THE OCCURRENCE AND DETECTION OF AFLATOXIN-MACROMOLECULAR CONJUGATES IN HUMANS

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To my father with fond memories and the most important lady in my life - my mother.

ABSTRACT

Aflatoxin B_1 (AFB_1), a highly toxic fungal metabolite (mycotoxin) of certain strains of *Aspergillus,* has long been known to be carcinogenic in animal species. Accumulation of epidemiological evidence led to its classification, in 1993, by the International Agency for Research on Cancer as a Group I human carcinogen. Aflatoxin B_1 contaminates the food supply in most tropical and sub-tropical countries, where it is associated with increased incidence of hepatocellular carcinoma (HCC). In these countries, AFB_1 is also linked to kwashiorkor, jaundice, and Rey's syndrome. The biological action of AFB_1 is through its oxidation to AFB_1-8,9 epoxide (AFB_1O). This epoxide binds to macromolecules like DNA, RNA and proteins as well as amino acids to form AFB_1-macromolecular adducts. Quantitation of these adducts is thought to be the most promising approach in the development of methods to measure levels of exposure to aflatoxins.

Aflatoxin B_1 was produced, isolated and purified using preparative thin layer chromatography (TLC). The toxin was oxidised to AFB_1O using dimethyldioxirane and the UV spectra of both the AFB_1 and AFB_1O were determined. Reaction of selected N_α-acetyl amino acids (AA) with AFB_1O was studied and UV spectrophotometry, TLC, high performance liquid chromatography (HPLC) and high performance capillary electrophoresis (CE) were used to characterise the reaction products. The epoxide was also reacted with albumin and DNA. Aflatoxin B_1-albumin reaction mixture was hydrolysed and characterised by TLC.

Spectrum measurement of the oxidative product of AFB_1 gave peaks at 266 and 367nm. Qualitative TLC and the epoxide spray reagents confirmed that epoxidation was successful. The *in vivo* reaction of selected N_α-acetyl AA with the epoxide gave peaks between 300 and 400 nm. N_α-acetyl-arginine, N_α-acetyl-lysine and N_α-acetyl-histidine showed reaction with AFB_1O with maximum wavelengths at 392, 397 and 391nm respectively. These results strongly suggest that AFB_1O is able to covalently bind to lysine, histidine and arginine in albumin.

A total of twenty nine blood samples were analysed by HPLC for the presence of AFB_1-lysyl adduct. Of the twenty nine samples, ten were from HCC patients, ten from control patients and nine from kwashiorkor patients. The results show that AFB_1-lysine does occur in patients at King Edward VIII Hospital (KEH) and the highest level was detected in HCC patients followed by kwashiorkor patients.

PREFACE

The research described in this study was carried out in the Department of Physiology, Medical Faculty, University of Natal, under the supervision of Professor Michael F. Dutton.

Where use was made of the work of others, it has been duly acknowledged in the text.

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LIST OF ABBREVIATIONS

AFB_1 .... aflatoxin B_1

A_λ .... absorbance at maximum wavelength

AA .... amino acid

AFG_1 .... aflatoxin G_1

AFB_1O .... aflatoxin B_1 epoxide

AFG_2 .... aflatoxin G_2

AFM_1 .... aflatoxin M_1

ARG .... arginine

ASN .... asparagine

BWA .... butanol : water : acetic acid (12:5:3)

CA .... chloroform : acetone (9:1)

CE .... capillary electrophoresis

CEI .... chloroform : ethylacetate : isopropanol (18:1:1)

CTLC .... cellulose thin layer chromatography plate

DNA .... deoxyribonucleic acid

ε_{248} .... extinction coeffitient at 248 nm

HIS .... histidine

HPLC .... high performance liquid chromatography

IWA .... isopropanol : water : ammonia (7:2:1)

LYS .... lysine

PDA .... potato dextrose agar

RPM .... revolutions per minute

SDS .... sodium dodecyl sulphate

SER .... serine

TLC .... thin layer chromatography

TRP .... tryptophan

TYR .... tyrosine

YES .... yeast extract sucrose

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CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

The role of mycotoxins in the well being of man is increasingly receiving attention, because of its negative impact on human nutrition and health (Smith *et al.*, 1995). In KwaZulu-Natal, South Africa, mycotoxins have been found to occur in cereals and animal feedstuffs (Dutton and Westlake 1985; Dutton and Kinsey, 1995). These surveys are consistent with the evidence from different studies that many foods, which are commonly eaten in the tropics and subtropics, e.g., ground nuts, maize, sorghum, rice etc., are contaminated by fungi which produce mycotoxins (Goldblatt, 1969; Nwokolo and Okonkwo, 1978). Furthermore, general screening of home-grown maize and other foodstuffs in South Africa revealed the frequent presence of the mycotoxins: deoxynivalenol, fumonisins, zearalenone and aflatoxins (Dutton *et al.*, 1993).

Studies from other parts of the world have shown that about 1.4 - 2.3% of ingested aflatoxin B_1 (AFB_1) becomes covalently bound to serum albumin (Gan *et al.*, 1988). These protein conjugates can be detected by chromatographic assays. Measurements of aflatoxin bound to serum albumin in children and adults from various countries show that between 12 and 100% of them contain aflatoxin-albumin adducts, with levels up to 350pg AFB_1-lysine equivalent/mg albumin (Wild *et al.*, 1990a). Members of the population in the greater Durban area, South Africa, may be exposed to dietary aflatoxins (Ramjee *et al.*, 1992), which is worrying because AFB_1 is now well known to be a human carcinogen (IARC, 1993).

Cancer is among the most feared diseases in the modern industrialised world and it is often a progressive fatal condition. It is one of the three leading causes of death in most countries. An estimated 80 - 90% of human cancer is linked to "extrinsic" or environmental factors, and its incidence could be minimised if the causative extrinsic factors could be identified and humans protected from them (Doll and Peto, 1981). This can either be achieved by elimination of exposure or by reduction of the host susceptibility. Diet presents a major source of human exposure to environmental carcinogens and epidemiological analyses indicate that 20 - 50% of all human cancer is associated with dietary factors (Wynder and Gori, 1977; Doll and Peto, 1981). Cancer usually delays for ten or more years after exposure to a chemical carcinogen and therefore retrospective epidemiological studies are difficult. Scientists have developed sensitive methods that quantitate an individual low-level

environmental exposure to a particular carcinogen. These methods can produce data that may establish a causal relationship between an agent and cancer (Chang *et al.*, 1994).

This project reports on a screening programme on people in Durban, South Africa. Aflatoxin B_1 was selected because of its high toxicity and carcinogenicity and also because of its high prevalence in South Africa. The basis for this project is that humans exposed to AFB_1 form conjugates of DNA and blood proteins through the epoxide of AFB_1, generated in the liver. These conjugates may be detected by suitable digestion and chromatography of the monomer-AFB_1, either lysyl-AFB_1 or guanyl-AFB_1 depending upon the source. Levels of these conjugates are proportional to the history of exposure of the subject to aflatoxin in the diet.

The objectives of this study were therefore to:-

* synthesise AFB_1-macromolecular conjugates
* develop chromatography methods for detection and routine screening of AFB_1-macromolecular conjugates
* determine which amino acids bind to AFB_1
* analyse human blood samples for the presence of AFB_1-lysine and other conjugates.

CHAPTER 2

LITERATURE SURVEY

2.1 MYCOTOXINS AND SECONDARY METABOLISM

Mycotoxins are mould secondary metabolites which, following consumption through contaminated food or feed, cause illness or death of man and animals (Smith and Moss, 1985). Disease caused by mycotoxins, which may be chronic or acute, is referred to as a mycotoxicosis.

Generalities which can be made about mycotoxins are:-

* The major number of known mycotoxins are produced by a few genera of moulds, including *Aspergillus*, *Penicillium* and *Fusarium* species.
* Many arise through the polyketide biosynthetic pathway.
* The liver is frequently the primary target organ in mammals, reflecting its detoxification function.

Secondary metabolites are natural products of incredible chemical diversity, obscure function and restricted taxonomic distribution that occur in chemical clusters or families. They usually require a narrower range of trace metal, pH and temperature for their production than is needed for the growth of the producing organism. They are normally synthesised after active growth has stopped, during the idiophase of the fungal life cycle (Bennett, 1985). Several propositions on the role of secondary metabolism centre on the suggestion that it is the process of secondary metabolism, not the secondary metabolites themselves, which are of importance to the organism (Haslam, 1985). An early idea concerning the function of secondary metabolism, was that advanced by Woodruff in 1966 (Weinberg, 1971) who envisaged secondary metabolism as an overflow process resulting from unbalanced growth and secondary metabolites as shunt metabolites, produced in order to reduce abnormal concentrations of normal intermediates. This theory has been modified by others and it has been suggested that secondary metabolism is a means of detoxification. When normal intermediates in metabolism reach concentrations toxic to the producing cell, the organism responds by diversion of the intermediate to a less metabolically active secondary product, which may be expelled into the environment or secreted in the cell. The third idea is that secondary metabolites are merely waste products of metabolism in

phylogenetically less advanced organisms. Bu'Lock (1980) has speculated that secondary metabolism serves to maintain basic metabolism in circumstances when its normal substrates, through nutritional imbalances and depletions, cannot be exploited for normal cellular growth and replication. There are a number of differences between primary metabolites and secondary metabolites viz., :-

Primary metabolites:

* They are widely distributed in nature.
* They are present during most of the life cycle of the organism.
* They have well defined physiological roles and are essential for growth.
* Examples include amino acids, lipids, nucleic acids, intermediates of citric acid cycle, and other such intermediary metabolites.

Secondary metabolites:

* They are of restricted taxonomic distribution.
* They are usually produced at only one stage of the life cycle.
* They have obscure biological function.
* Unlike primary metabolites, secondary metabolites are not essential for growth.
* Examples of secondary metabolites are toxins and antibiotics (Bennett, 1985).

Mycotoxins exhibit a wide array of biological effects because of the diversity of their chemical structures. Individual mycotoxins can be: mutagenic e.g., AFB_1, sterigmatocystin, citrinin, zearalenone, penicillic acid and patulin; carcinogenic e.g., aflatoxins, sterigmatocystin, penicillic acid, patulin, luteoskyrin, rugulosin, citrinin and ochratoxin A; teratogenic e.g., AFB_1, ochratoxin A, rubratoxin B, T-2 toxin and certain ergot alkaloids; and oestrogenic e.g., zearalenone (Smith and Moss, 1985; Steyn and Vleggaar, 1986).

Mycotoxins have also been classified according to the specific organs and tissues they primarily damage (Hayes, 1981; Smith and Moss, 1985), i.e.,: hepatotoxins, e.g., AFB_1 and ochratoxin A; nephrotoxins, e.g., ochratoxin A, citrinin, AFB_1, rubratoxin B and sterigmatocystin; neurotoxins e.g., citreoviridin and patulin; dermatoxins e.g., trichothecens, T-2 toxin, diacetoxyscipenol, and sporodesmins.

Mycotoxins affect the basic metabolic processes such as carbohydrate metabolism, mitochondrial function, lipid metabolism, and the biosynthesis of proteins and nucleic acids. The four mechanisms causing these effects are:-

* inhibiting key enzymes,
* reacting with DNA and RNA to inhibit protein synthesis,
* interacting with biological membranes, and
* reacting with enzyme cofactors (Keeler, *et al.*, 1992).

In the early 1940s and 1950s research on fungal metabolism was mainly concerned with the discovery of new antibiotics. During this time, although several mycotoxins were first discovered, they were systematically disregarded. Interest grew in antibiotics because of their health and commercial benefit, whereas mycotoxins were not considered useful because of their toxic properties (Bennett, 1985). Consequently no attention was paid to the implied threat to human and animal health by mycotoxins, and mycotoxicosis has been referred to as "the neglected disease" (Forgacs, 1962; Forgacs and Carll, 1962).

In 1960 a poultry disease, "Turkey X" disease, which killed over 100 000 turkey poults in the United Kingdom was traced to mould-contaminated feedstuffs (Goldblatt, 1969). This triggered new interest in the study of mycotoxin and ultimately led to the subsequent identification of over 250 toxic fungal metabolites. The etiological agent responsible for "Turkey X" disease was associated with the mould contamination of Brazilian groundnut meal and shown to be the aflatoxins (Allcroft and Carnaghan, 1962). These were later also identified as potent naturally occurring carcinogens (Goldblatt, 1969). Later it was discovered that cyclopiazonic acid was also involved in "Turkey X" disease (Cole, 1986) and consequently this condition is the first example of a multi-mycotoxicosis.

2.2 AFLATOXINS

Aflatoxins are toxic metabolites produced by moulds widely encountered in most parts of the world (McLellan *et al.*, 1994) and are produced by some strains of *Aspergillus flavus, A. nomius* and *A. parasiticus* (Chu and Ueno, 1977; Hesseltine, 1986). The name aflatoxins is derived from *Aspergillus flavus* **toxins.** They are a major problem facing feed and food producers (Conning and Lansdown, 1983), particularly in third world countries because, while attempting to alleviate malnutrition in such population, individuals may suffer severe liver and other injury. This is the case

because important protein sources such as peanuts and cereals, especially maize may be contaminated by mycotoxin-producing fungi.

Aflatoxins are among the most common fungal contaminants of agricultural commodities in tropical and sub-tropical areas, where the warmth and humidity provide optimal conditions for their growth. The general life cycle of aflatoxin-producing fungi is shown in Fig. 2.1 Spores infect the seed in the field where they germinate, penetrate the seed coat and produce toxin inside the seed (Keeler *et al.*, 1992). This has also been shown to be the case in some other plants.

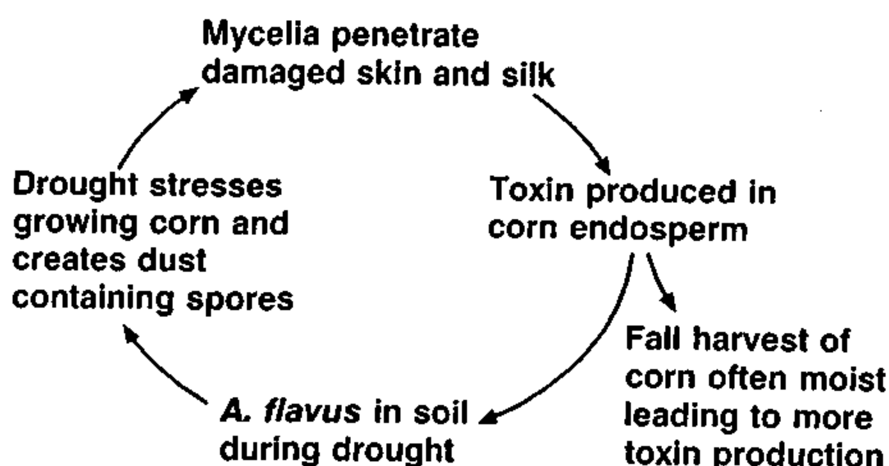


Figure 2.1 General life cycle of *Aspergillus flavus*, a mycotoxin-producing fungi. *Aspergillus flavus* can produce aflatoxins on maize in the field, after harvest, in storage or during processing of foods (Keeler *et al.*, 1992).

The ingestion of aflatoxins by animals results in acute liver toxicity and hepatoma (Coulter,1987). Researchers have been able to elucidate a family of related aflatoxin compounds. These compounds share the common structural features of a bisfuran ring system fused to a substituted coumarin nucleus, (Fig. 2.2). The major aflatoxins (AFs) are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) as shown in Fig. 2.2. They were given these names because of their respective blue and green fluorescence under long-wave ultra-violet (UV) light, and their chromatographic mobilities.

②

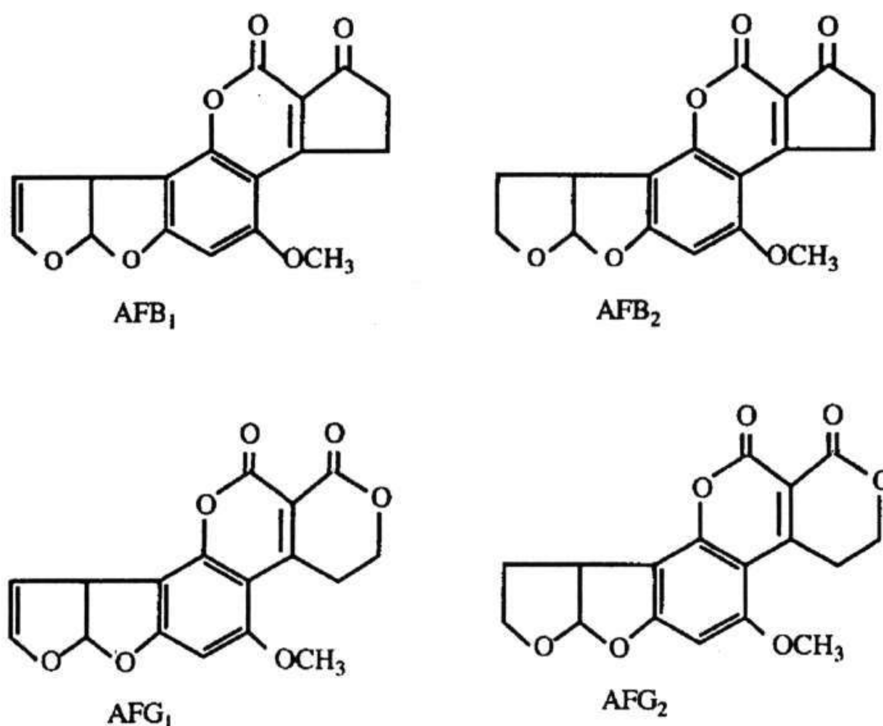


Figure 2.2 Chemical structures of Aflatoxins (AFs) B_1, AFB_2, AFG_1, and AFG_2 (Eaton and Gallagher, 1994).

Aflatoxin B_1 is an extremely potent carcinogen. In rats, it is one of the most active hepatocarcinogens known. Not only is AFB_1 the most potent hepatocarcinogen of the aflatoxins, but it is the most abundant commodity (Goldblatt, 1969). For these reasons, most research has concentrated on AFB_1.

Aflatoxin B_1 has been classified by the International Agency for Research on Cancer (IARC) as a Group I human carcinogen (Autrup *et al.,* 1991); and one of its metabolic products found in milk, AFM_1, as a Group 2B possible human carcinogen, (IARC, 1993). This spells the potential dangers of aflatoxins to the well-being of man.

2.3 FACTORS AFFECTING PRODUCTION OF AFLATOXINS IN NATURAL SUBSTRATES

The most important factors affecting the development of aflatoxin concentration are the fungus, the substrate, temperature and moisture (Schroeder, 1969).

2.3.1 Fungus

The *A. flavus* group of fungal species includes: *A. flavus* and *A. parasiticus*, which produce aflatoxin, as well as *A. oryzae* and *A. tamarii*, which do not (Goldblatt 1969). The type of aflatoxin produced *in vitro* by isolates of *A. flavus* and *A. parasiticus* vary widely, depending on natural substrates. *Aspergillus parasiticus* is reported to produce both the B and the G aflatoxins whereas *A. flavus* produces the Bs only (Klich and Pitt, 1988).

2.3.2 The Substrate

Early investigators reported relatively low yields of aflatoxins with chemically defined media. Natural materials were, therefore, evaluated as suitable substrates for producing large quantities of aflatoxin with fewest interfering substances. Natural substrates are typically moistened with water, sterilised, inoculated, and incubated at 25 - 30°C for 5 to 14 days.

2.3.3 Relative Humidity and Moisture

The most important factor in growth and aflatoxin production by *A. flavus* is the moisture content of or relative humidity (RH) of a natural substrate. A temperature range of 25 to 35°C and moisture contents at adsorption equilibrium with relative humidities of 80 to 100 % are favourable for the production and accumulation of the toxin in stored field crops (Schroeder, 1969). At 70% RH very few fungi will grow, this is the safe storage moisture content equivalent for seeds and other natural substrates (Goldblatt 1969). *Aspergillus flavus* is a mesophyte with a minimum of 80% RH for growth and 85% RH for sporulation (Goldblatt, 1969).

2.3.4 Temperature and Time

Temperature is one of the most important environmental factors influencing the growth and toxin production of toxigenic fungi. Boller and Schroeder (1973) observed that at 30°C, invasion and colonisation of rice inoculated with *A. parasiticus* increased with storage humidity. At relative humidity (RH) of 70, 75, and 80%, 15 to 30% of the kernels were infected. More rapid invasion and colonisation by *A. parasiticus,* greater accumulations of aflatoxins, and greater activity by species of the natural mycoflora accompanied increases in the storage RH. Diener and Davis (1970) studied the limiting temperature and relative humidity for aflatoxin production by *Aspergillus flavus* in stored

peanuts, and found that aflatoxin was formed in sound mature kernels between 40°C and 14°C and in broken kernels down to 13°C, but none was formed at 41°C after days or at 12°C after 84 days in 98± 1% RH. The limiting temperatures for aflatoxin formation in peanut kernels with intact shell were 41°C for 21 days and 16°C for 84 days of incubation. It was found that the limiting RH at 30°C for aflatoxin production in sound mature kernels was 84%, in broken mature kernels and immature kernels limiting RH was 83% and in kernels from unshelled peanuts it was 86% for 84 days of incubation. The limiting RH at 20°C for sound and broken mature kernels was 83%, 86% for immature kernels and 92% for kernels from unshelled peanuts.

