 460.

Prince, A.M. 1981. 'Hepatitis B virus and hepatocellular carcinoma: Molecular biology provides further evidence for an etiologic association,' Hepatology 1, 73-75.

Prince, L.O. and Campbell, T.C. 1982. 'Effects of sex difference and dietary protein on the binding of aflatoxin B_1 to rat liver chromatin proteins *in vivo*,' Cancer Res. 42, 5053-5059.

Purchase, I.F.H. and Goncalves, T. 1971. 'Preliminary results from food analysis in the Inhambane area,' In Mycotoxins in Human Health. London: Macmillan Press, pp. 263-269.

Purves, L.R. 1973. 'Primary liver cancer in man as a possible short duration seasonal cancer,' S. Afr. J. Sci. 69, 173-178.

Quisenberry, W.B. 1960. 'Sociocultural factors in cancer in Hawaii,' Ann. New York Acad. Sci. 84, 795.

Raj, H.G., and Lotlikar, P.D. 1984. 'Urinary excretion of thiol conjugates of aflatoxin B_1 in rats and hamsters,' Cancer Letters, 22, 125-133.

Reddy, J.K. and Svoboda, D.J. 1975. 'Aflatoxin B_1-induced liver tumours in *Tupaia glis* (tree shrews), a non-human primate,' Fed. Proc. 34, 827.

Richard, J.L. 1978. In Toxins: Animal, Plant and Microbial. P. Rosenberg (ed.) Oxford: Pergamon, P. 810.

Robinson, P. 1967. 'Infantile cirrhosis of the liver in India with special reference to probable aflatoxin etiology,' Clin. Pediat. 6, 57-62.

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

Rohring, T.P. and Yourtee, D.M. 1983. '*In vitro* metabolism of aflatoxin Q_1 by rat liver post-mitochondrial homogenates,' Res. Comm. Chem. Path. Pharmacol. 40, 457-464.

Sargeant, K., Sheridan, A., O'Kelly, J. and Carnaghan, R.B.A. 1961. 'Toxicity associated with certain samples of groundnuts,' Nature 192, 1096-1097.

Schuller, P.L. and Van Egmond, H.P. 1981. 'Detection and determination of mycotoxins (in particular aflatoxins) in food and feed. A concise manual of thin layer chromatography laboratory techniques. In Workshop on Mycotoxin Analytical Methods, Cairo, Egypt, September 9-16, 1981.

Schuller, P.L., Verhulsdonk, C.A.H. and Paulsch, W.E. 1973. 'Analysis of aflatoxin M_1 in liquid and powdered milk,' Pure Appl. Chem. 35, 291-296.

Serck-Hansen, A. 1970. 'Aflatoxin-induced fatal hepatitis? A case report from Uganda,' Arch. Environ. Health 20, 729-731.

Shafritz, D.A. and Kew, M.C. 1981. 'Identification of hepatocellular carcinoma,' Hepatology 1, 1-18.

Shafritz, D.A., Shouval, D., Sherman, H.I., Hadziyannis, S.J. and Kew, M.C. 1981. 'Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma: studies in percutaneous liver biopsies and *post mortem* tissue specimens,' New Eng. J. Med. 305, 1067-1073.

Shank, R.C. 1977. 'Metabolic activation of mycotoxins by animals and humans: An overview,' J. Toxicol. Environ. Health 2, 1229-1244.

Shank, R.C., Bourgeois, C.H., Keschamras, N. and Chandaviomol, P. 1971. 'Aflatoxins in autopsy specimens from Thai children with an acute disease of unknown aetiology,' Fd. Cosmet. Toxicol. 9, 501-507.

Shank, R.C., Gordon, J.E. Wogan, G.N., Nondasuta, A. and Subhamani, B. 1972. 'Dietary aflatoxins and human liver cancer. Field survey of rural Thai families for ingested aflatoxins,' Fd. Cosmet. Toxicol. 10, 71-84.

Skinhoj, P., Hart Hanse, J.P., Nielsen, N.H. and Mikkelsen, F. 1978. 'Occurrence of cirrhosis and primary liver cancer in an Eskimo population hyper endemically infected with hepatitis B virus,' Am. J. Epidemio. 108, 121-125.