The cycling of temperature between 33°C and 15°C has been found to favour AFB_1 accumulation, whereas cycling between 25°C and 15°C favours AFG_1 production. This could possibly be explained through the control of the enzyme systems responsible for relative production of AFB_1 and AFG_2 (Lin *et al.,* 1980). Schroeder and Hein (1967) also found that more AFG was produced or accumulated in relation to AFB at low temperatures (within optimal range). Maximal production of aflatoxin occurred at 24°C and maximal growth of *A. flavus* isolates occurred between 29°C and 35°C. The yellow colour of chloroform extracts appeared to be directly correlated with aflatoxin concentration (Schindler *et al.,*1967). Recent work by Karunaratne and Bullerman (1990) has indicated that it is possible that the faster growing mycelia at 35°C are capable of breaking down the preformed aflatoxin and this has an important implication on the detoxification process of aflatoxins.

2.4 METABOLISM AND BIOCHEMICAL EFFECTS OF AFLATOXIN B_1 METABOLISM

Chemical methods based primarily upon their fluorescence properties have been applied to study the metabolism of aflatoxin by living systems.

2.4.1 Toxic Metabolites in Milk

In some early investigations, animals were fed with toxic rations to investigate the metabolic fate of aflatoxins. Allcroft and Carnaghan (1963) reported that cows fed with toxin contaminated peanut meals excreted in their milk, a factor that was toxic to ducklings. Methanol-chloroform extracts of the milk were administered to ducklings and caused liver lesions or death. The liver lesions were similar to those caused by authentic samples of aflatoxins.

In further studies it was found that the toxic principle was only in the rennet-precipitated casein fraction of the milk. De Iong *et al.* (1964) demonstrated the presence of "milk toxin" in the milk of lactating rats treated with chromatographically pure AFB_1. They concluded that the toxic principle in milk represented a metabolic product of AFB_1 that retained the toxic properties of the parent compound and this was called AFM_1.

Aflatoxin mixtures were administered to animals and the compounds themselves or derivatives of them appeared in urine, faeces and milk. Only small proportions of the compounds administered were excreted without metabolic conversion to derivative form. Some of the metabolic conversions of AFB_1 are:-

* hydroxylation of the bis-furan ring which gives rise to the monohydroxylated derivative AFM_1 (Figure 2.3); this has been found in milk and livers of several animal species. Recently it has been shown that AFM_1, like AFB_1, also forms epoxides (Bujons *et al.*, 1995) and this is suspected to be responsible for its possible carcinogenicity.
* o-demethylation, a major degradative pathway in the rat, which gives rise to a phenolic derivative whose subsequent metabolic fate is unknown.

These processes take place principally in the liver of treated animals, this is a fact related to the retention of the compound by that organ and also to the localisation of histological and biochemical effects of the compound in that tissue. Less is known about the metabolic fates of AFB_2, AFG_1, and AFG_2, but they probably undergo similar changes.

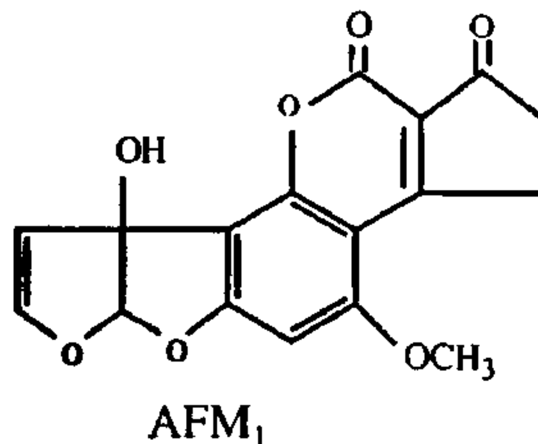


Figure 2.3 Chemical structure of aflatoxin M_1 (Van Egmond, 1989).

2.4.2 Metabolic Effects Of Aflatoxins

The most potent member of the aflatoxin series with regard to toxicity to animals, is AFB_1. Biochemical changes occurring immediately after exposure of animal and cell cultures to aflatoxins have revealed a general pattern of responses (Croy and Wogan, 1981) which, because of their early and consistent occurrence and their central position in certain cellular metabolic pathways, are thought to play an important role in the toxicity of the compound. Responses involved in these exposures concern alterations induced by aflatoxins in nucleic acid and protein metabolism.

The following is an outlined sequence of events in which aflatoxins interfere with nucleic acid and protein metabolism:-

* interaction of aflatoxin(s) with DNA (an initial critical event in the sequence)
* interaction interferes with DNA transcription,
* failure of DNA transcription results in impairment of both DNA and RNA synthesis (inhibition of the polymerases responsible for the respective synthesis),
* inhibition of protein synthesis.

All of these responses have been observed in one or more *in vivo* experimental systems in which animals were exposed to effective doses of AFB_1, and several have also been demonstrated with *in vitro* cell cultures.

2.4.3 Interaction of AFB_1 With DNA

Aflatoxin B_1 requires activation in order to react with DNA or proteins (Croy *et al.*, 1978). Its biochemical mechanism indicates that its bioactivity is associated with the vinyl moiety. Aflatoxin B_1 is converted by cytochrome P_{450} enzymes to the reactive electrophilic 8,9-epoxide. The reaction of 8,9-epoxide with liver DNA and serum albumin forms AFB_1-N^7-guanine (AFB_1-N^7-gua) and lysine adducts, respectively. It has been found that the binding of AFB_1 with one of the purine or pyrimidine bases in DNA, indicated in figure 2.4, causes mutations which can contribute to carcinogenesis. Experimental animals developed liver, kidney, and colon tumours when they were fed AFB_1. Some of the criteria which have been applied to demonstrate aflatoxin-DNA binding includes alterations in the aflatoxin absorption spectrum that occur upon interaction with DNA.

There is a shift in absorption maximum (from 363nm to 366-368nm) upon equilibration of AFB_1 with calf thymus DNA in phosphate buffer, (Sporn *et. al.*, 1966). This shift is accompanied by marked hypochromism at 362nm. It was found by Sporn *et al.* (1966) that 600 moles of native DNA-phosphorus equivalent bound 1 mole of AFB_1. Denatured DNA bound AFB_1 in a molar ratio of 170 : 1. Black and Jirgensons (1967) found that aflatoxin binding resulted in gross conformational changes in the histone and DNA molecules.

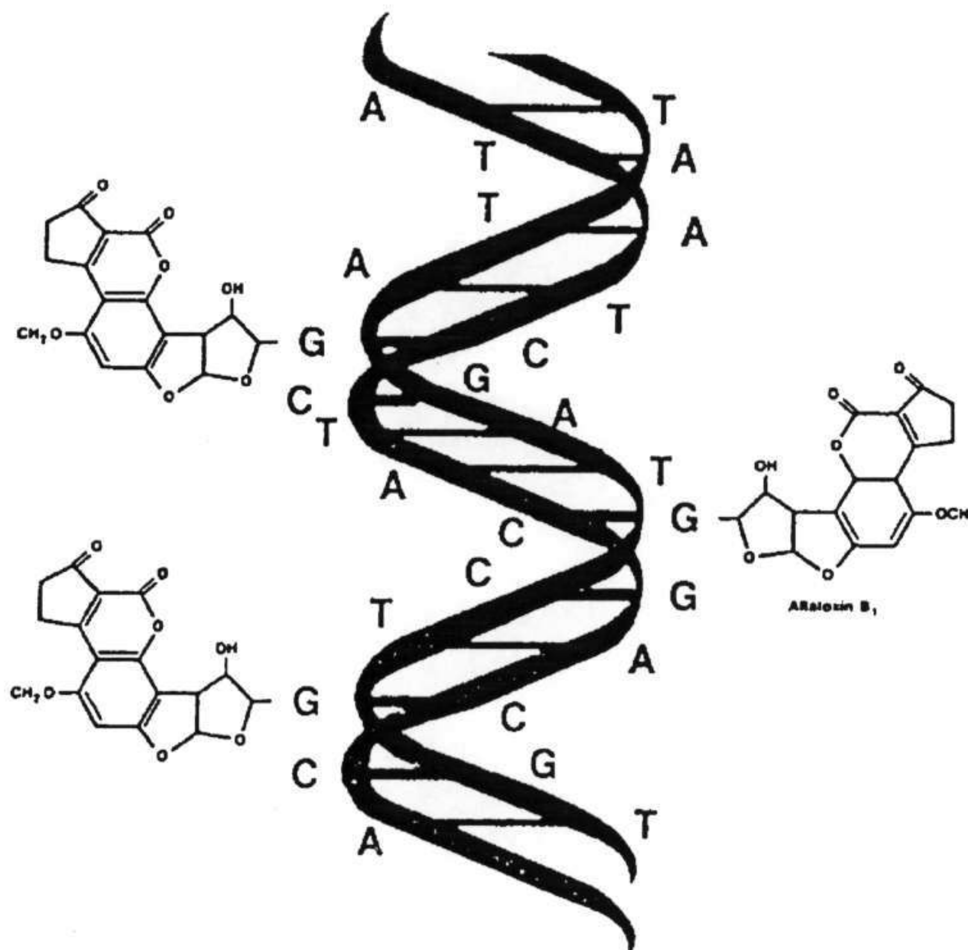


Figure 2.4 Illustration of aflatoxin B_1 bound to DNA at the guanine base derived from liver (Keeler *et al.*, 1992).

Phase II reactions of toxicants are very important in the detoxification of xenobiotics. They involve the joining together of a substrate compound with another species that occurs normally in (is endogenous to) the body, they are therefore referred to as conjugation reactions (Manahan, 1990).

Two important enzymes involved in the detoxification of AFB_1-epoxide in the hepatocytes are the microsomal epoxide hydrolase (EPHX) and glutathione S-transferase M_1 (GSTM1). Epoxide

hydrolase converts the epoxide into 1.2-dihydrodiols and GSTM1 conjugates the epoxide to glutathione, a crucial conjugating agent in the body. Lack or diminution of these enzymes might leave more of the epoxide available to bind to DNA, RNA and/or proteins. By binding to DNA, the epoxide causes an AGG-to-AGT (arginine to serine) transversion mutation at the third base of codon 249 in exon 7 (McGlynn *et al.,* 1995). In proteins, activated AFB_1 has been shown to interact mostly at the lysine part of albumin thereby inhibiting protein synthesis. In DNA it interacts with guanine thereby causing mutations.

2.4.4 Biochemical effects of aflatoxins in the liver

In animals treated with aflatoxins, the effects of the toxins have been reported to be virtually tissue-specific, affecting only the liver, with few exceptions (Wogan, 1966). This generalisation is applicable to the toxicity following acute dosing and to the carcinogenicity resulting from chronic exposure. Sequential histologic alterations manifest in the hepatotoxicity of AFB_1 in rat liver have been described by Butler and Barnes (1964).

Carcinogens are either electrophilic molecules or are biologically activated by cytochrome P_{450} dependent mixed function oxygenase (MFO). The electrophiles are capable of binding *in vivo* with the informational macromolecules, thus inducing cancer (Ames *et al.,* 1973). Inactivated AFB_1 (procarcinogen) has no reactivity towards nucleophiles. It shows only weak *in vitro* association with DNA. Activated AFB_1 (ultimate carcinogen) binds to the macromolecules (Coles *et al.,* 1980).

Using rat studies, many investigators have shown a linear relationship between dietary aflatoxin dose and binding to serum albumin and liver DNA (Wild *et al.,* 1986). Dietary AFB_1 dose also correlated well with urinary and liver AFB_1-N^7-gua levels. Mutational properties of the primary AFB_1-DNA adduct show a predominant G-T transversions, identical to the principal mutation in human liver tumours associated with aflatoxin (Bailey *et al.,* 1996). Aflatoxin B_1-hepatic DNA adduct levels in rats and rainbow trout correlate well with incidence of liver tumours. These results demonstrate that AFB_1-albumin and urinary AFB_1-N^7-gua levels can serve as surrogates for the liver DNA adduct levels.

2.5 AFLATOXIN B_1-EPOXIDE: THE ULTIMATE CARCINOGEN

The 8,9-epoxide (Fig. 2.5) has been identified as the active form of AFB_1 (Baertschi *et al.*, 1988; Rajkumar and Harris, 1993). Other researchers (Swenson *et al.*, 1977; Jacobsen and Humayun, 1986) have called it the 2,3-oxide, but in this study it will be referred to as the 8,9-epoxide. The 8,9-epoxide can be synthesised *in vitro* activation by crude metabolic enzyme preparation (Garner *et al.*, 1972). There are also two *in vitro* chemical procedures for the formation of 8,9-epoxide. One involves oxidation with the mild organic oxidant chloroperbenzoic acid and the other oxidation with dioxirane. The synthesis of AFB_1-8,9-dichloride (AFB_1-Cl_2) is also important as it is an electronic analogue of AFB_1-8,9-epoxide. It has been shown that AFB_1-Cl_2 is more carcinogenic than AFB_1. Aflatoxin B_1-dibromide (AFB_1-Br_2) is however more susceptible to nucleophilic attack at C-8 than the dichloride and thus it was a better model to investigate the action of AFB_1-8,9-epoxide before its availability by *in vitro* synthesis.

Gorst-Allman *et al.* (1977) compared the rates of hydrolysis of AFB_1 dichloride and dibromide in aqueous solution at pH 7.4. The rate of hydrolysis was taken as an indication of the ease of attack at the electronegative C-8. This was done by following the bathochromic shift from 360 to 390nm due to the formation of the phenolate anion. The $t_{1/2}$ of dibromide reaction was found to be 30s at 20°C compared with that of the dichloride which was 185s at 20°C. As the 8,9-epoxide has not been isolated or detected in biological systems, its role has been inferred from structures of adducts with DNA and other bioreceptors. Raney *et al.* (1992) pointed out that the biologically relevant species is the exo-8,9-epoxide, which is highly reactive, as opposed to the endo isomer.

The epoxide, which can be detoxified *in vivo* by conversion into the *trans*-diol by epoxide hydrase (McGlynn *et al.*,1995), is extremely labile under non-biological conditions. The epoxide intermediate will, therefore, require protection by a hydrophobic environment, if produced on the endoplasmic reticulum, during its migration to the site of action with genetic material (RNA or DNA). Alternatively, the epoxidation may occur in the nucleus but the presence of suitable cytochrome activity has not been demonstrated in this organelle.

The 8,9-epoxide of AFB_1 has been prepared using dimethyldioxirane as the oxidant (Baertschi *et al.*, 1988; Ferrer *et al.*, 1996) and the properties of dimethyldioxirane have been determined by Adam *et al.*,1986. Aflatoxin B_1 8,9-epoxide is stable for at least 12 hours at room temperature in the solid form or in solutions of acetone and methylene dichloride and can be stored for long periods at -

10°C (Baertschi *et al.*, 1988). The preparation of the epoxide of AFB_1 contains both the exo- and the endo-8,9-epoxides (Fig. 5) in the ratio of 10:1 respectively (Raney *et al.*, 1992).

The exo isomer was found to be strongly mutagenic in a base-pair reversion assay employing *Salmonella typhimurium*, whilst the endo isomer was found to be essentially non mutagenic (Iyer *et al.*, 1994). Apart from *S9* fraction epoxidation by *A. flavus in vitro* (Saxena *et al.*, 1991) oxidation has been shown to occur by lipoxygenase purified from human term placenta and intra-uterine conceptal tissues. This supports the speculation that AFB_1 may be a carcinogen produced by the placenta.

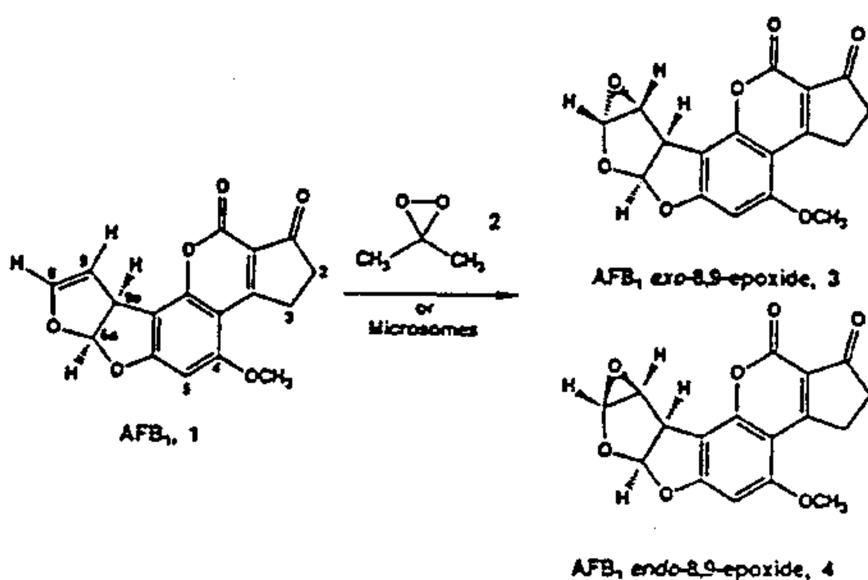


Figure 2.5 Formation of aflatoxin B_1 exo- and endo- epoxides (adapted from Raney *et al.*, 1992)

Tjalve *et al.* (1992) showed that microsomal preparations of the bovine olfactory mucosa have a much higher ability than liver microsomes to induce covalent binding of AFB_1 to calf thymus DNA and to microsomal proteins. Therefore, inhaled aflatoxins may also have detrimental effects.

2.6 AFLATOXIN B_1-ADDUCTS

2.6.1 Introduction

Researchers have explored new approaches to recognising and predicting toxic exposures of humans to aflatoxins and their potential consequences (Wild *et al.*, 1990b). These approaches require the development of indicators (biomarkers) which will enable the identification and predictions to be made with some certainty (Groopman *et al.*, 1990). The laboratory assay must give a long term exposure assessment in a sample obtained non-invasively. Biological markers (biomarkers) can be used to recognise reactions, responses, or adverse changes in a biological system at an early stage and with certainty and hence biomarker research may be of great value to human health care (Ashby, 1987). Macromolecular adducts can be detected and quantified using sensitive and sophisticated analytical methods (Wild *et al.*, 1990b).

Previous approaches, using the measurement of unbound aflatoxins, had the disadvantage of reflecting relatively short term (a few days) exposure. Such previous approaches were used to measure aflatoxin metabolites and/or nucleic acid adducts in human urine, serum, and milk (Groopman *et al.*, 1988; Wild *et al.*, 1988; Groopman *et al.*, 1993).

Dose-dependent binding of aflatoxins to DNA (Appleton *et al.*, 1982) and serum albumin (Wild *et al.*, 1986) have been shown to occur and this provides the experimentally based rationale to investigate such interaction as biomarkers of toxin exposure in people. Wild *et al.* (1990b) have evaluated the methods for quantitation of aflatoxin-albumin adducts. They suggested a combination of the hydrolysis enzyme linked immunosorbant assay (ELISA) for large scale screening followed by confirmatory analysis in positive samples.

The two important macromolecular adducts are the DNA adducts (Jennings *et al.*, 1992; Tjalve *et al.*, 1992) and the protein adducts (Sabbioni *et al.*, 1987; Wild *et al.*, 1990a). Each of these adducts can be studied using different methodologies. Aflatoxin B_1-lysine adduct is formed via the same metabolic activation that leads to AFB_1-DNA adduct formation. Therefore, the level of albumin adduct in experimental animals reflects the degree of DNA damage in the liver, which is the target organ for experimental AFB_1 carcinogenesis (Wild *et al.*, 1986; Sabbioni *et al.*, 1987).

2.6.2 Proposed mechanism for the formation of the AFB_1 adduct

Sabbioni *et al.* (1987) have proposed that the active species *in vivo* or *in vitro* is the 8,9-dihydro-8,9-diol which is produced rapidly by hydrolysis of the epoxide or dibromide respectively. It was found that at physiological pH the dihydrodiol is in equilibrium with the hydroxydialdehyde. Like aldoses and glyceraldehydes, alpha-hydroxyaldehydes combine with primary amino groups in proteins to form Schiff bases which undergo Amadori rearrangement to alpha-amino ketones. The amino ketone is converted by chemical transformations to the pyrrolidine-3-one. This completes the adduct formation (Fig. 2.6).

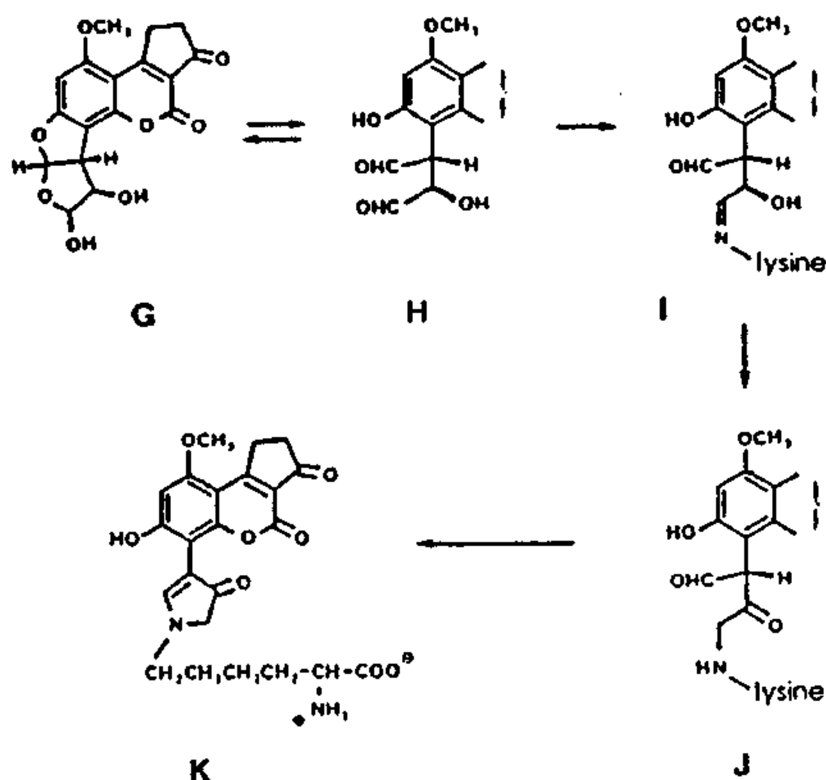


Figure 2.6 Proposed mechanism for the formation of AFB_1 adducts (Sabbioni *et al.*, 1987).

2.6.3 DNA adducts

DNA adducts are significant in chemical carcinogenesis. Among the analytical methods currently used for the detection and quantitation of DNA adducts are: immunological methods (Candlish *et al.*, 1991), fluorimetric methods, and ^{32}P-postlabeling (Gupta *et al.*, 1982). These methods extend the detection for DNA adducts to 1 per 10^6 - 10^{10} normal nucleotides. Each method

has advantages and disadvantages, therefore they play different and sometimes complementary roles in biomarker and human biomonitoring.

2.6.4 Protein adducts

Protein adducts have been found to be invaluable biomarkers of exposure because they are quantitatively better dosage indicators than are DNA adducts and plasma albumin has long been known to bind AFB_1 (Rao *et al.*, 1968). Furthermore, blood proteins are readily accessible (Sabbioni *et al.*, 1990). Overall reactivity of tissue proteins to common monofunctional alkylating agents like ethylene oxide is 1-5 times higher than that of DNA. Proteins of most importance in toxin conjugation are those that circulate in blood, i.e., haemoglobin and albumin. Serum albumin and haemoglobin are abundant in blood and can be isolated easily. Haemoglobin adducts tend to persist for the lifetime of circulating erythrocytes (120 days in humans). Even after several months of last exposure period, haemoglobin adducts may still be detected. Albumin adducts are more abundant than those of haemoglobin but can only be used for assessing recent exposure, as albumin has a faster turnover than haemoglobin (i.e., a half life of 20-25 days), and exists freely in serum. (Albumin is synthesised in the liver endoplasmic reticulum, a major site of xenobiotic activation).

Wild *et al.* (1990a) have reported that between 12 and 100% of aflatoxin bound to serum albumin in children and adults from various African countries, contain aflatoxin albumin adducts. They found the level of up to 350pg AFB_1-lysine equivalent/mg albumin. In Thailand the levels of this adduct was lower and no positive sera were detected from France or Poland. The levels of AFG_1-albumin adducts were found to be 5.7 and 2.8 fold lower than that of AFB_1. It was therefore suggested that AFG_1 could be used as an internal standard for methods using the measurement of aflatoxin-albumin adducts to quantitate human exposure to aflatoxin (Sabbioni and Wild, 1991).

2.6.5 Analysis of adducts

Three general methods have been used for the chemical analysis of protein adducts:

* The adducted protein of interest is isolated and subjected to mild acid- or base-catalysed hydrolysis to release the adducting residue from the protein. The free molecule is isolated and quantified by conventional analytical methods such as UV absorption spectroscopy.

* Another method starts with a harsh and complete acid hydrolysis of the protein adduct to individual amino acids, which are analysed by conventional means.
* The third method is specifically for the release of adducted N-terminal valines in haemoglobin using a modified Edman degradation method with pentafluorophenyl isothiocyanate (high sensitivity, mild degradation conditions).

The method of choice for isolating albumin from plasma is generally affinity chromatography. However, a significant loss of the major human lysine AFB_1-adduct occurs in this method because of instability of adduct during the cibracon blue affinity chromatography step used in the isolation. A more rapid ammonium sulphate precipitation step is, therefore, preferred (Wild *et al.*, 1990b).

2.7 METHODS OF ANALYSIS FOR DNA AND PROTEIN ADDUCTS

2.7.1 Immunological methods

Antibodies recognising specific DNA adducts are available and enable researchers to monitor the toxicity, mutagenicity, and carcinogenic properties of these adduct. Immunological methods for DNA adduct quantitation are competitive radioimmunoassay (RIA) (Gan *et al.*, 1988), solid-phase competitive or non-competitive enzyme-linked immunosorbent assay (ELISA) (Wild *et al.*, 1990b) and ultrasensitive enzymatic radioimmunoassay (USERIA). Antibodies have also been used in the development of various immunopurification techniques.

In the RIA technique, the ability of antigen in a sample to compete with a fixed amount of radiolabelled antigen for a limiting quantity of antibody is measured. This gives the concentration of antigen in the sample. Enzyme-linked immunosorbent assay employs the solid phase-bound antigen and a second antibody conjugated to an enzyme. The second antibody conjugated to an enzyme increases assay sensitivity (one molecule of enzyme can hydrolyse many substrate molecules). Ultrasensitive enzymatic radioimmuno assay is similar to ELISA but the substrate for the second antibody is radiolabelled. Sensitivity of USERIA is equivalent or even more than that of ELISA (Chang *et al.*, 1994).

There are two different ways of relating sensitivity to immunoassays. When the amount of a given DNA adduct required for precise quantitation is involved, it is said to be absolute sensitivity. Relative sensitivity denotes the lowest relative modification level to be detected in DNA. At a given

absolute sensitivity, the relative sensitivity of a method depends on the amount of DNA required by the system. Immunoassay methods are and have low costs in analysing large numbers of samples. The disadvantage of immunological methods, however, is the production of antibodies which require extensive development efforts. This also requires prior knowledge of the structure of the DNA adduct under investigation. Antibodies are also valuable in immunopurification. Monoclonal antibodies have been used in various affinity chromatography procedures to eliminate interfering substances prior to analysis by either immunoassays or non-immunological assays (Wild *et al.*, 1990b).

2.7.2 Fluorimetric methods

The inherent fluorescent properties of many polycyclic aromatic hydrocarbons (PAHs) and compounds with extended heterocyclic structures is of high value in fluorimetric methods (Chang *et al.*, 1994).

Synchronous scanning fluorescence spectrophotometry (SFS) is one such approach. In SFS, simplified spectra are generated by scanning both excitation and emission simultaneously with a fixed wavelength difference. Synchronous scanning fluorescence spectrophotometry provides a sensitive means for the detection of selected DNA adducts but has a limited ability to distinguish between closely related compounds. Therefore, SFS has not sufficient resolving power to determine a single adduct in a complex mixture. Synchronous scanning fluorescence spectrophotometry is also vulnerable to interference by other fluorescence-emitting substances. This implies a need for an additional sample cleanup step prior to SFS analysis. Alternatively, HPLC coupled with fluorescence detection could be used (Wild *et al.*, 1990b).

2.7.3 ^{32}P-Postlabeling assay

^{32}P-Postlabeling assay uses a sequence of steps:

* enzymatic DNA digestion to the deoxyribonucleoside-3'-monophosphates (dNps),
* labelling the adducted nucleotides with ^{32}P ATP and T_4 polynucleotide kinase (T_4PNK) to produce the deoxyribonucleoside-3',5-bisphosphates (dpNps),
* enrichment of adducted nucleotides by the elimination of normal nucleotides and
* separation and detection by high resolution thin layer chromatography (TLC) and autoradiography respectively (Beach and Gupta, 1992).

Advantages of ^{32}P-postlabeling.

- Sensitivity: the detection limit of one adduct in 10^6-10^8 normal nucleotides is readily attained.
- Assay requirements: minimum DNA requirements for the assay is as low as 1- 10μg
- Versatility: prior knowledge of adduct composition is not necessary, therefore, multiple adducts resulting from exposure to complex mixtures can be detected.

Disadvantages of ^{32}P-postlabeling

- It is not possible to detect absolute quantitation of an unknown adduct because the efficiency of the T_4 PNK reaction differs with various DNA adducts.
- The method will only quantify, reliably, those adducts that can be chromatographically separated.
- The most crucial phase of the ^{32}P-postlabeling assay, which is adduct enrichment, has been overlooked.
- Presently researchers use the technique of butanol extraction (Gupta, 1985) and nuclease P_1-mediated enrichment (Reddy and Randerath, 1986).

2.8 EXCISED MACROMOLECULAR ADDUCTS

Under normal physiological conditions, nucleic acids and proteins are broken down by various catabolic processes. The adducted portion of the degraded macromolecule is then excreted through urination. The relatively large volumes of urine that can easily be obtained from exposed individuals aid in the analysis of the extremely low concentrations of the breakdown products. Although excised macromolecular adducts only reflect the most recent exposures to xenobiotics, molecular epidemiology information has been derived using these biomarkers in human populations. Like in DNA and protein adducts, conventional analytical techniques and immunoassays can be used for the quantitation of excised DNA adducts (Chang *et al.*, 1994).

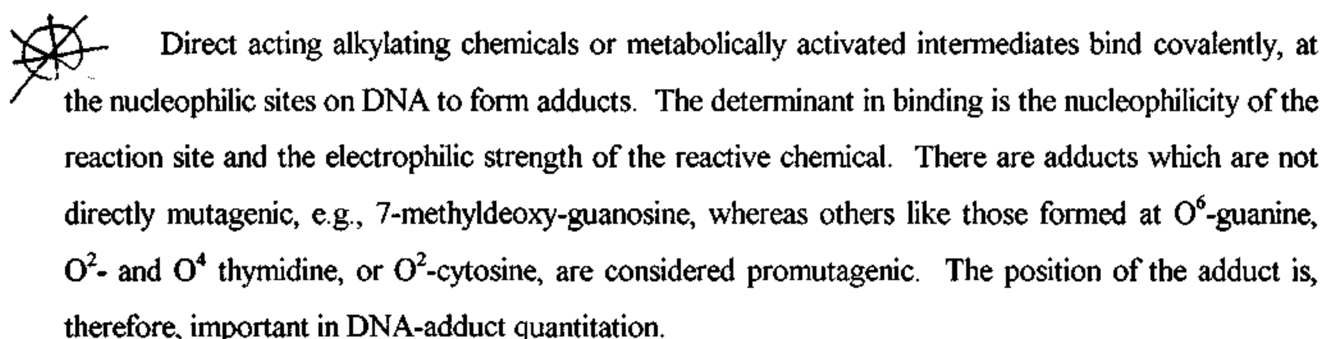
2.9 MYCOTOXIN DOSAGES, ADDUCTS AND CARCINOGENESIS.

2.9.1 Internal Dose

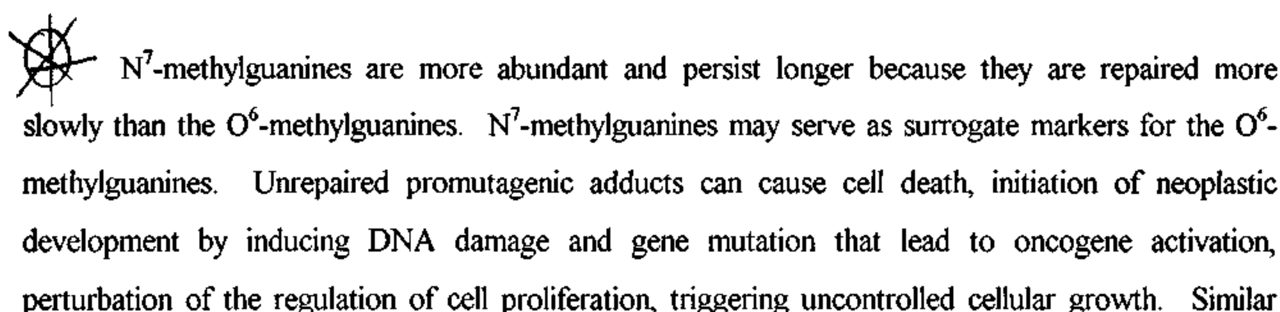
Internal dose refers to the actual amount of xenobiotic absorbed into the organism. It is determined by measuring the xenobiotic or its metabolite in the body fluids, tissues, or excretory products. Blood and urine are most commonly analysed because of the accessibility and quantity, e.g., AFB_1 in urine serves as an indication of exposure to dietary aflatoxins. The advantage of internal dose is that it avoids the uncertainties of determining the concentration of the toxicant in the environment, the root of exposure, and the toxicant absorption rate. The disadvantages of internal dose are that it overestimates the biological effect of chemicals, that require activation e.g., AFB_1. This means that the metabolic rate of the chemical is not taken into account. Internal dose also underestimates the biological effect of chemicals that act directly, i.e., these chemicals act on the tissue of first contact.

2.9.2 Biologically Effective Dose

Biological effective dose is the amount of chemical needed to induce a biological response or consequence. The interaction of electrophiles with nucleophiles is the primary step in carcinogenesis. Adducts are invaluable as biomarkers, because they represent the amount of material interacting with the critical subcellular, cellular, and tissue targets. Adducts are therefore, considered to be the biologically effective dose of xenobiotics.



Direct acting alkylating chemicals or metabolically activated intermediates bind covalently, at the nucleophilic sites on DNA to form adducts. The determinant in binding is the nucleophilicity of the reaction site and the electrophilic strength of the reactive chemical. There are adducts which are not directly mutagenic, e.g., 7-methyldeoxy-guanosine, whereas others like those formed at O^6-guanine, O^2- and O^4 thymidine, or O^2-cytosine, are considered promutagenic. The position of the adduct is, therefore, important in DNA-adduct quantitation.



N^7-methylguanines are more abundant and persist longer because they are repaired more slowly than the O^6-methylguanines. N^7-methylguanines may serve as surrogate markers for the O^6-methylguanines. Unrepaired promutagenic adducts can cause cell death, initiation of neoplastic development by inducing DNA damage and gene mutation that lead to oncogene activation, perturbation of the regulation of cell proliferation, triggering uncontrolled cellular growth. Similar

effects may also be produced through repair errors. Deoxyribonucleic acid adducts are, therefore, a relevant indicator of the biologically effective dose of a carcinogen (Chang *et al.*, 1994).

Protein adducts in haemoglobin and albumin and urinary (DNA base) adducts have been used as surrogates for exposure estimates. Both haemoglobin and albumin adducts are accessible, plentiful, persistent and stable (not subject to repair). Their quantity is also dose related and correlates well with DNA adducts. Some substances that form adducts in the body are the N-nitrosamines, PAHs, aromatic amines and AFB_1. This study concentrates on AFB_1-adducts.

2.10 AFLATOXIN AND HUMAN HEALTH

There is a growing concern over the impact of aflatoxins and other mycotoxins on human health because, besides AFB_1 having been a proven human carcinogen, aflatoxins are postulated to:

* play a role in the aetiology of kwashiorkor,
* increase neonatal susceptibility to infection and malignant diseases,
* compromise immune response to prophylactic immunisation and
* may play a role in the pathogenesis of diseases in heroin addicts (Smith *et al.*, 1994; Smith *et al.*, 1995).

The role of aflatoxins in human disease includes: epidemiological correlations between aflatoxins in food and hepatoma in Africa, i.e., Kenya, Swaziland, Mozambique and South Africa (Peers *et al.*, 1987); in Thailand (Munoz and Bosch, 1987; Autrup *et al.*, 1987); and in the Peoples Republic of China (Yea *et al.*, 1989); severe liver disease in children in India after ingestion of aflatoxin-contaminated foods, and epidemiological and clinical correlations between Reye's syndrome in children and dietary exposure to aflatoxin in Thailand. The young and malnourished are susceptible to the effects of aflatoxins. There is, therefore, a need for the investigations of long-term exposure to aflatoxins in children especially in areas where protein energy malnutrition is endemic.

Saad *et al.* (1995) investigated exposure of infants to AFM_1 from mother's breast milk in Abu Dhabi. Their results demonstrate that exposure to aflatoxins is not a problem solely in rural communities of developing countries, with low incomes, but may also be a problem for relatively wealthy countries, if they lack the legislative measures and the educational infrastructure to highlight and control the level of aflatoxin in food and feeds. The importance of legislative measures is

confirmed by the observation that countries with stringent quality control, e.g., Singapore, have low degree of individual exposure to aflatoxins (Chao *et al.*, 1994).

Multiple factors are involved in the interaction of aflatoxin with albumin. Compounds with 4-hydroxycoumarin ring bind to serum albumins and the introduction of a hydrophobic group to a coumarin compound greatly increases its anticoagulant action and its binding to albumin. Aflatoxin B_1 is much more effective than 4-hydroxycoumarin in prolonging the blood clotting time of rats. The comparative effective doses were 56×10^{-6}mM AFB_1 to 93×10^{-3}mM 4-hydroxycoumarin (Bassir and Bababunmi, 1972).

Kwashiorkor is a serious disorder of children which is widespread in the tropics. Kwashiorkor was previously thought to be caused by protein deficiency but over the past fourteen years a steady accumulation of evidence incriminating aflatoxins in the aetiology of kwashiorkor has been observed. Speculations are that kwashiorkor might result from chronic aflatoxin poisoning (Anon, 1984), but whether it is prime factor for the development of kwashiorkor is still unknown (Abdulmagid, 1993). It is not clear whether the fundamental difference in the metabolism of aflatoxins in kwashiorkor and non-kwashiorkor patients is causally related to kwashiorkor or is a consequence of the disease but it is speculated that aflatoxins causes kwashiorkor by damaging the liver, which becomes unable to manufacture albumin. Low level of albumin then lead to the disease (Read, 1990).

Protein energy malnutrition (PEM) is a dominant type of malnutrition world-wide and embraces all disorders attributable to lack of protein and calories. These disorders include marasmus and kwashiorkor. Protein energy malnutrition serves as a euphemism for starvation. There is evidence that PEM is very widespread and is a major associated cause of death in early childhood.

There are no mysteries in the pathogenesis of marasmus because it is the childhood equivalent of starvation and the features are characterised by varying degrees of wasting (Hendrickse, 1982). On the other hand the pathogenesis of kwashiorkor remains obscure. It was earlier thought that it is caused by severe protein deficiency with relative carbohydrate excess, but this is no longer tenable. It is now known that there are no essential differences in the protein/energy ratios in the diets of children who develop marasmus or kwashiorkor. Clinical features of kwashiorkor range from slight oedema, in a depressed, unhappy child, to a gross oedema, extreme misery and apathy, grotesque skin lesions and hair changes in children whose biochemistry is in a state of chaos and whose immunity is depressed (Hendrickse *et al.*, 1982).

There is no record of kwashiorkor in Europe when other forms of malnutrition in children were rife. Whether kwashiorkor is purely a nutritional disease or whether malnutrition renders children susceptible to etiologic factors which precipitate the syndrome remains unexplained.

Hendrickse *et al.* (1982) investigated blood and urine samples for their aflatoxin content by HPLC. They used kwashiorkor, marasmic kwashiorkor, marasmus and normally nourished children. Aflatoxins were detected more often, and at higher concentrations, in sera from children with kwashiorkor than in the other malnourished and control groups. In the kwashiorkor children, they also detected aflatoxicol (a metabolite of AFB_1 and AFB_2) which was not detected in the controls. That showed a difference in metabolism between children with kwashiorkor and the other groups. They concluded that either the children with kwashiorkor have a greater exposure to aflatoxins or that their ability to transport and excrete aflatoxins is impaired by the metabolic rearrangements associated with kwashiorkor.

Hendrickse *et al.* (1982) screened a total of 177 sera. They detected aflatoxin in the serum of children in all nutritional groups included in their study. Aflatoxins were detected least in the controls (15.9%), marginally more often in children with marasmus (19.3%) and marasmic kwashiorkor (21.9%) and most often in those with kwashiorkor (36.4%). A similar trend has also been observed by Ramjee *et al.*, 1992. Aflatoxins were more often detected in boys than in girls (in the three malnourished groups). They had evidence of a positive association between poor nutritional state and the presence of aflatoxins. The serum concentrations of the individual AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, and aflatoxicol were highest in children with kwashiorkor and lowest in controls.

A total of 250 urine specimens were screened for aflatoxins and aflatoxicol was not detected. Children with aflatoxins had lower concentrations of aflatoxin in their urine than the other groups. The highest concentrations occurred among children with marasmus and marasmic kwashiorkor. This was firm evidence that children in the Sudan are exposed to aflatoxins in their diets.