Steiner, P.E. and Davis, J.N.P. 1957. 'Cirrhosis and primary liver carcinoma in Uganda, Africa,' Br. J. Cancer 11, 523-534.

Stora, C., Dvorackova, I. and Ayrand, N. 1981. 'Characterization of aflatoxin B_1 in human liver cancer,' Res. Comm. Chem. Pathol. Pharmacol. 31, 77-84.

Stubblefield, R.D. 1979. 'Rapid method for the determination of aflatoxin M_1 in dairy products,' J. Amer. Oil Chemists' Soc. 56, 800-803.

Stubblefield, R.D., Kwolek, W.F. and Stoloff, L. 1982. 'Determination and thin layer chromatographic confirmation of identity of aflatoxins B_1 and M_1 in artificially contaminated beef livers: Collaborative study,' J. Assoc. Off. Anal. Chem. 65, 1435-1444.

Stubblefield, R.D. and Shotwell, O.L. 1981. 'Rapid method for the determination of aflatoxins in animal tissues,' J. Assoc. Off. Anal. Chem. 64, 964-968.

Summers, J. and Mason, W.S. 1982. 'Properties of the hepatitis B liver viruses related to toxonomic classification,' Hepatology 2, 615-665.

Swenson, D.H., Lin, J.K., Miller, E.C. and Miller, J.A. 1977. 'Aflatoxin B_1-2,3-oxide as a probable intermediate in the covalent binding of aflatoxins B_1 and B_2 to rat liver DNA and ribosomal RNA *in vivo*,' Cancer Res. *37, 172-181.*

Swenson, D.H., Miller, E.C. and Miller, J.A. 1974. 'Aflatoxin B_1-2,3-oxide, evidence for its formation in rat liver *in vivo* and by human liver microsomes *in vitro*,' Biochem. Biophys. Res. Commun. 60, 1036-1043.

Swenson, D.H., Miller, J.A. and Miller, E.C. 1975. 'The reactivity and carcinogenicity of aflatoxin B_1-2,3-dichloride, a model for the putative 2,3-oxide metabolite of aflatoxin B_1,' Cancer Res. 35, 3811-3823.

Szmuness, W., Stevens, C.E., Harley, E.J. and Zang, E.A. 1980. 'Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high risk population in the United States,' New Eng. J. Med. 303, 833-841.

Tabor, E., Gerety, R.J., Vogel, C.L., Bayley, A.C., Anthony, P.P., Chan, C.H. and Barker, L.F. 1977. 'Hepatitis B infection and primary hepatocellular carcinoma,' J. Natl. Cancer Inst. 58, 1197-1200.

Temcharoen, P., Anukarahanonta, T. and Bhamarapravati, N. 1978. 'Influence of dietary protein and vitamin B_{12} on the toxicity and carcinogenicity of aflatoxins in rat liver,' Cancer Res. 38, 2185-2190.

Tilak, T.B.G. 1975. 'Induction of cholangiocarcinoma following treatment of a rhesus monkey with aflatoxin,' Fd. Cosmet. 13, 247-249.

Trucksess, M., Richard, J., Stoloff, L. and McDonald, J. 1983. 'Absorption and distribution patterns of aflatoxicol and aflatoxins B_1 amd M_1 in blood and milk of cows given aflatoxin B_1,' Am. J. Vet. Res. 44, 1753-1756.

Tung, H.T., Wyatt, R.D., Thaxton, P. and Hamilton, P.D. 1975. Toxicol. Appl. Pharmacol. 34, 320.

Ueno, I., Friedman, L. and Stone, C. 1980. 'Species differences in the binding of aflatoxin B_1 to hepatic macromolecules,' Toxicol. Appl. Pharmacol. 52, 177-180.

Van Egmond, H.P., Paulsch, W.E. and Schuller, P.L. 1978. 'Confirmatory test for aflatoxin M_1 on a thin layer plate,' J. Assoc. Off. Anal. Chem. 61, 809-812.

Van Egmond, H.P., Sizoo, E.A., Paulsch, W.E. and Schuller, P.L. 1981. 'Rapid method for the semi-quantitative determination of aflatoxin M_1 in milk,' unpublished.