In kwashiorkor children there may be a greater exposure to aflatoxin or there may be an impairment of the ability to transport and excrete these substances consequent on the metabolic derangements associated with kwashiorkor. It is therefore, important to measure aflatoxin levels in food cooked in the homes (food on the plate) of all groups of patients. However, it is clear that children with kwashiorkor are at greater risk from aflatoxin than normal children. It might occur that

after initial insult to the liver, impaired ability to handle and excrete aflatoxins creates a vicious cycle, in which serum aflatoxin values rise, as the ability to handle these substances progressively declines.

Aflatoxins were detected in the livers of a small number of children who died of kwashiorkor in South Africa and Nigeria. This invites speculation about the significance of these findings in relation to the unanswered questions about the aetiology and pathogenesis of kwashiorkor (Hendrickse, 1982). There has been a recent report of the occurrence of kwashiorkor in a fully breast-fed infant and in an identical twin (Oyelami *et al.*,1995). This does not agree with the belief that the aetiology of kwashiorkor is wholly nutritional. Moreover, aflatoxins were detected in the organs of these children on post-mortem examination.

Most doctors are poorly informed about the environmental hazards constituted by aflatoxins and other mycotoxins because these toxins have been ignored by the medical profession (Hendrickse, 1982). Some of the toxic metabolic effects of aflatoxins in animals include: fatty degeneration of the liver, depressed albumin synthesis, depressed enzyme synthesis, immunosuppression, haemorrhagic phenomena, and depressed glucose metabolism. Light microscopic histology of the liver in acute toxicity have demonstrated bile duct proliferation and fatty infiltration, while ultrastructural studies have revealed marked changes in the endoplasmic reticulum, mitochondria and nucleoli (Coulter, 1987).

Investigations in Sudan have shown that many commonly eaten foods are contaminated by aflatoxins which can be detected in the blood and urine of local children. Such findings support a hypothesis that aflatoxins (and possibly other mycotoxins) may be factors in the pathogenesis of kwashiorkor. It is not easy to reconcile this concept with the classical, purely nutritional explanations for the syndrome, but it accords with the geographical distribution, seasonal incidence, dietary associations and patho-physiology of kwashiorkor (Hendrickse *et al.*, 1982).

Apeagyei *et al.* (1986) analysed the autopsy liver specimens from 22 children in Ghana who had died of kwashiorkor. They used both TLC and HPLC and of the subjects, 20 had AFB_1 at a concentration range of 62 to 4409*pg/g* of tissue. Two of the liver specimens showed the presence of aflatoxicol, 12*pg/g* and 99*pg/g* respectively. This confirms that AFB_1 and aflatoxicol is present in livers of children with kwashiorkor. Apeagyei *et al.* (1986) did not detect any of the oxidative metabolites of AFB_1 (AFM_1, AFP_1, and AFQ_1). This implies an impaired microsomal mixed function oxidase system in kwashiorkor patients. Aflatoxins cannot, therefore, be detoxified by oxidation and removed from

the liver as would normally occur. It must however be emphasised that the nature of association between kwashiorkor and aflatoxins remains unexplained, although an association has been established.

2.11 MONITORING EXPOSURE TO AFB_1 USING AFB_1-LYSINE, AFB_1-ALBUMIN AND AFB_1-PROTEIN ADDUCTS

Exposure to aflatoxins has been monitored using different methods. In correlation studies the method was by extrapolation from analysis of food sample contamination whilst in the case-control studies the method was by questionnaire. In all these studies exposure assessment was relatively imprecise (Wild *et al.*, 1990b). While such data is important, it only measures the internal dose, i.e., the amount of a particular chemical agent ingested by an individual. Among the factors influencing the internal dose are the intake, distribution, metabolic (in)activation and excretion, all of which are not accounted for in the internal dose. This lead to the development of new approaches to the determination of exposure assessment of aflatoxin. Aflatoxin B_1 has been shown to bind to albumin in a quantitative manner (Skipper *et al.*, 1985, Wild *et al.*, 1986, Dirr and Shabort 1986, Sabbioni *et al.*, 1987). The major adduct formed in the binding of AFB_1 to albumin is the AFB_1-lysine adduct.

Different methods have since been used to quantitate the level of aflatoxin bound to albumin. Such methods include the measurement of:

* aflatoxin B_1-albumin intact adduct
* aflatoxin B_1-lysine adduct
* aflatoxin G_1-lysine adduct
* protease digest of AFB_1-albumin.

Gan *et al.* (1989) used the intact albumin adducts in their fluorimetry experiments (excitation and emission set at 320 and 420nm respectively). The intact albumin was chosen because of the instability of AFB_1-lysine adduct during isolation steps ($t_{1/2}$ = 8hrs). In their experiment they found that non-adducted native serum albumin also has a fluorescent profile when excited by 320nm light (i.e., a background fluorescence from native albumin is present). Their laser induced fluorescence (LIF) was unable to conduct the time-resolved LIF study on the elimination of background fluorescence from sample fluorescence with signal subtraction method. Their alternative was, therefore, to record and digitally store the fluorescence of native albumin, measure the fluorescence of adducted albumin and subtract the background by standard routines.

Sheabar *et al.* (1993) used the digested proteins method. They found that increasing protease to protein ratio up to 0.1 (w/w) resulted in enhanced detection of AFB_1 adducts. Higher ratios did not show further increases. Incubation of serum proteins with protease (4.1 : 1 w/w) for more than 15 hrs at 37% showed no significant improvement in detection of protein adducts. Approximately 30% of the highly polar aflatoxin-protein adducts are lost during immunoaffinity chromatography of serum protein digest. Only 20-35% recovery of AFB_1-albumin adduct are reported due to losses during chromatographic isolation of albumin as well as the AFB_1-lysine adduct (Sheaber *et al.*, 1993). Losses as high as 95% of the AFB_1-lysine adduct have been reported (Wild *et al.*, 1990b) due to a large number of experimental steps (Sheaber *et al.*, 1993).

Sabbioni *et al.* (1990) suggested that human serum could be prepared for aflatoxin-lysine analysis by direct pronase digestion without prior specific albumin isolation. The mechanism of decomposition of AFB_1-lysine remains obscure (Sabbioni *et al.*, 1987, Sabbioni *et al.*, 1990). Wild *et al.* (1990b) indicated that AFB_1-lysine is probably not the only aflatoxin containing molecule present after hydrolysis, other aflatoxin-peptides may occur. Thus, it may be of value to screen for conjugates of aflatoxins and amino acids other than lysine.

CHAPTER 3

PRODUCTION OF AFLATOXINS AND PREPARATION OF AFLATOXIN B_1 -EPOXIDE

3.1 PRODUCTION OF AFLATOXINS

3.1.1 Introduction

Since the discovery of aflatoxins in the 1960s, a number of studies have been conducted to determine conditions for their maximum production. This included the production of aflatoxins by different strains of *Aspergillus flavus* and *A. parasiticus* on semi-synthetic (Davis *et al.*, 1966) and natural media. It was soon apparent that strains of these species had the ability to produce relatively large amounts of the aflatoxins. For example, Diener and Davis (1966) screened isolates of *A. flavus* for aflatoxin production on groundnuts, peanuts and on a nutrient liquid medium. Ninety percent of the isolates produced AFB_1. Reddy *et al.* (1971) have shown that as high as 30mg of aflatoxin per 100ml of a defined medium, containing sucrose, asparagine, and salts in both stationary and shaken cultures can be produced. High yields have also been achieved using yeast extract-sucrose medium (YES) (Park and Bullerman, 1981).

Isolation and purification of aflatoxins is an important aspect in view of the fact that other toxins can be co-produced with aflatoxins, especially cyclopiazonic acid (Gallagher *et al.*, 1978; Trucksess *et al.*, 1987; Blaney *et al.*, 1989; Gqaleni *et al.*, 1996). Purification of aflatoxins can be achieved by preparative TLC separation on silica gel G60 plates (Grimeno, 1979; Stahl, 1988). The techniques used to produce pure aflatoxins for subsequent use in this project are outlined in this chapter.

3.1.2 Materials and Methods

All reagents were of analytical grade unless stated otherwise. Thin layer silica gel G chromatography plates (20 x 20cm on aluminium backing) and silica gel G 60 were purchased from Merck.

The fungal culture

Aspergillus parasiticus (NIX) was maintained on potato dextrose agar (PDA, appendix1b). A spore suspension was prepared from one Petri dish of culture by adding 10ml of (0.1%) sterile sodium dodecyl sulphate and agitating with a sterile glass rod. This was vortexed and filtered through sterile glass wool to produce a spore inoculum.

Culture flasks were prepared by dispensing 100ml yeast extract sucrose medium (YES, appendix 1b) into 250ml Erlenmeyer flasks, plugging with cotton wool and autoclaving at 15psi for 15 minutes. Each flask was inoculated with 1ml of inoculum and incubated in a shake incubator at 28°C at 180 revolutions per minute for 48 hours. The culture fluid from 10 flasks was then filtered to remove mycelium and pooled. The filtered fluid was then extracted with an equal amount of chloroform twice. The chloroform extracts were dried by passing through sodium sulphate and then rotary evaporated to dryness, leaving a yellow powder which was redissolved in 15ml of chloroform and purified by preparative TLC as described below.

Preparation of preparative thin layer chromatography plates

Glass silica TLC plates, 20 x 20cm, were washed thoroughly with detergent, hot water, distilled water and finally dried with acetone. The plates were placed in a spreader and a slurry of pouring consistence was prepared by mixing 30.0g silica with distilled water (70ml). The slurry was poured into the spreader (set to a gap of 0.3mm) and the plates were spread evenly by smoothly pushing the spreader over the plates. The layer was allowed to set and the coated plates were placed in a rack and air dried. They were then activated overnight in an oven, at 110^{0}C.

Streaking of plates and extraction of AFB_1 from silica

Important: Aflatoxin B_1 is a human carcinogen, therefore, great care was exercised to avoid exposure to AFB_1 and its derivatives. Caution was exercised when AFB_1 was extracted from silica to avoid inhalation !

The plates were evenly streaked with the crude aflatoxin solution on an origin 1cm from one edge of the plate using a Pasteur pipette. The streak was repeated thrice to concentrate the sample. The plates were developed by ascending chromatography in chloroform/acetone (90:10), after which they were dried in a fume cupboard. The aflatoxins were then visualised under long wave

UV light for the presence of fluorescent bands. The R_f value of each toxin was determined according to the equation: R_f = distance migrated by spot/distance migrated by solvent front. Under UV light, each fluorescent band was scored using a sharp needle and the silica gel containing the band was scraped, using a spatula, into a sintered glass funnel and washed with portions of acetone (25ml in total) and the silica discarded. The acetone was evaporated on a rotary evaporator under vacuum, with the temperature set at 60^0C. The residue was dissolved in a minimum amount of chloroform and rechromatographed until the toxin was chromatographically pure.

Quantitation of aflatoxin B_1

The purified toxin was dissolved in 500μl acetone and a 10μl aliquot was transferred to another vial and evaporated to dryness. The residue was dissolved in 2ml spectrophotometric grade ethanol and the solution scanned (200 - 700nm) using a Milton Roy Spectrophotometer. The concentration of AFB_1 was calculated from the absorbance at a specified wavelength using its molar extinction coefficient (Cole and Cox, 1981).

3.1.3 Results

The purified toxins were recognised with respect to their R_f values, which were as follows: AFB_1 = 0.46 ; AFB_2 = 0.36 ; AFG_1 = 0.31 ; AFG_2 = 0.24 and their absorption spectra (see Fig. 3.1 for that of AFB_1). Aflatoxin B_1 was the toxin of major importance because it was required for the synthesis of AFB_1-8,9 epoxide.

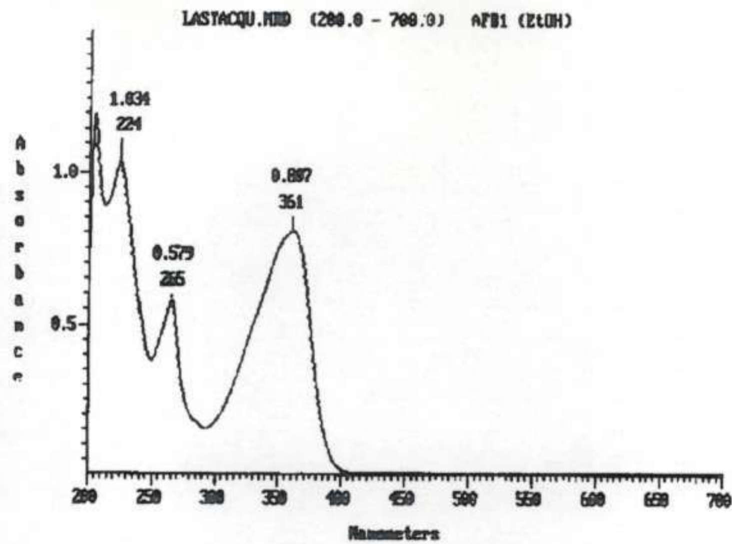


Figure 3.1 Spectrophotometric scan of AFB_1 in ethanol (spectrosol) monitored at 200 - 700nm.

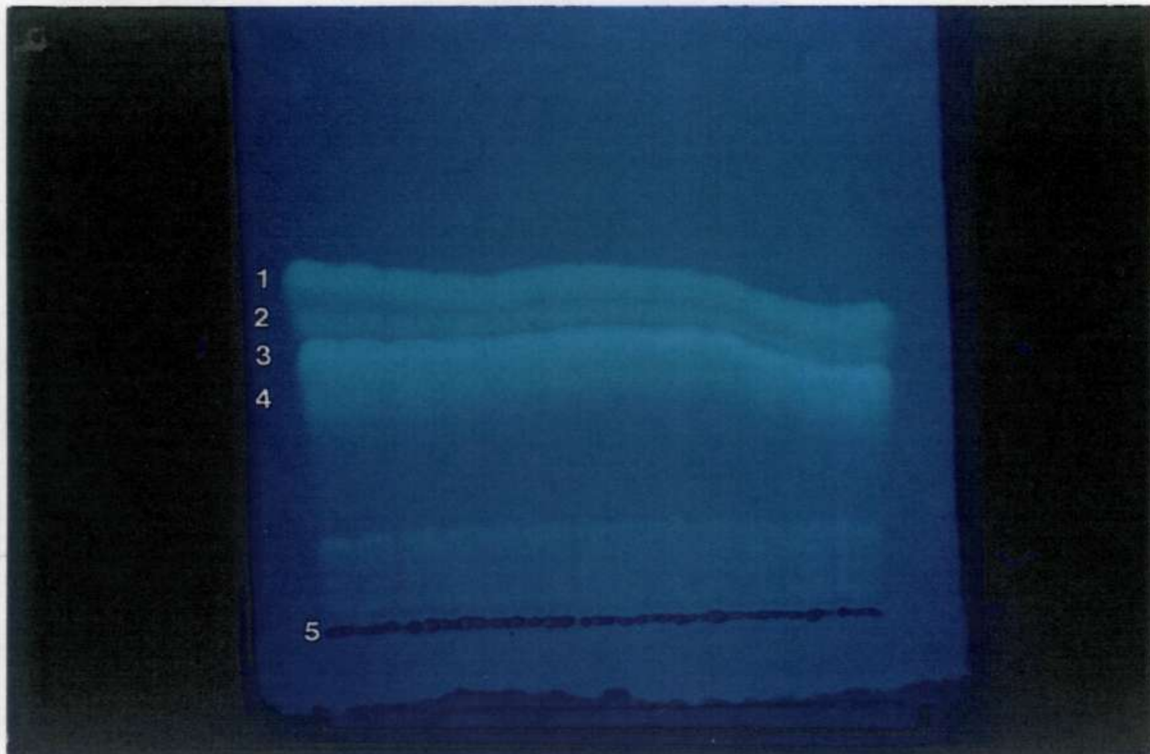


Plate 3.1. Aflatoxin extracts streaked on silica TLC plate and viewed under UV at 366nm. The chromatogram was developed in chloroform : acetone (90 : 10). 1=AFB_1, 2 = AFB_2, 3 = AFG_1, 4 = AFG_2 and 5 represents the origin.

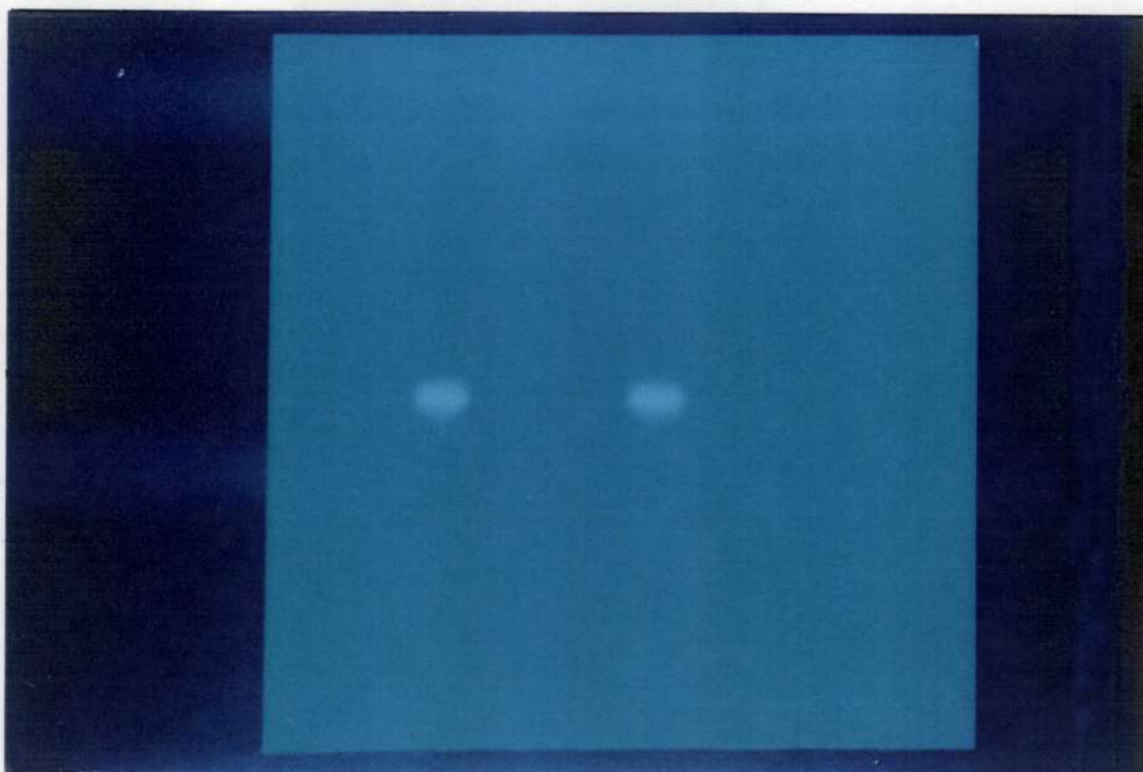


Plate 3.2 Aflatoxin B_1 spotted on silica TLC plate in duplicate and developed in chloroform : acetone (9 : 1). The plate was viewed under UV at 366nm.

The amount of AFB_1 recovered was calculated as follows:-

Concentration (c) = A/ε x molar mass of AFB_1 (g/l)

$$= \frac{1000 \times A \times 312\ (\mu g/ml)}{21800}$$

$$= \frac{1000 \times 1.474 \times 312}{21800}$$

$$= 21 \mu g/ml$$

(A is absorbance and ε is the molar extinction coefficient)

Therefore, 42 μg in the 2ml ethanol solution, which means:

42 μg in 10μl of the original sample. Therefore, in 0.5ml there were:

$$\frac{42 \mu g \times 0.5 ml}{0.01 ml}$$

$$= 2100 \mu g$$

3.1.4 Discussion

In order to obtain aflatoxins of adequate purity from cultures of *A. parasiticus*, preparative TLC was used, as the four major aflatoxins are very similar in chemical structure. The best solvent system to separate the toxins on preparative silica gel plates was found to be chloroform/acetone (90:10) which gave sufficient separation of the toxins as four fluorescent bands (Plate 3.1) and removes fungal lipids to the solvent front. The R_f values obtained were consistent with those reported in the literature for chloroform based solvent systems (Dutton, 1993) and gave a pattern on TLC similar to that reported by Yabe *et al.* (1988) for extracts from culture material of *A. parasiticus*.

Two problems were encountered during the isolation procedure. One was the presence of orange pigments, which often co-chromatographed with the aflatoxins. These are probably anthraquinone metabolites related to aflatoxin biosynthetic intermediates, which have been reported before (Donkersloot *et al.*, 1972; Stahl, 1988; Chuturgoon, 1990). They were also isolated for other studies and were effectively separated from the aflatoxins by addition of small amounts (10µl) of acetic acid to the TLC solvent system.

Another problem associated with preparative TLC of aflatoxins is the high ratio of silica gel to aflatoxin required to affect a separation. This results in grams of silica gel being scrapped from the plates with micrograms of toxin present. Losses are inevitable due to decomposition of the toxin on the silica and strong binding of an aflatoxin residue that could not be dislodged even with excessive washing with polar solvents. Losses of toxin impregnated silica gel were minimised by the use of glazed paper to pool silica scrapings and washing glassware well with the appropriate solvents.

The amount of pure AFB_1 obtained was 2.1mg/l of original YES culture fluid and, although this is low as compared to other workers (30mg/100ml of culture fluid, Yabe *et al.*, 1988), it was adequate for some of the investigations to be done. The UV spectra of the isolated toxins were similar to that reported in the literature (Cole and Cox, 1981) and indicated that the isolated toxins were of the required purity.

3.2 PREPARATION OF AFLATOXIN B_1-EPOXIDE

3.2.1 Introduction

Epoxidation is an important means of activating AFB_1 making it more susceptible to nucleophilic attack. It occurs by adding an oxygen atom between two unsaturated carbon atoms to form a mixture of two isomers, the exo- and endo forms. Exo-epoxidation of AFB_1 has an effect of increasing the toxicity of the parent compound by this activation, because it can react with important cellular macromolecules (McLean and Dutton, 1995; Manahan, 1990). In living systems AFB_1 is converted into the exo-epoxide by the so called phase I reactions mediated by the cytochrome P450 in the endoplasmic reticulum. In cellular detoxification by the phase II reaction, the epoxide readily undergoes conjugation with other species naturally present in the body e.g., glutathione, to form a substance (conjugation product) that can be readily excreted (Stark, 1980).

The epoxide may be synthesised *in vitro*, chemically, using either *m*-chloroperbenzoic acid or dioxirane reagent. Although epoxidation by *m*-chloroperbenzoic acid is usually simple, the dimethyldioxirane procedure remains the method of choice for preparation of exo-epoxide in crystalline form. This is because fewer impurities need to be removed during the crystallisation process and epoxidation with dimethyldioxirane is efficient and rapid (Iyer and Harris 1993). The objective reported here was to prepare the epoxide using dimethyldioxirane, so that its binding properties, especially with amino acids, could be evaluated.

3.2.2 Materials and Methods

All reagents were of analytical grade unless stated otherwise. Aflatoxin B_1 was either purchased from CSIR, Pretoria S. A. or produced in the laboratory. Cellulose TLC plates 20 x 20cm were obtained from Merck. Potassium monoperoxysulphate was purchased from Sigma Chemical Co. (St. Louis, U.S.A.), dimethylenedichloride (30ml) was dried over 50mg anhydrous sodium sulphate.

Preparation of dimethyldioxirane

Dimethyldioxirane was prepared from potassium monoperoxysulphate as a distilled solution in acetone (Murray and Jeyaraman, 1985) as follows:-

A mixture of distilled water (10ml), acetone (6ml), sodium bicarbonate (12mg) was prepared in a 500ml three-necked round bottom flask with constant stirring (magnetic stirring bar). The round bottom flask was equipped with a solid addition flask containing potassium peroxymonosulphate (24mg). An air condenser was connected to a dry ice condenser which was attached to a receiving flask (100ml), cooled in ice water. The solid potassium peroxysulphate was added in small portions (for 1 minute) with stirring to commence the reaction. After three minutes, a slight vacuum was applied to the reaction assembly. The yellow-coloured dimethyldioxirane-acetone solution collected primarily in the receiving flask. The solution, 8ml, was stirred briefly with 50mg anhydrous $MgSO_4$, filtered, and stored in a freezer for subsequent use.

Preparation of aflatoxin B_1 epoxide

Aflatoxin B_1 (10mg), was dissolved in 2ml dry acetone and 1ml dioxirane reagent was added. After twenty minutes (at room temperature), 5µl of the solution was spotted on a silica TLC plate against AFB_1 standard and developed as stated below (to check if epoxidation had taken place). The solvent and excess reagent were removed from the rest of the reaction mixture by evaporation under a stream of nitrogen and 1.5ml dry dimethylenedichloride was added. A fraction of the solution (4µl) was mixed with 4µl 1M HCl (for *trans*-diol experimentation). The remaining solution of the epoxide was stored at -20 ^{0}C for subsequent use.

Confirmation of the presence of the aflatoxin B_1 -8,9 epoxide

The silica TLC plate, spotted with the reaction mixture (epoxide), was developed in chloroform : ethylacetate : isopropanol (CEI) 18:1:1. The TLC plate was dried under a stream of warm air and tested for the presence of the epoxide as follows:-

The chromatograms were immersed in epoxide staining solution I (Appendix I), for 1 to 2 seconds and heated to 130°C for 25 min. They were then cooled to room temperature and immersed for 1 to 2 seconds in epoxide staining solution II (Appendix 1).

3.2.3 Results

The R_f value of the epoxide, which gave a green chromophore with the epoxide stain, was found to be 0.16 in CEI, whilst that of AFB_1 was 0.54 under the same conditions (Fig. 3.2). The spectrophotometric scan (Fig. 3.2) showed that almost all AFB_1 was converted to the epoxide. Aflatoxin B_1-epoxide showed a maximum wavelength at 266 and 367nm (Plate 3.3). The scan of AFB_1O (Fig. 3.2) was different from that of AFB_1 (Fig. 3.1, page 32).

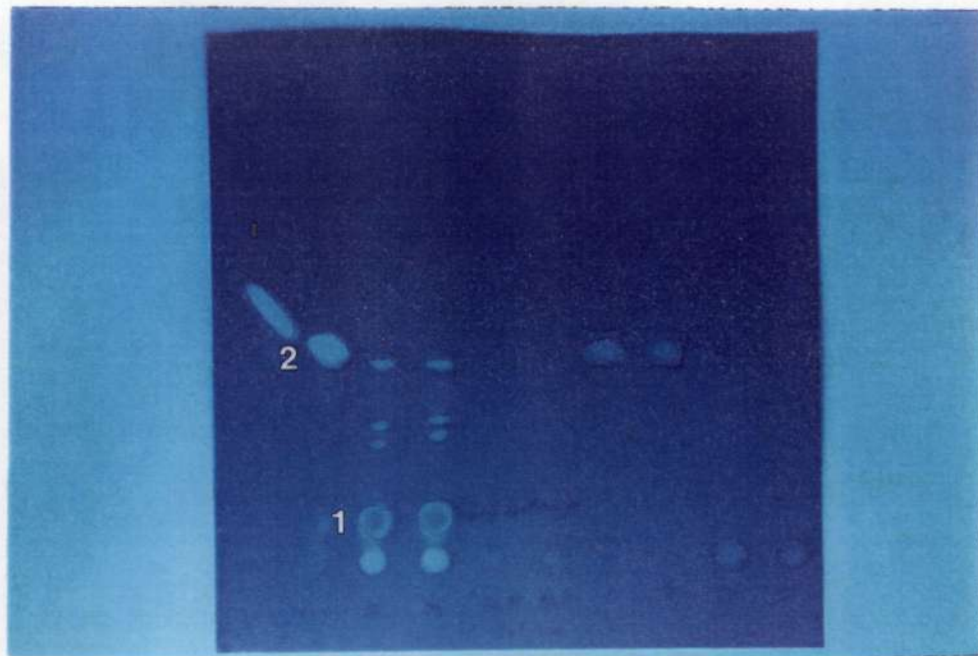


Plate 3.3 Aflatoxin B_1 - epoxide reaction mixture (1) spotted against AFB_1 (2) on silica TLC plate and developed in chloroform : ethyl-acetate : isopropanol (18 : 1 : 1). The plate was viewed under long wave UV at 366nm.

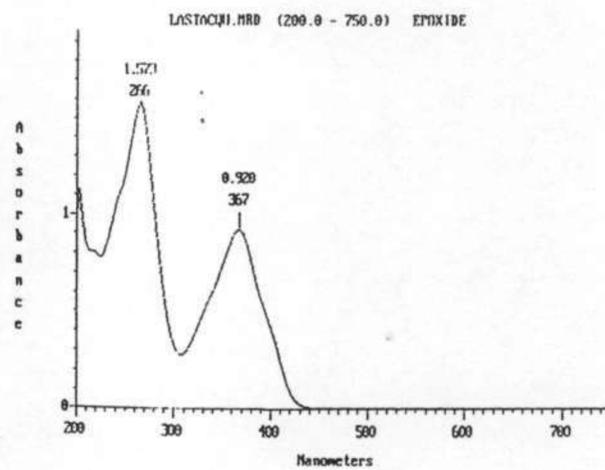


Figure 3.2 Spectrophotometric scan of AFB_1O (200 - 750nm) in ethanol.

3.2.4 Discussion

Initial attempts to prepare the dimethyldioxirane reagent were not successful. The liquid that distilled over was colourless, an indication that dimethyldioxirane was too diluted (should be yellow) and did not have the distinctive odour of dioxirane. It was found from further experimentation that the vacuum generated to allow for distillation at reduced temperatures was critical, the optimum being around 50mm of mercury. Although the reagent is reported to be stable for up to five months when stored at -20°C, in this case it was used on the same day of production to synthesise AFB_1O.

Examination of the reaction mixture from treated AFB_1 by TLC showed a single spot at R_f 0.16 in the CEI solvent system as compared to the parent AFB_1 at R_f 0.54. This change in R_f value is to be expected as the epoxide is more polar than AFB_1 and has a higher molecular mass 328 as compared with 312 for the parent AFB_1 toxin. That it was an epoxide was confirmed by its reaction with the epoxide detecting reagent to give a green colour. Aflatoxin B_1 does not react with this reagent.

Unfortunately it was not possible to use TLC to further purify the epoxide as it had to be eluted with highly polar solvents, e.g., methanol, from the silica gel which resulted in the formation of a methoxy decomposition product. No attempts were made to separate the endo from the exo form of the epoxide, as this was unnecessary in the further series of reactions to be done.

CHAPTER 4

REACTION OF SELECTED N_α-ACETYL AMINO ACIDS WITH AFLATOXIN B_1-8,9 EPOXIDE

4.1 Introduction

Amino acids (AA) are the basic structural units of proteins. They contain an amino group, a carboxyl group, a hydrogen atom and a distinctive R group bonded to a carbon atom, which is called the alpha (α) carbon. Twenty kinds of side chains varying in size, shape, charge, hydrogen bonding capacity, and chemical reactivity, are commonly found in proteins including serum albumin and therefore, are available for reaction with AFB_1-8,9 epoxide (AFB_1O).

The bound AA likely to react are: lysine (LYS), which has already been identified in an adduct form, arginine (ARG), and asparagine (ASN); tyrosine (TYR); serine (SER); histidine (HIS); and tryptophan (TRP). In order to prepare amino acid-AFB_1-adducts, N_α acetyl AA were reacted with AFB_1O. N_α acetyl AA were used because they have the alpha amino group blocked, ensuring that this group could not react, and that any derivatisation would occur on the side chain. Methods to detect and monitor the product formed between an N_α acetyl AA and AFB_1O include TLC and UV spectrophotometry.

4.2 Materials and Methods

The N_α acetyl derivatives of: LYS, SER, HIS, TRP, TYR, ARG and ASN were obtained from Sigma Chemical Co. (St. Louis, USA). Aflatoxin B_1 epoxide was prepared as described in Chapter 3.

4.2.1 Preparation of the Adduct

An N_α-acetyl AA (10 mg) was dissolved in 4ml of 0.05M sodium phosphate buffer pH 7.4, and 0.4mg of AFB_1O, dissolved in 100µl acetone, was slowly added with mixing. The development of a yellow coloured complex was noted on the addition of the epoxide. The reaction was allowed to continue at 35°C, with shaking, for 86 hours.

4.2.2 Qualitative Analysis

Spectrophotometric scan

After 86 hours the UV scan of the N_α-acetyl amino acid-epoxide mixtures were taken (200nm - 700nm) and compared. These complexes were also spotted on cellulose thin layer chromatography (CTLC) plates and developed in butanol/water/acetic acid (BWA) (12:5:3). The plates were dried and viewed under long wave (366nm) UV light and the presence/absence of AFB_1O noted. The plates were then treated with chlorine and sprayed with tolidine reagent (appendix I) to confirm the presence/absence of AFB_1-8,9-epoxide- N_α-acetyl AA complexes.

4.3 Results and discussion

Thin layer chromatography coupled with UV spectroscopy offers an important complementary tool for monitoring the formation of aflatoxin adducts with biochemical molecules. The former technique is mainly used for qualitative evaluation and to a lesser extent semi-quantitation. In these studies reaction mixtures of N_α-acetyl AA and AFB_1O were spotted onto cellulose plates as these are far less polar than silica gel layers and allow chromatographic movement of amino acids, and their derivatives in suitable solvent systems such as IWA and BWA. In this case BWA was chosen as the ammonium hydroxide in the IWA system reacts with the AFB_1O adduct to form yellow decomposition products.

Although AFB_1O adducts should fluoresce due to the coumarin chromophore in the system the fluorescence is diffuse and is over laid by streaking due to unreacted and decomposed AFB_1O. In order to visualise the adducts the chromatograms were sprayed with tolidine after

treatment with chlorine gas. Although N_α-acetyl AA in theory could react in this system, only the adducts did to any degree and this was a convenient way to decide when reaction had taken place. On TLC of the various reaction mixtures it was found that only amino acids with reactive nitrogen atoms in their side chains had formed adducts (Fig. 4.1). These were the N_α-acetyl derivative of arginine, lysine and histidine. From observation of the intensity of the spot on the TLC plate the degree of reaction was in that order. It seems that the more basic AA react more favourably with the epoxide than histidine, which is more likely, as they are more nucleophilic in nature but the actual yields were found to be in the descending order of arginine, histidine and lysine. It also seems that both the exo and endo forms of the epoxide react under these conditions as opposed to the exo- form only, *in vivo*. Although it is not apparent from the TLC plate the spots did exhibit diffused fluorescence under UV light which confirmed their identity as adducts, because no corresponding fluorescenses were observed in the chromatograms of the other AA reaction mixtures.

The use of spectroscopy in these studies was also useful and allowed for the quantitation of the adducts from their absorption at selected wavelengths and the application of literature extinction coefficients. Amino acids normally absorb at 210nm (Fig. 4.2, 4.4 and 4.6) with aromatic AA absorbing at 280nm (Apendix II). Aflatoxin B_1 absorbs at 365nm with its derivative, AFB_1O absorbing at 266 and 367nm (Fig. 3.2). The adduct of AA with AFB_1O absorb at 395nm and it follows that other adducts should absorb in the range 390 - 400nm at neutral pH. Normally this form is only generated in alkaline pH but in this case it is due to the presence of the open ring form of the bisfuran system due to the formation of the adduct (Fig. 2.6). All three acetyl AA shown to react with AFB_1O gave this characteristic peak (Fig. 4.3, 4.5, and 4.7). Arginine adduct absorbed at 392nm and using the lysyl-AFB_1 extinction value gave a concentration of 0.21mg/ml in the original phosphate buffer; histidine absorbed at 391nm and gave a concentration of 0.31mg/ml and lysine at 397 and gave a concentration of 0.18mg/ml. The lysyl adduct (Fig. 4.5) also showed a small peak at 349nm which is possibly due to unreacted epoxide or its decomposition product. The reaction mixtures containing N_α-acetyl serine, tyrosine, and tryptophan (Appendix II) showed no absorption at 390nm, only those characteristic of the amino or epoxide.

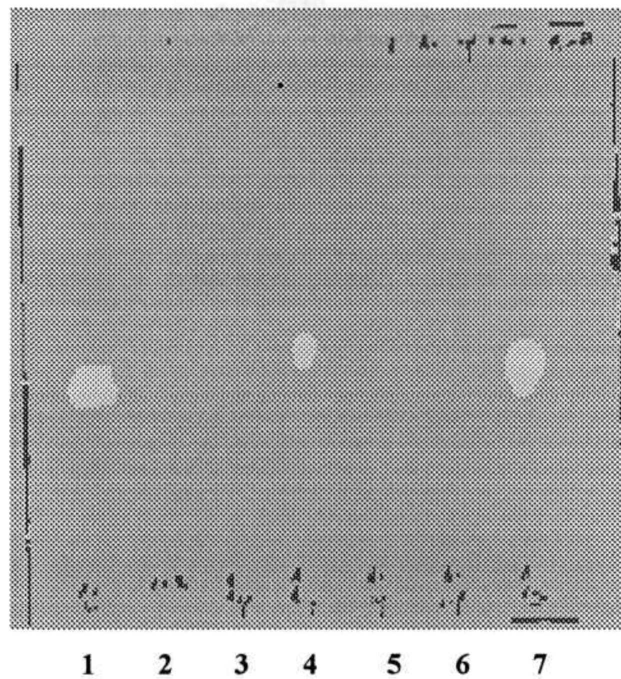
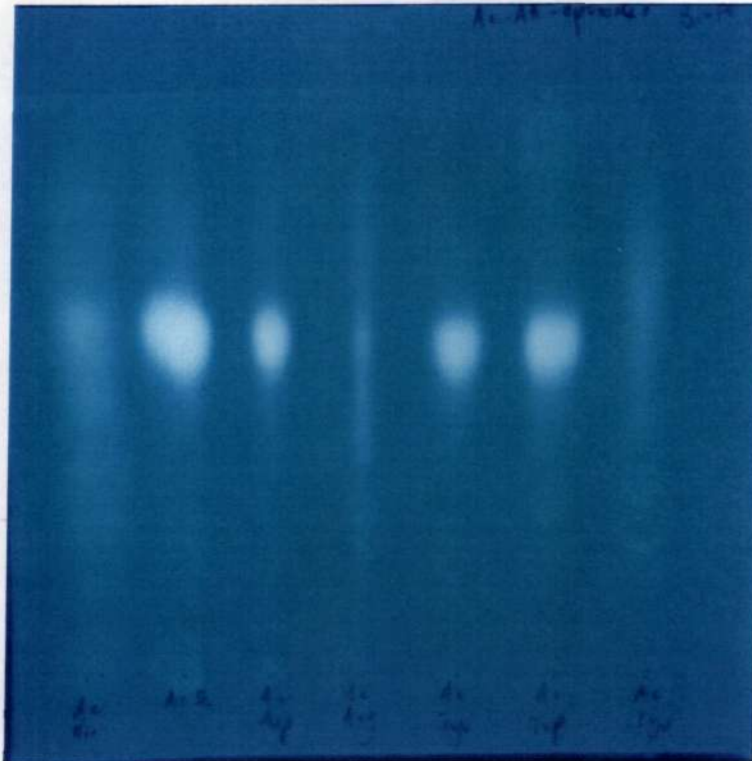


Figure 4.1 Thin layer chromatograms of N_α acetyl AA-AFB$_1$O mixtures (developed in BWA) after treatment with chlorine and spraying with tolidine reagent. Compounds with N acyl linkages show up as coloured spots. N_α acetyl AA used were :

Lane 1 N_α-acetyl arginine,
Lane 2 N_α-acetyl serine,
Lane 3 N_α-acetyl asparagine,
Lane 4 N_α-histidine,
Lane 5 N_α-acetyl tyrosine,
Lane 6 N_α-acetyl tryptophan and
Lane 7 N_α-acetyl lysine.



1 2 3 4 5 6 7

Plate 4.1 Photograph of CTLC plate (Fig 4.1) viewed under UV (366nm) before chlorination - treatment and tolidine spray reagent. The bright spots at 2, 3, 5 and 6 are free AFB_1O indicating negative reaction with the acetyl amino acids.

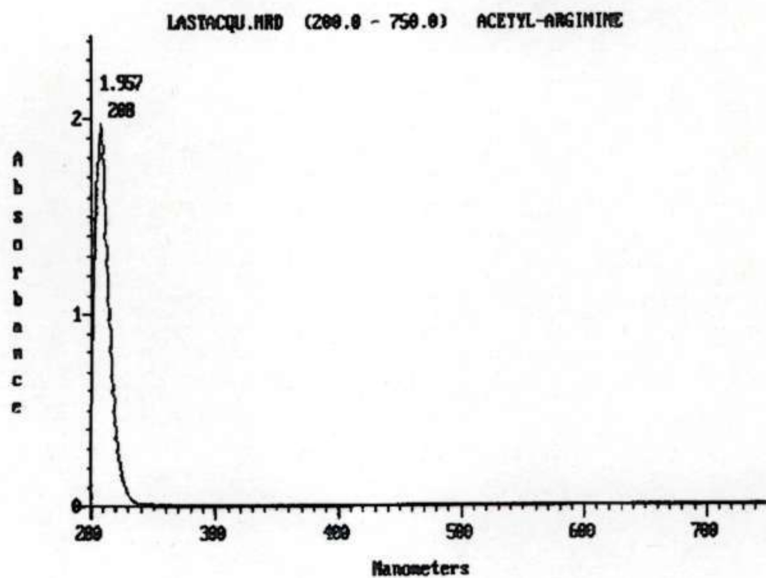


Figure 4.2 Spectrophotometric scan of N_α-acetyl-arginine monitored at 200 - 750nm in 0.1M sodium phosphate buffer pH 7.4.