Van Rensburg, S.J. 1977. 'Role of epidemiology in the elucidation of mycotoxins in health,' In Mycotoxins in Human and Animal Health. J.V. Rodricks, C.W. Hesseltine and M.A. Mehlman (eds.) Park Forest South, Ill.: Pathotox Publishers Inc., pp. 699-711.

Van Rensburg, S.J., Kirsipuu, A., Pereira Continho, L. and Van der Watt, J.J. 1975. 'Circumstances associated with the contamination of food by aflatoxin in a high primary liver cancer area,' S. Afr. Med. J. 49, 877-883.

Van Rensburg, S.J., Vander Watt, J.J., Purchase, I.F.H., Pereira Coutinho, L. and Markham, R. 1974. 'Primary liver cancer rate and aflatoxin intake in a high cancer area,' S. Afr. Med. J. 48, 2508a-2508d.

Velasco, M., Sorensen, R., Daiber, A., Carmona, A. and Katz, R. 1971. 'Primary carcinoma of the liver associated with Australia antigen,' Lancet 1, 1183-1184.

WHO. 1977. 'Advances in viral hepatitis,' Technical Report Series No. 602. Geneva: World Health Organization.

WHO. 1983. 'Prevention of liver cancer,' Technical Report Series No. 691. Geneva: World Health Organization.

Wieder, R., Wogan, G.N. and Shimkin, M.B. 1968. 'Pulmonary tumours in strain A mice given injections of Aflatoxin B_1,' J. Natl. Cancer Inst. 40, 1195-1197.

Wogan, G.N. 1973. 'Aflatoxin Carcinogenesis,' In Methods in Cancer Research VII, H. Busch (ed.) New York: Academic Press, pp. 309-344.

Wogan, G.N. and Newberne, P.M. 1967. 'Dose - response characteristics of aflatoxin B_1 carcinogenesis in the rat,' Cancer Res. 27, 2370-2376.

Wolf, H. and Jackson, E.W. 1963. 'Hepatoma in rainbow trout: descriptive and experimental epidemiology,' Science 142, 676-678.

Wong, Z.A., Decad, G.M., Byard, J.L. and Hsieh, D.P.H. 1979. 'Conversion of aflatoxicol to aflatoxin B_1 in rats *in vivo* and in primary hepatocyte culture,' Fd. Cosmet. Toxicol. 17, 481-486.

Wong, J.J. and Hsieh, D.P.H. 1976. 'Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential,' Proc. Natl. Acad. Sci. 73, 2241-2244.

Wong, Z.A. and Hsieh, D.P.H. 1978. 'Aflatoxicol: Major aflatoxin B_1 metabolite in rat plasma,' Science 200, 325-327.

Wong, Z.A. and Hsieh, D.P.H. 1980. 'The comparative metabolism and toxicokinetics of aflatoxin B_1 in the monkey, rat and mouse,' Toxicol. Appl. Pharmocol. 55, 115-125.

Yu, F.L. 1983. 'Preferential binding of aflatoxin B_1 to the transcriptionally active regions of rat liver nucleolar chromatin *in vivo* and *in vitro*,' Carcinogenesis 40, 889-893.

Yu, F.L., Cass, M. and Rokusek, L. 1982. 'Tissue, sex and animal species specificity of aflatoxin B_1 inhibition of nuclear RNA polymerase II activity,' Carcinogenesis 3, 1005-1009.

Zambia Tumour Registry Records, Ministry of Health, Government of the Republic of Zambia.

Zuckerman, A.J. 1977. 'Symposium on liver carcinoma, hepatocellular carcinoma and hepatitis B,' Trans. Roy. Soc. Trop. Med. Hyg. 71, 459-461.

Zuckerman, A.J. 1982. 'Primary hepatocellular carcinoma and hepatitis B virus,' Trans. Roy. Soc. Trop. Med. Hyg. 76, 711-718.

Zumla, A. and Voller, A. 1982. 'A serological study on primary hepatocellular carcinoma in Zambia,' Trans. Roy. Soc. Trop. Med. Hyg. 76, 546-551.