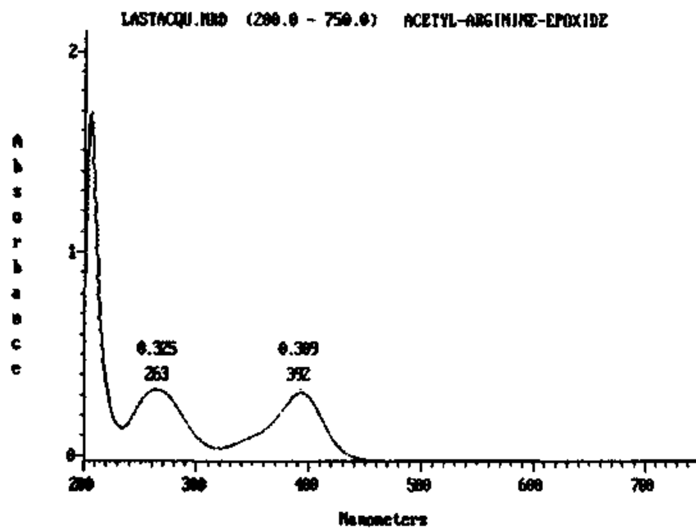


Figure 4.3 Spectrophotometric scan of N_{α}-acetyl-arginine-AFB_1O mixture monitored at 200 - 750nm in 0.1M sodium phosphate buffer pH 7.4.

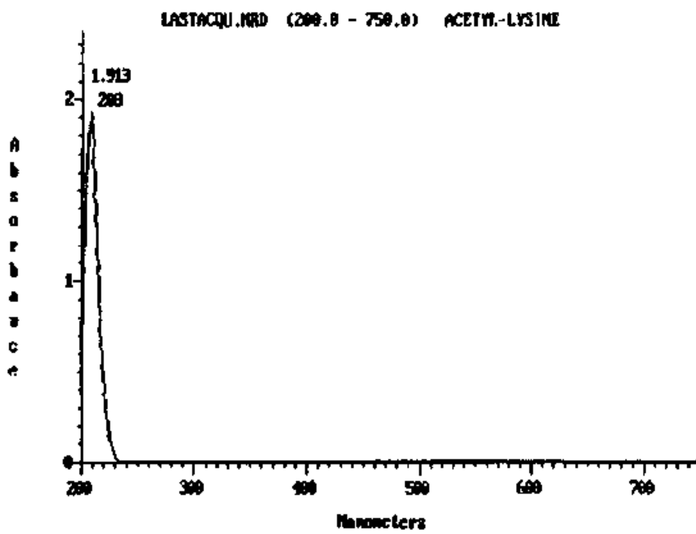


Figure 4.4 Spectrophotometric scan of N_{α}-acetyl-lysine monitored at 200 - 750nm in 0.1M sodium phosphate buffer, pH 7.4.

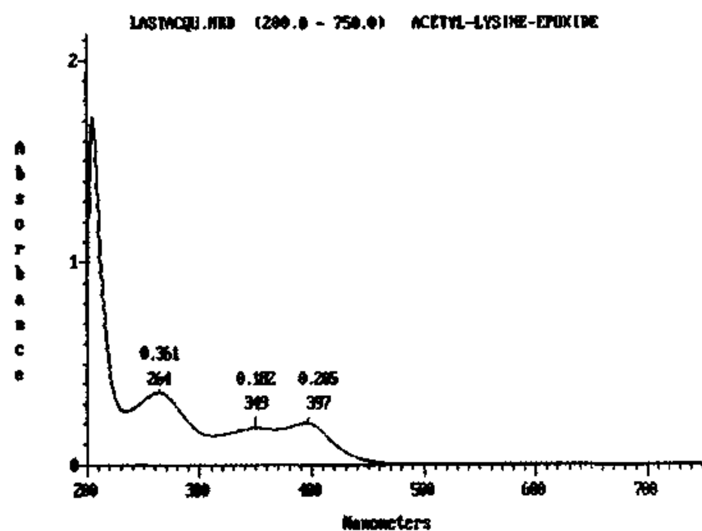


Figure 4.5 Spectrophotometric scan of N_{α}-acetyl-lysine-AFB_1O mixture monitored at 200 - 750nm in 0.1M sodium phosphate buffer, pH 7.4

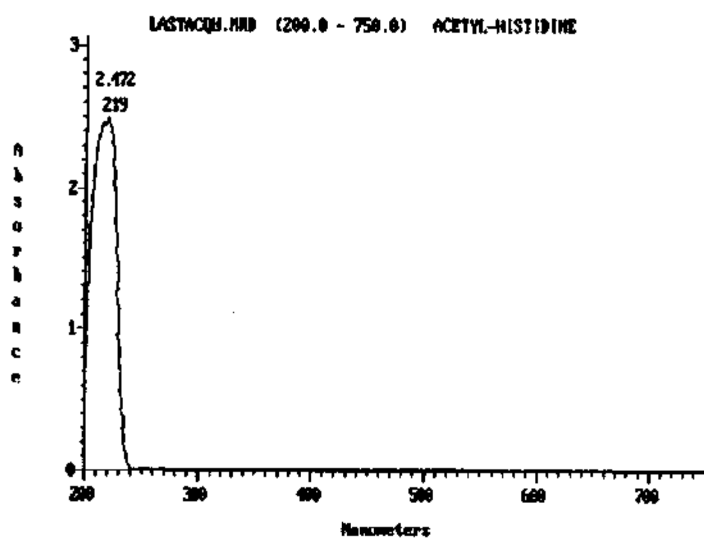


Figure 4.6 Spectrophotometric scan of N_{α}acetyl-histidine monitored at 200 - 750nm in 0.1M sodium phosphate buffer, pH 7.4

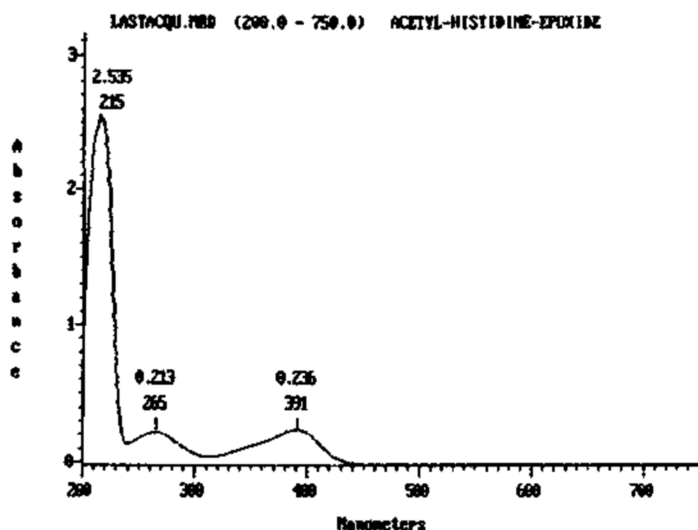


Figure 4.7 Spectrophotometric scan of N_{α}-acetyl-histidine-AFB_1O monitored at 200 - 750nm in 0.1M sodium phosphate buffer pH 7.4.

4.4 Conclusion

From this work it has been shown that AFB_1O combines with more than one amino acid *in vitro*. This was supported by both TLC and spectroscopic data. It therefore, follows that the epoxide would react with more than one amino acid residue *in vivo*. This is similar to the situation with DNA, which was thought for some time to react with AFB_1O through its guanine residues but cytosine (Yu *et al.*, 1991) and more recently (Iyer *et al.*, 1994) adenine have also been found to react. Other workers (e.g., Wild *et al.*, 1990b) have also suggested that adducts other than lysine are formed *in vivo*, as peptides formed from albumin enzyme digests not containing lysine have been shown to have adducted AFB_1. It seems highly probably that these will be adducts of histidine and arginine as shown by preliminary studies (Myeni *et al.*, 1997).

CHAPTER 5

PREPARATION OF AFLATOXIN B_1-ADDUCTS OF DNA, LYSINE AND ALBUMIN

5.1 Introduction

A number of assays may be used to recognise and quantitate the product of the interaction of AFB_1O with either DNA or protein. These methods have been reviewed in section 2.7. In this study, assays that quantitate defined adducts of DNA or protein, have been opted for, because they are specific as far as adduct detection. These methods require the initial preparation of pure and chemically defined adducts, which are then searched for in the somatic tissue of exposed individuals. Such methods require substantial chemical and biochemical preparations and involve the selection of DNA or protein adducts most worthy of study. Aflatoxin B_1-guanyl, AFB_1-DNA, AFB_1-lysyl and AFB_1-albumin adducts were selected because it has been shown that AFB_1 interacts mostly with the lysine residues of albumin and with the guanine moiety of DNA.

Aflatoxin B_1 adducts may be detected using high performance liquid chromatography (HPLC), high performance capillary electrophoresis (CE) and UV spectrophotometry. High performance capillary electrophoresis is a separation technique based on electrophoresis in narrow-base capillaries, usually 25 - 75μm inner diameter (id). Capillaries are usually filled with only buffer and the advantages of capillaries are mostly related to the Joule, since they have a high electrical resistance. This enables the application of very high electrical fields (100 to 500V/cm), with minimal heat generation. The heat that is generated is efficiently dissipated by the large surface area-to volume ratio of the capillary. High electrical fields result in short analysis times and high efficiency and resolution. An important aspect of CE practice is electro-osmotic, or electroendosmotic flow (EOF). This is the bulk flow of liquid in the capillary and is caused by the surface charge on the interior capillary wall.

Electroendosmotic flow causes movement of almost all species, regardless of charge, in the same direction. Normally, the flow is from the anode to the cathode. Anions will be flushed towards the cathode since the magnitude of the flow can be more than an order of magnitude greater than their electrophoretic mobilities. Cations, neutrals, and anions can, therefore, be

electrophoresed in a single run because they all migrate in the same direction. Cations migrate fastest, neutrals are all carried at the velocity of the EOF but are not separated from each other, anions migrate slowest because of their attraction to the anode but are still carried by the EOF toward the cathode (Heiger, 1992).

High performance capillary electrophoresis is a rapidly growing separation technique, with a diverse application range. It has been used on a number of analyses of different compounds like haemoglobin variants (Castagnola *et al.*, 1995); human serum proteins and apolipoproteins (Lehmann *et al.*, 1995); seed albumins (Salmanowicz, 1995); and underivatized amino acids (Guo *et al.*, 1995). It offers simpler method development, minimal sample volume (1 to 10nl) and lack of organic waste.

High performance liquid chromatography was selected because when coupled with fluorescence detection it is more sensitive than CE. The columns for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressures of up to 5.5×10^7 Pa (8000 p.s.i.). Straight columns of 20 to 50cm in length and 1 to 4mm in diameter are generally used though smaller capillary columns are available. The choice of mobile phase to be used in any separations will depend on the type of separation to be achieved. Isocratic separations may be made with a single solvent, or two or more solvents mixed in fixed proportions. Alternatively a gradient elution system may be used where the composition of the developing solvent is continuously changed by use of a suitable gradient programmer. Generally this involves use of two pumps. One of the most important features of an HPLC system is the pumping system.

It is imperative that the sensitivity of the detector system is sufficiently high and stable, since the quantity of material applied to the column is frequently very small. Commonly the detector is a variable wavelength ultraviolet-visible spectrophotometer, fluorimeter, a refractive index monitor or an electrochemical detector. Recently there has been an interfacing of HPLC to a mass spectrometer.

In this study CE was used to analyse the products of the reactions of macromolecules with AFB_1O and HPLC the monomer and hydrolysis products of the macromolecular adducts.

5.2 Materials and Methods

5.2.1 *Materials*

N_α-acetyl-l-lysine, proteinase K, and diazald were all purchased from Sigma Chemical Co. (St. Louis, U.S.A.), albumin, DNA, acylase I and amberlite (IR 120, Na) standard grade particle size 1.18mm were supplied by BDH Laboratory Supplies (England). Tolidine and ninhydrin spray reagents were prepared as indicated in appendix Ia.

Spray Reagents

Ninhydrin and tolidine spray reagents were used for detection of free amines and nitrogen containing compounds which can be converted into chloramines respectively.

5.2.2 *Preparation of Adducts and Derivatives*

Aflatoxin B_1 Albumin

Aflatoxin B_1 8,9-epoxide (0.8mg, prepared as described in Chapter 3) was added to 40mg albumin (fraction V) in 0.05M sodium phosphate buffer (4ml), pH 7.4 and incubated at 37°C for 24 hours. The UV spectrum of the product of reaction was determined at a 4-hour interval, by mixing 10µl of the AFB_1 albumin sample with 3ml phosphate buffer. The AFB_1 albumin solution was also examined by CE to follow the course of the reaction.

After 24 hrs at 37°C, 5µl of the sample was spotted on cellulose TLC plate, and developed two dimensionally in (1) BWA both ways and (2) IWA/BWA to check for fluorescence. The plate was observed under UV and sprayed with ninhydrin. The remaining sample was digested with proteinase K as follows: proteinase K (6.7mg, dissolved in 2ml sodium phosphate buffer, 0.05M, pH 7.4) was added to 2ml of reaction mixture and incubated at 37°C overnight, with shaking. The digest was then spotted on cellulose TLC plates and developed in BWA. The plates were observed under UV, sprayed with ninhydrin and different amino acids identified using standard R_f values (Jones and Heathcote, 1966). The sample was then filtered and examined by HPLC.

Aflatoxin B_1 Lysyl Adduct

N_α-acetyl-L-lysine (23.5 mg), was dissolved in 2ml sodium phosphate buffer, 0.05 M, pH 7.4 and incubated at 37°C with 0.8mg AFB_1-epoxide. After 24 hours the reaction mixture was freeze dried and extracted with acetone (10ml) to remove acetone soluble residues. The acetone was pooled, and run on a silica TLC plate, developed in CEI/TEF. The non-acetone soluble residue was dissolved in a minimum amount (300μl) of buffer and 5μl was spotted on a CTLC plate and developed in two separate solvent systems: the first solvent system was IWA for the first direction and BWA for the second direction; and the second solvent system consisted of BWA for both directions. Tolidine and ninhydrin spray reagents (appendix I) were used for detection of N_α-acetyl lysine derivatives. The sample was then incubated with acylase I (2.5mg, dissolved in 4ml 0.1M phosphate buffer, pH 7.4) for 24 hours at 37°C.

Clean Up of Adduct By Ion Exchange

Amberlite resin, IR 120, Na standard grade particle size 1.18mm, was packed half way a 27 x 50mm glass column which had been previously filled with distilled water. Care was taken to make sure that no air spaces were in the column. The amberlite column, was then prepared by washing with distilled water (2000ml) until it was neutral. The column was washed with 1M HCl (20ml), to convert the resin to its acid form and then it was washed with distilled water (20ml) until the effluent was neutral. The N_α-acetyl-lysine-AFB_1 mixture was freeze-dried in a Virtis freeze dryer. The powder was dissolved in 4ml of distilled water and run on the column, elution of N_α-acetyl-lysine-AFB_1 was effected with 25ml distilled water. Excessive N_α-acetyl-lysine was washed off with ammonia (2M, 15ml). The column was regenerated by washing with 1M NaOH (20ml) followed with distilled water (1000ml) until the effluent was neutral and reconstituted into its acid form as above.

Methylation of N_α-acetyl-Amino Acids

This reaction was carried out to block the carboxyl group of the N-terminal AA to ensure that only the side chain functional group reacted. Two methods were used:

(i) Diazomethane Method

Ethanol (95%, 25ml) was added to a solution of potassium hydroxide (5g) in 8ml water in a 100ml distillation flask fitted with dropping funnel and an efficient condenser for distillation. A solution of 21.5g diazald in 200ml of the ether was added through a dropping funnel over a period of about 25 minutes to the ethanolic potassium hydroxide solution warmed to 65°C in a water bath. The yellow distillate was passed onto a receiver flask cooled to 0°C in an ice bath. After the addition, a further 40ml of ether was added and slowly distilled until the distillate was colourless. The etherical solution contained about 3g of diazomethane. Because of the toxic and explosive nature of diazomethane, the apparatus was kept in a fume cupboard and the reaction mixture was topped up with dry ether at all times. The following N_α-acylated amino acids were reacted with the diazomethane:

Nonpolar/hydrophobic amino acid i.e., tryptophan; *polar but uncharged amino acids* i.e., serine, cysteine, tyrosine, asparagine and lysine; *positively charged R group amino acids* i.e., arginine and histidine; *negatively charged amino acid* i.e., aspartic acid.

(ii) Esterification Method

N_α-acetyl-l-lysine, 20mg, was weighed, suspended in 400µl of high purity methanol and reacted with thionyl chloride (three drops). After an hour at room temperature, the reaction mixture was neutralised with solid sodium bicarbonate (6mg). Dry analar acetone (2ml) was used to extract the ester. A sample of the acetone extract (5µl) was spotted on a CTLC plate and developed in two dimensional chromatography to check for methylation of N_α-acetyl-l-lysine: 2 x BWA; and IWA/BWA. The acetone was evaporated and the ester was again spotted as mentioned above.

Reaction of Methylated Amino Acids With Aflatoxin B_1 Epoxide

Methylated N_α-acetyl-l-lysine was dissolved in phosphate buffer at pH 7.2 and reacted with AFB_1-epoxide and spotted on TLC. Different solvent systems in two dimensions were used, i.e., IWA/BWA, 2x BWA and 2x CEI to characterise the product by CTLC. The reaction was repeated at pH 5.6 and 8.6.

Reaction of DNA With Aflatoxin B_1 Epoxide

A solution of AFB_1-epoxides (0.2mg), in anhydrous acetone, 100μl was added with stirring to 2mg calf thymus DNA dissolved in 1.0 ml sodium phosphate buffer, pH 7.2 at 10°C. After thirty minutes at room temperature the reaction mixture was acidified to pH 2.0 with 0.1 M HCl. The reaction mixture was heated at 80°C for thirty minutes and filtered through 0.45 μm filter and subjected to UV spectrophotometry. Guanyl AFB_1O adduct was prepared in exactly the same way, substituting guanine for DNA.

Preparation of Albumin and Serum Protein Adducts

Serum (80μl) pH 7 was added to 0.1mg epoxide (dissolved in 40μl acetone) and incubated overnight at 37°C. Albumin (20mg) was dissolved in 0.05M sodium phosphate buffer, pH 7.4. The reaction product and albumin solutions were each (5μl) examined by Poly-Acrylamide Gel Electrophoresis (PAGE) and a Serum Protein Electrophoresis (SPE) kit.

5.2.3 Determination of Thin Layer Chromatographic Characteristics of Products

The following compounds in addition to N_α-acetyl lysine were reacted with AFB_1O: lysine, methyl N-acetyl lysine albumin and albumin digest. Each of these compounds (0.11g) was dissolved in 4ml phosphate buffer (pH 7.4; 0.1M) and 20μl was spotted on CTLC plates. Cellulose plates were run two-dimensionally using either HS/BWA, or IWA/BWA solvent system (Haworth and Heathcote, 1969). The compounds were also spotted on silica plates and developed uni-dimensionally on CEI. The plates were sprayed with 0.2% ethanolic ninhydrin.

5.2.4 Instrumental Analysis of Reaction Products

High Performance Liquid Chromatography of Aflatoxin B_1 lysyl Adduct

Two HPLC chromatographic instruments were used to analyse the lysyl AFB_1 adduct depending upon the exact conditions used and availability of instrumentation, namely a Spectra Physics fitted with a C_{18} reverse phase (RP) Merck column and fluorescence detector. The other instrument used was a Hewlett Packard 1090 HPLC system fitted with a C_8 RP Phenomenox column.

Two systems were used:

(i) The chromatography consisting of a 2 minute linear gradient solvent system similar to that used by Sabbioni *et al.* (1990) with a C_{18} RP column (Merck).

(ii) An isocratic solvent system consisting of 0.01M sodium phosphate buffer pH 7.2/methanol (50 : 50 or 70 : 30). In later analyses a C_8 Phenomenox column (Merck) was used with sodium phosphate buffer at 0.1M pH 3.1.

In all cases the flow rate was set to 0.5ml per minute and injections were repeated at least three times to check reproducibilty.

High Performance Capillary Electrophoresis of Macromolecular Adducts

Analysis was performed on Hewlett Packard CE using a UV detector. Aflatoxin B_1 8,9-epoxide formed as described in Chapter 3, was reacted with an albumin solution (20mg albumin, dissolved in 0.05M sodium phosphate buffer pH 10). The reaction was allowed to continue for 12 hours at 37°C. The mixture was then filtered and analysed using CE under the following conditions:- 0.1M sodium phosphate buffer pH 3, injection at 200.0 mbars, voltage set at 15 Kv. The CE wavelengths were set at 210, 260, 280, 293, and 400nm. The adduct was detected. An albumin solution was used as a standard and was also analysed using CE conditions similar to those for the adduct.

5.3 Results and Discussion

Having digested AFB_1-albumin mixture, a number of AA residues (Table5.1) were identified using standard R_f values. Some of the AA residues showed slight fluorescence under UV light, which indicated their positions (R_f values), which was an indication of AFB_1O binding. It was not possible to identify all the AA as the R_f values did not correspond to known values. This could be explained partly due to the possible interaction between AFB_1O and AA. A better separation of the residues was effected using two dimensional TLC in BWA both ways. This solvent system was used to avoid possible decomposition of adducts by NH_3 in the IWA system.

For purification purposes, N_α-acetyl-lysine-AFB_1O mixture was passed through a cation exchange column. This was done so that N_α-acetyl-lysine could be separated from N_α-acetyl-lysine-AFB_1O. The principal feature underlying ion exchange chromatography is the attraction between oppositely charged particles. Amino acids, like many biological, molecules have ionisable groups and the fact that they may carry a net positive or negative charge was utilised in separating mixtures of such compounds. N_α-acetyl-lysine-AFB_1O would be less basic than N_α-acetyl-lysine and as cation exchangers possess acid groups there would be no attraction and the adduct would pass through the column. N_α-acetyl-lysine, however has both a basic and acidic group and would bind to the column.

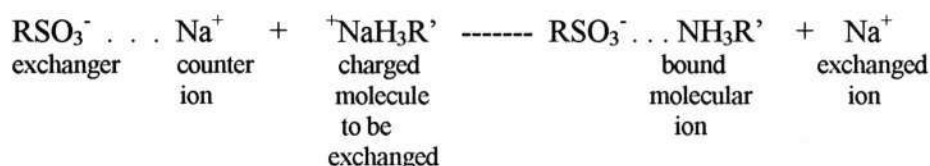
Hydrolysis of aflatoxin B_1-albumin

After the hydrolysis of the protein as mentioned above, the following amino acids were identified using standard R_f values:-

Table 5.1 R_f values of the hydrolysate of AFB_1-albumin.

R_f in BWA	R_f in IWA	AMINO ACID
0.05	0.18	cysteine
0.16	0.22	arginine
0.17	0.37	glutamine
0.25	0.45	threonine
0.36	0.53	proline
0.42	0.65	unidentified
0.52	0.65	methionine
0.59	0.73	phenylalanine
0.66	0.82	isoleucine
0.60	0.57	unidentified
0.42	0.23	unidentified
0.30	0.27	unidentified
0.20	0.67	unidentified

Exchange of ions at the exchange site is thought to occur instantaneously and is an equilibrium process:



Although other workers (Sabbioni *et al.*, 1990) have recommended the use of N_α-acetyl lysine to avoid the reaction of the α amino group with AFB_1O, initial work on this reaction indicated that a certain amount of decomposition of the adduct took place particularly under the conditions of TLC. A possibility was that the α carboxyl group also took part in the reaction and in order to exclude this it was decided to methylate the N_α-acetyl AA. Initially methylation was done using diazomethane but yields were low and the alternative method using esterification with methanol and thionyl chloride proved more effective. The reaction of methylated amino acids with

AFB_1O was found to be favoured at pH 8.6 and 7.2, but not at pH 5.6. this suggests that lysyl-AFB_1 production is not well promoted at pH 5.6. This information is essential when preparing AA-AFB_1 adducts. When Guanine was incubated with AFB_1, at pH 7.2, a shift in absorption maximum was observed. The shift was from 362nm to 365nm (appendix 1c). Similar shifts have been observed by Sporn *et al.*, 1966. A similar trend was observed with DNA.

Initially difficulties were encountered on the HPLC of the AFB_1O adduct because of the build up of pressure in the pumping system. This was traced to the use of the gradient solvent system which caused the polar components of the reaction mixture to precipitate out on the top of the column when the solvent mixture became methanol rich. In order to overcome this an isocratic system was used (phosphate buffer/methanol 50 : 50) but this also caused a steady build-up of pressure, again possible because of slow precipitation. On increasing the buffer content to 70% the intensity of the adduct peak decreased and the retention was substantially increased.

These problems were finally overcome when a C_8 Phenomenox column was employed together with 0.1M phosphate buffer (pH 3.1). Under these conditions the adduct had a retention time of 3.5 minutes (Fig. 5.1) on the Spectra Physics system and 3.2 minutes (Fig. 5.2) using the Hewlett Packard 1090 instrument. The respective values of AFB_1 were 6.4 minutes on the Spectra Physics (Fig. 5.4) and 7.4 minutes (Fig. 5.3) minutes using the Hewlett Packard 1090 instrument. The peaks were found to be reproducible within a range of ±0.2 minutes for repeated injections. Under acid conditions (pH 3.1) the UV absorption spectrum of AFB_1-lysine adduct shifted from 390nm to 370nm (Fig.5.5). Similar pH dependency of the UV spectra has been observed by Garner *et al.*, 1979; Sabbioni *et al.*, 1987; and Sabbioni and Wild, 1991.

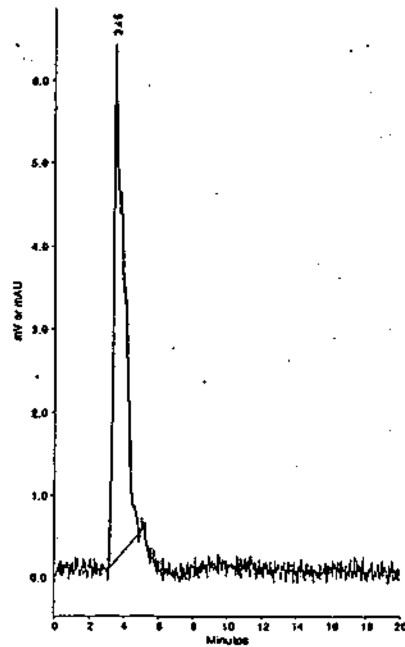


Figure 5.1 HPLC profile of AFB_1-lysine conjugate. The conditions were:- 0.02M sodium phosphate buffer, pH7.2

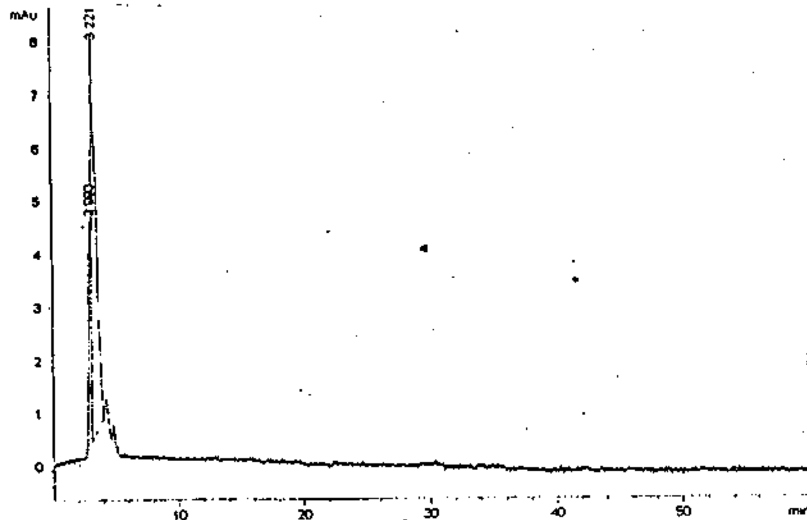


Figure 5.2 HPLC profile of AFB_1-lysine conjugate. The elution conditions were:- 50% methanol : 50% 0.1M sodium dihydrogen phosphate pH 3.1. The flow rate was set at 0.5ml/min, using a C_8 reverse phase prodigy, 5µl (150 x 4.6mm) column (Phenomenex).

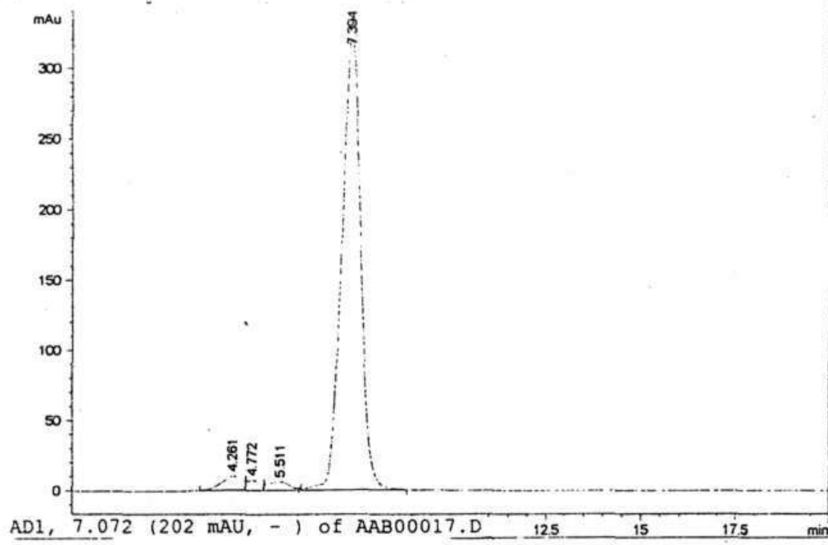


Figure 5.3 HPLC (1090 Hewlett Packard) profile of AFB_1 (2.2μg/ml).

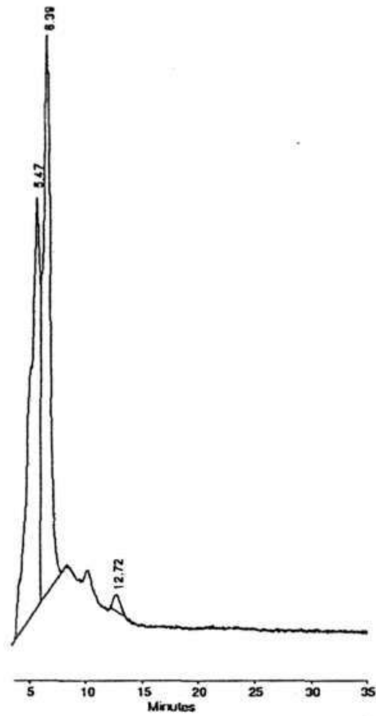


Figure 5.4 HPLC (Spectra Physics) profile of AFB_1

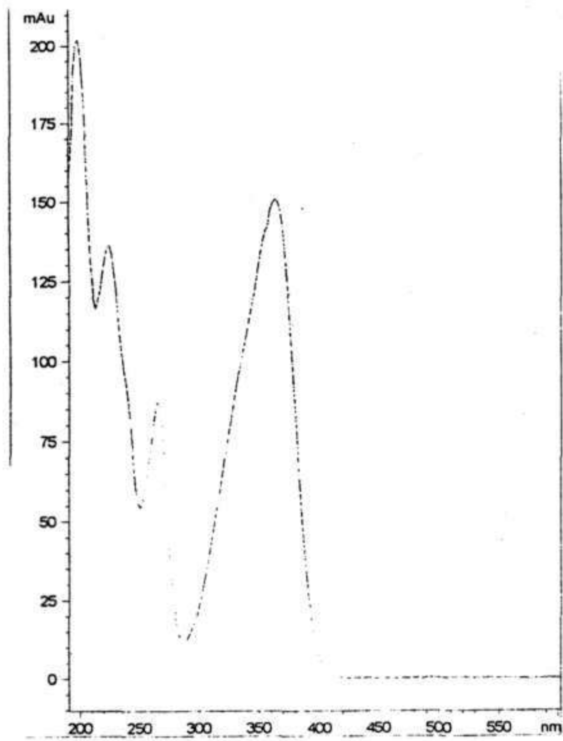


Figure 5.5 UV absorption spectrum of AFB_1

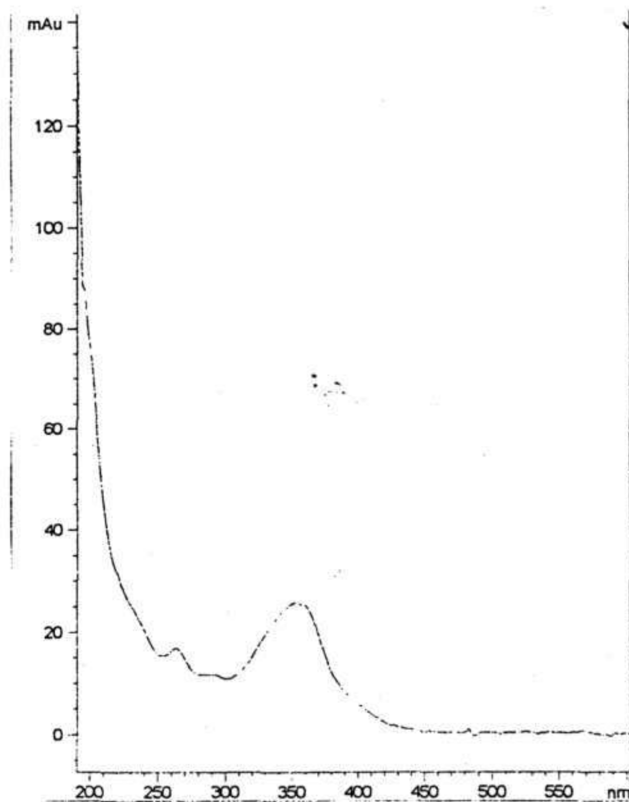


Figure 5.6 UV absorption spectrum of AFB_1-lysine adduct monitored at 200 - 600nm.

Capillary Electrophoresis

Both standard albumin and albumin conjugated with AFB_1O were separated on CE with retention times of 10.32 minutes and were detected at their UV absorbance at 210nm (Fig. 5.8). On monitoring these molecules at 400nm no response was observed for the free albumin but a substantial signal was found for the adduct (Fig. 5.7). Presumably this absorbance is due to the coumarin chromophore in the AFB_1 residues. This is an important finding as it should be possible to directly examine blood serum for the presence of albumin conjugates that absorb at 400nm. Whilst other adducts may contribute to such a signal it could perhaps act as an elimination screen for patients being monitored for exposure to AFB_1 in their diet. Such patients include children with kwashiorkor who routinely have higher AFB_1 adduct levels in their blood. Clearly a rapid bulk screening method for such patients would be advantageous.

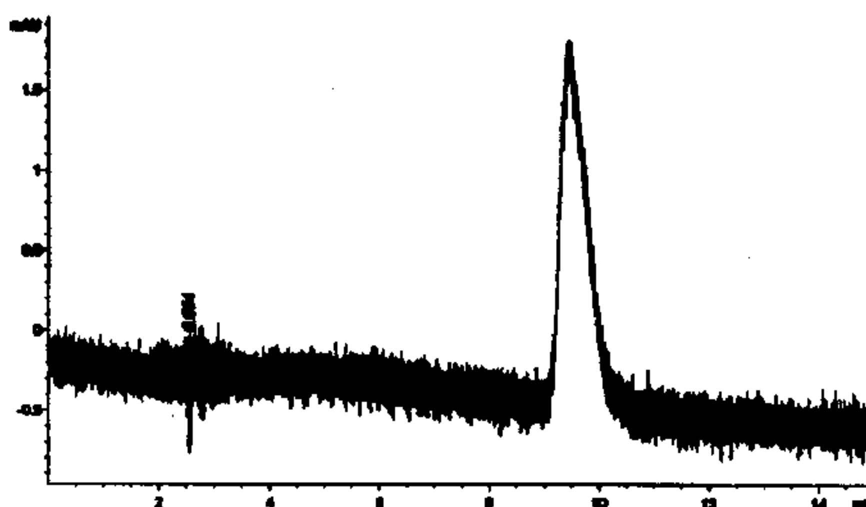


Figure 5.7 Capillary electrophoresis of aflatoxin B_1 -albumin adduct monitored at 400nm. Unbound albumin does not show absorbance at this wavelength.

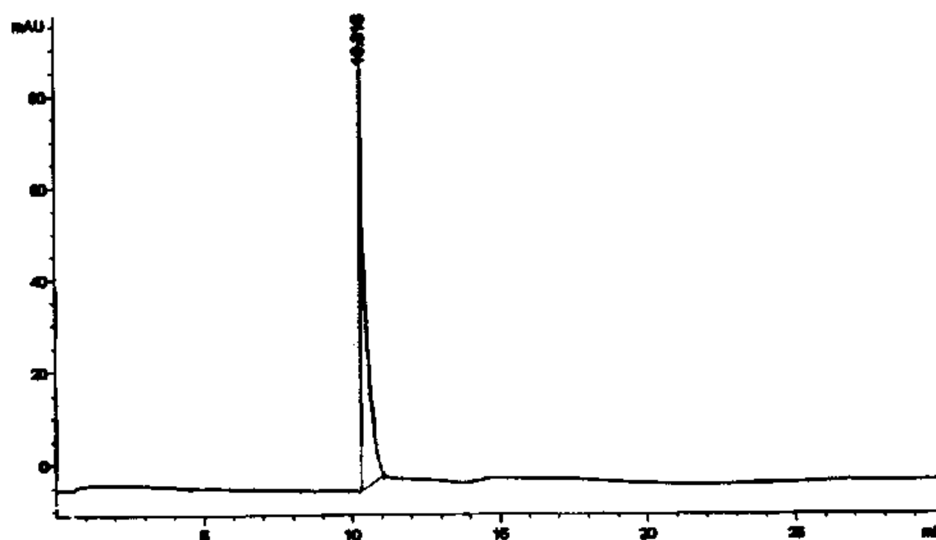


Figure 5.8 Capillary electrophoresis of aflatoxin B_1-albumin adduct at 210nm. Unbound albumin also exhibit absorption at this wavelength.

It can be seen from Plate 5.1 that AFB_1O binds covalently to albumin and that there was no evidence from the electrophoretogram to suggest that other proteins do so *in vitro*. This may be due to the lack of suitable amino acid residues in their primary structure in comparison to albumin, which would, therefore, react preferentially. The *in vivo* situation is likely to be different for various reasons, (see Chapter 6) e.g., AFB_1O seems to bind to DNA in preference to proteins (Garner *et al.* 1979). There is also difference in the binding of AFB_1 and AFG_1 epoxides to DNA, the former binding more strongly than the latter in kidney (Garner *et al.* 1979).

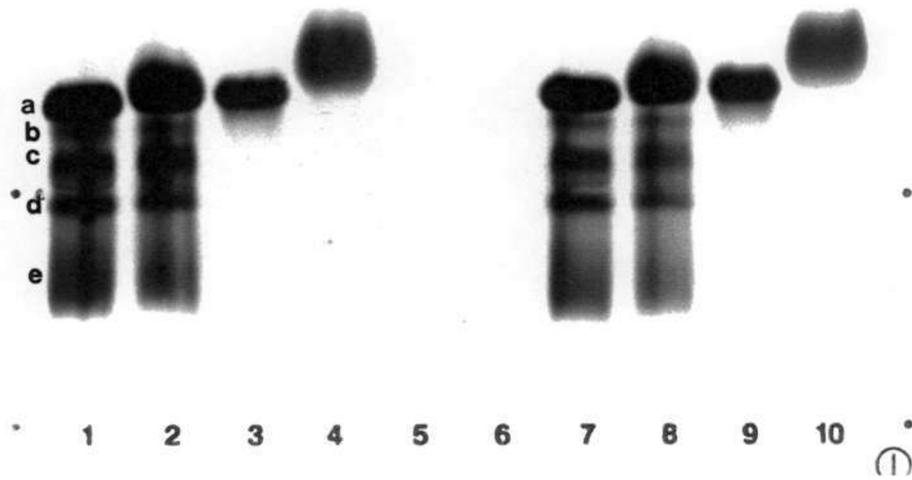


Plate 5.1 Serum protein electrophoresis of 1 : serum; 2 : serum-AFB_1-epoxide; 3 : albumin; and 4: albumin-AFB_1-epoxide. The fractions are:- a (albumin), b (α_1 globulin), c (α_2 globulin), d (β globulin), and e (γ globulin).

CHAPTER 6

DETECTION AND MEASUREMENT OF AFLATOXIN B_1-LYSINE ADDUCT IN HUMANS

6.1 Introduction

In the previous chapters it was shown that AFB_1 conjugates could be detected using HPLC by fluorescence as well as UV detection and by CE. Moreover, their absorbance characteristics could be determined using UV absorption spectrophotometry. The number of cases in which AFB_1 has been detected in liver biopsies of HCC patients and blood and urine samples in children with kwashiorkor in King Edward Hospital VIII (KEH), Durban, is high (Bux, *et al.*, 1994; Ramjee, 1990). As a follow-up to such studies it has been of importance to make a brief survey and investigate whether such patients would have AFB_1-lysine conjugates which would indicate the extent to which AFB_1 had been present in the patients' bodies.

The study presented in this chapter was, therefore, aimed at searching for the presence of AFB_1-lysine adducts from human blood samples. The detection was carried out so as to compare the presence of such conjugates in patients suffering from kwashiorkor and liver cancer to control patients (those patients suffering from neither of these conditions). Ethical approval for this study was obtained from the Ethics Committee, University of Natal, Medical Faculty and consent was sought from concerned patients or their parents.

6.2 Materials and methods

6.2.1 Materials

All reagents were of analytical grade unless stated otherwise. Whole blood (3 - 5ml) was collected from KEH at the Paediatrics Out patient Department (POPD) and in the wards, using heparin free tubes. Proteinase K (EC 3.4.21.64) and Serva Blue G were obtained from Sigma Chemical Co. (St. Louis, U.S.A.).

6.2.2 Sample preparation

Blood was kept at 37°C to facilitate the process of clotting. Serum was obtained by centrifugation and immediately aliquoted into 1.5ml Eppendorf tubes, in volumes of 0.5ml. The samples were properly labelled and stored frozen at -20°C until they were required. Twenty nine blood samples, divided into nine kwashiorkor, ten liver cancer and ten control patients were analysed by HPLC for the presence of AFB_1-lysine conjugates. Control samples were collected from patients who had been admitted to KEH for conditions other than liver cancer or kwashiorkor. Twenty nine blood samples were analysed for the presence of free AFB_1. The samples were divided into fifteen kwashiorkor, six HCC and eight control patients obtained as indicated above.

Total protein determination in the serum

Protein content of serum was determined by the Bradford method (Bradford, 1976) as described below.

Preparation of Bradford reagent

Serva Blue G (50mg) was dissolved in 24 ml of absolute ethanol and transferred into a 500ml volumetric flask. Phosphoric acid, 85% (w/v), 50ml, was slowly added with shaking and the final volume was brought to 500ml with distilled water. The Bradford reagent was stored in the dark at room temperature until it was required.

Albumin standard (1.5mg/ml)

Albumin (15mg) was weighed and completely dissolved in 10ml of distilled water.

* A sample of serum, 15μl, was diluted to 1.0ml with distilled water.
* From the diluted, sample a small portion (100μl) was transfered into a 10ml test tube.
* A series of standards were prepared as follows:

Standard albumin, µl (1.5mg/ml)	0	20	40	60	80	100
Distilled water, µl	100	80	60	40	20	0
Corresponding to the following in mg albumin /ml serum	0	20	40	60	80	100

- The Bradford reagent (5ml) was added to each tube and vortexed gently.
- Absorbance was read at 595nm after 2 minutes. The albumin content of the samples were calculated from the standard curve and corresponds to the concentration (mg albumin/ml serum) given in the above table.

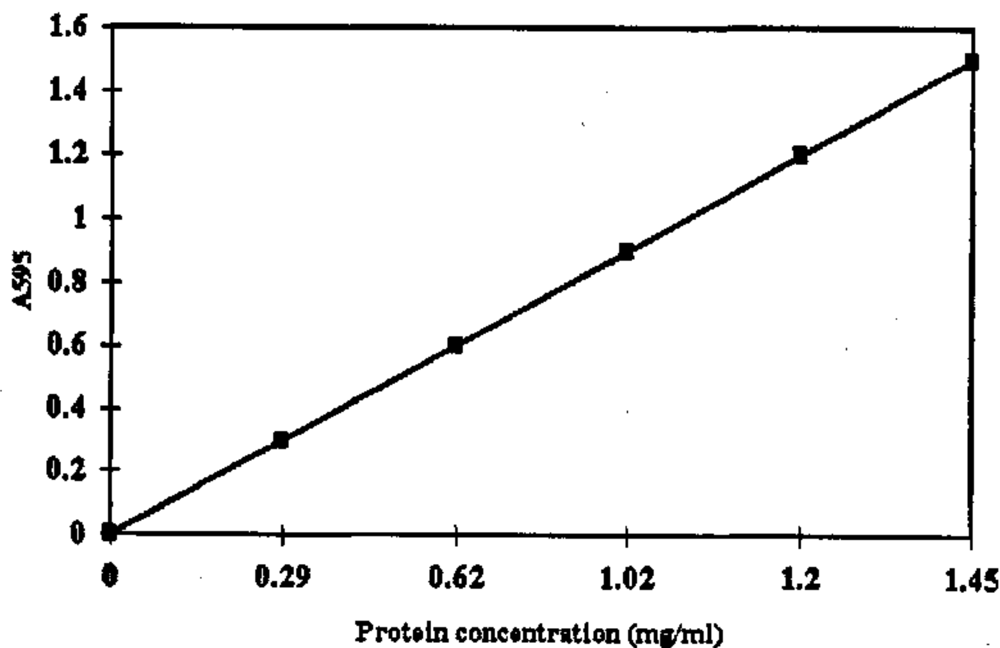


Figure 6.1. A protein standard curve using albumin and Bradford assay.

Albumin extraction

- Sera (0.5ml) were placed in a water bath at 55°C for 45 minutes to inactivate any human immunodeficiency virus.
- Samples were cooled on ice for 5 minutes and 1.5 volumes of cold saturated ammonium sulphate were added drop-wise.
- The mixture was vortexed and centrifuged at 9000 x g for 15 minutes, at 0°C to precipitate immunoglobulins.

* The supernatant containing albumin was removed and 100µl of 1M acetic acid per 500µl serum volume was added to adjust the solution to pH 5 and precipitate the albumin.

* Albumin was collected by centrifugation as above and immediately, following removal of supernatant for TLC determination of the free aflatoxins, the albumin was redissolved in a volume of phosphate buffered saline (PBS, 0.1M, pH 7.4) equivalent to twice that of the starting volume of sera.

Albumin hydrolysis

Determination of aflatoxin B_1-lysine from albumin

The solutions of albumin in phosphate buffered saline, (2mg/ml, as determined from the standard curve) were digested with proteinase K, digesting 3mg albumin with 1mg enzyme. The digests were centrifuged at 2 700 RPM at 0°C and the supernatants were filtered through 0.22µm membrane filters before they were analysed by HPLC using UV detection as described in chapter 5 on page 56. The concentration of lysine-AFB_1 (ng/ml) in the standard was calculated using (extinction coefficient) ε at pH 4 (appendix Ic) values of Sabbioni *et al.* (1987).

Free Aflatoxins

For free aflatoxin determination, blood samples (0.5 ml) were mixed with an equal volume of chloroform, and the mixture vortexed for three minutes. The chloroform layer was removed and fresh chloroform was added and the procedure was repeated three times. The chloroform extracts were pooled together and evaporated to dryness. The residue was reconstituted in 100µl of chloroform. The chloroform extracts were then spotted on silica TLC plates and developed in chloroform : acetone (9:1) to check for the presence of unbound aflatoxins. The number of samples that were analysed for free aflatoxins were as follows : 6 obtained from liver cancer, 15 kwashiorkor, and 8 control patients.

6.3 Results and Discussion

Figure 6.2 illustrates that one of the six samples from the HCC patients had unbound AFB_1 whereas one of the fifteen kwashiorkor and none of the control patients showed the presence of AFB_1 by TLC. The concentration of free AFB_1 in the HCC patient was higher than that in the kwashiorkor patient, by visual comparison of the TLC plate. However, it must be borne in mind

that TLC is mainly a qualitative technique, with low sensitivity as compared to HPLC. It is interesting that no control patients revealed the presence of free aflatoxins, and one out of the six HCC patients was positive for AFB_1.

The results for free AFB_1 in blood samples, however, do not tell us much concerning long term exposure of the individuals to aflatoxins. The reason is that once aflatoxins are ingested, they undergo biological changes which may result in excretion of the toxin. So, exposure to aflatoxins does not necessarily mean that an individual will have free toxins in their systems for any length of time. However, the presence of free AFB_1 implies the exposure of the individual to this toxin. A major possibility during biological changes of AFB_1 in the body, is the formation of AFB_1O. This epoxide rapidly conjugates with macromolecules to form AFB_1-macromolecular adducts. Because these macromolecular adducts have much longer life in the body, they are a better dosage indicator of individual exposure to aflatoxins than a measurement of free aflatoxins. Consequently, free aflatoxin determination may be used for a short term exposure while AFB_1-macromolecular conjugate determinations may be used for long term exposure assessment.

The processes controlling the formation of aflatoxin adducts in the human body are several, inter-related ones and therefore, give rise to a complex situation. Initially the process was over simplified in that, it was thought that a certain amount of aflatoxin in the diet gave rise to a certain level in the blood, which then damaged various cellular functions and was then excreted. However, early studies on rats revealed that AFB_1 was a powerful carcinogen and probably gave rise to hepatocellular carcinoma in humans when ingested over long periods at a chronic level (Munoz and Bosch, 1987). Consequently simple absorption, circulation and excretion of the toxin was unlikely. It became apparent (Wild *et al.,* 1986) that AFB_1 could covalently link with blood proteins and tissues and this, coupled with the observation that the cytochrome P_{450} system of the liver converted AFB_1 to its true active form, the epoxide (Ramsdel *et al.,*1991) gave a basis for understanding its action in the body.

This now leads to a much more complicated situation when considering the relation of absorption, circulation and excretion of the toxin in the body. In particular it presents difficulties in understanding and interpreting the presence and concentration of albumin-toxin adducts in the blood. In its most simplest forms it might be argued that the adduct merely acts as a bio-marker and the higher the level the greater the immediate exposure of the patient to AFB_1 in their diets. The formation of AFB_1O-albumin adducts is controlled by two main processes: the uptake of AFB_1 by the blood from the digestive processes, which in turn depends on the level in the diet and; the

activity of the cytochrome P_{450} which is a heterogeneous system with certain molecular types having the ability to generate the epoxide and others not. In addition it is subject to induction so a patient being exposed to a sudden large dose of toxin may not in fact produce much of the epoxide.

The subject of AFB_1 excretion is also complicated. Free toxin may be excreted in the urine (Gan *et al.*, 1988; Groopman *et al.*, 1993), as well as adducts, including conjugation with guanine (main detoxification system (Degen and Newman 1978; 1981)) nucleic acid bases (Groopman *et al.*, 1985) and amino acids (Sabbioni *et al.*, 1987). The enzymes microsomal epoxide hydrolase (EPHX) and glutathione S-transferase M_1 (GSTM1) are also very important, as their diminition leaves more of the epoxide available for binding to DNA, RNA and/or proteins (McGlynn *et al.*, 1995). The activity of the detoxification process could therefore, greatly influence the levels of both free and conjugated toxin. The appearance of base and amino acid conjugates presumably reflects the presence of macromolecular adducts and their subsequent catabolism.

The findings in this limited study indicate that patients with HCC have the highest mean level of albumin adduct in their blood (3.48ng lysyl-AFB_1 / mg albumin) followed by kwashiorkor patients (1.18ng lysyl-AFB_1 / mg albumin). In its most simplistic form this would indicate that the highest degree of exposure was in HCC patients. From the consideration outlined above it could mean other things, e.g., higher cytochrome P_{450} activities, perhaps as a result of genetic predilections, less efficient glutathione conjugating abilities or slower turnover of albumin in the body or all of these. It seems that in kwashiorkor patients, they are less capable of metabolising AFB_1 which leads to higher levels of free toxin in their blood (Hendrickse *et al.*, 1982). Why they should then have higher levels of adduct than the control samples is not clear.

A more worrying aspect of this study is the presence of adduct in all the controls, although at a lower mean level (0.83ng lysyl-AFB_1 / mg albumin). Previous work (Ramjee, 1990) on maize, the food staple of most Black people entering KEH shows only sporadic contamination with AFB_1, which is certainly not at a level and frequency to explain the general presence of AFB_1 conjugates in their blood. Clearly more work is needed in order to identify the source of AFB_1 in the diet, which is more likely to be in oil seeds such as groundnuts.

King Edward VIII Hospital admits patients from as far as the rural areas of Northern KwaZulu-Natal where the population still depends on home grown maize as their main source of diet. It is in this context then that the presence of lysine-AFB_1 even among the control patients should be looked at. Some of these patients may have, at one stage in their life history, been

exposed to aflatoxins, which is evidenced by the presence of AFB_1-lysine conjugates even among the control patients. Wild *et al.* (1990a) has also indicated that between 12 and 100% of sera collected from children in various African countries contained aflatoxin-albumin adducts. The adverse effects of aflatoxins in the body are numerous but liver suffers the most. Therefore, it is possible that impairment of detoxification mechanism in both kwashiorkor and HCC patients, explains accumulation of the adducts in the blood of such patients.

Methods for AFB_1 macromolecular conjugate determinations are, however, still new. They still need some further developments. All the twenty nine samples that were analysed showed the presence of AFB_1-lysyl conjugate. Using the extinction coefficients of lysine-AFB_1, the standard was found to contain 61.47 ng/ml lysyl-AFB_1 (Table 6.1). Figure 6.3 shows that the mean lysyl-AFB_1 levels were highest in HCC patients (Table 6.4), followed by kwashiorkor patients (Table 6.3) and were least in control patients (Table 6.2). There was a mean 1.4 fold increase in levels of lysine-AFB_1 from control to kwashiorkor patients and the increase was 4.2 from control to HCC patients (Fig. 6.4). The method of mycotoxin-macromolecular adduct determination has potential, particularly because it has been shown that the mycotoxin, AFB_1 binds with more than one AA *in vitro*. Aflatoxin B_1-macromolecular adduct determination can, therefore, be used to search for the presence of the other AFB_1-AA conjugates *in vivo*.

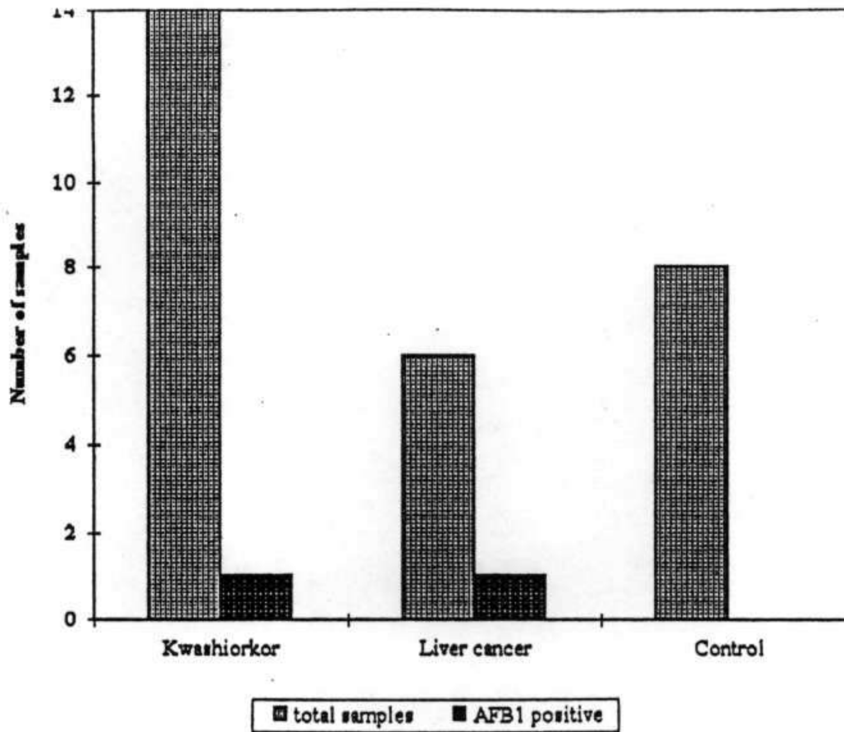


Figure 6.2 A chart comparing the presence of unbound aflatoxin B_1 in kwashiorkor, liver cancer and control patients.

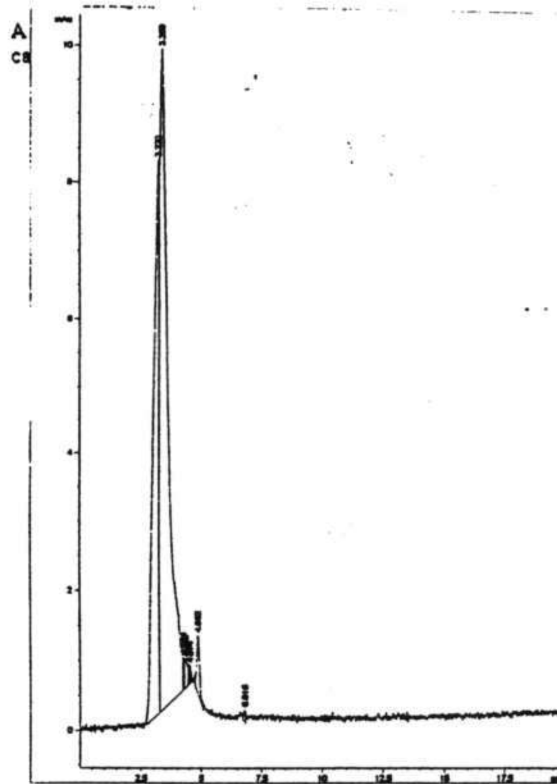


Figure 6.3 High performance liquid chromatography profile of AFB_1-lysine conjugate from a blood sample of a liver cancer patient. 0.1M sodium phosphate buffer, pH 3.1.

Table 6.1 Amount of lysyl-aflatoxin B_1 adduct derived from HPLC results (Fig. 5.2 on page 58).

Peak area	Amount of lysyl-AFB_1 (ng/ml in buffer)*	Amount of lysyl-AFB_1 (ng lysyl-AFB_1 / mg N_α-acetyl lysine)[1]	Percentage yield (%)[2]
129.70	*61.47	24.59	40.25

* Concentration of lysyl-AFB_1 = $\frac{A_{370} \times \text{molar mass} \times 1000\ \mu g/ml}{\varepsilon_{248}}$

$$= \frac{0.002 \times 501 \times 1000\ \mu g/ml}{16\,300}$$

$$= 0.06147 \mu g/ml$$

$$= 61.47\ ng/ml$$

1 ng/ml standard x 4.0ml /10mg N_α-acetyl lysine)

Total amount of lysyl-AFB_1 in 4ml of buffer = 61.47ng/ml x 4ml

= 245.9ng

2 ng adduct/ng AFB_1 O x 100% =

$$\frac{\frac{245.9\text{ng adduct}}{\text{MW adduct}} \times 100\%}{\frac{0.4 \times 10^3 \text{ ng } AFB_1O}{\text{MW } AFB_1O}}$$

$$= \frac{\frac{245.9\text{ng adduct}}{501} \times 100\%}{\frac{0.4 \times 10^3 \text{ ng } AFB_1O}{328}}$$

$$= 40.25\%$$

Table 6.2 Lysyl-aflatoxin B_1 adduct in the blood albumin control patients as determined by HPLC.

Sample (Control patients)	Peak area	Amount of lysyl-AFB_1 (ng/ml in buffer)*	Amount of lysyl-AFB_1 (ng lysyl-AFB_1 /mg albumin)**
1	2.30	1.09	0.36
2	3.24	1.54	0.51
3	5.51	2.61	0.87
4	6.66	3.16	1.05
5	6.20	2.94	0.98
6	6.93	3.28	1.09
7	6.80	3.22	1.07
8	3.38	1.60	0.53
9	5.60	2.65	0.88
10	6.02	2.85	0.95
Mean	5.26	2.49	0.83 ± 0.26

* $\frac{\text{peak area of sample} \times \text{concentration of standard in ng/ml}}{\text{peak area of standard}}$

$= \frac{\text{peak area of sample}}{129.70} \times 61.47\text{ng/ml}$

** concentration of sample in ng/ml in 1ml sample is equivallent to 3mg of digested albumin

Table 6.3 Lysyl-aflatoxin B_1 adduct in the blood albumin kwashiorkor patients as determined by HPLC.

(Sample) kwashiorkor patients	Peak area	Amount of lysyl-AFB$_1$ (ng/ml in buffer)*	Amount of lysyl-AFB$_1$ (ng lysyl-AFB$_1$ /mg albumin)**
11	7.56	3.58	1.19
12	7.94	3.76	1.25
13	8.94	4.24	1.41
14	8.02	3.80	1.27
15	8.59	4.07	1.36
16	8.51	4.03	1.34
17	8.44	4.00	1.33
18	8.54	4.05	1.35
19	8.54	**4.05**	1.35
Mean	8.34	3.95	1.32 ± 0.067

* $\frac{\text{peak area of sample}}{\text{peak area of standard}} \times \text{concentration of standard in ng/ml}$

$= \frac{\text{peak area of sample}}{129.70} \times 61.47\text{ng/ml}$

** concentration of sample in ng/ml in 1ml sample is equivallent to 3mg of digested albumin

Table 6.4 Lysyl-aflatoxin B_1 adduct in the blood albumin HCC patients as determined by HPLC.

Sample (HCC patients)	Peak area	Amount of lysyl-AFB_1 (ng/ml in buffer)*	Amount of lysyl-AFB_1 (ng lysyl-AFB_1/mg albumin)**
20	10.25	4.85	1.62
21	9.67	4.58	1.53
22	16.36	7.75	2.58
23	9.89	4.69	1.56
24	8.93	4.23	1.41
25	9.96	4.72	1.57
26	124.81	59.15	19.72
27	10.53	4.99	1.66
28	9.99	4.73	1.58
29	10.05	4.76	1.59
Mean	22.04	10.45	3.48 ± 5.71

* $\frac{\text{peak area of sample}}{\text{peak area of standard}} \times$ concentration of standard in ng/ml

$= \frac{\text{peak area of sample}}{129.70} \times 61.47$ ng/ml

** concentration of sample in ng/ml in 1ml sample is equivallent to 3mg of digested albumin

Toxins other than AFB_1 have also been shown to conjugate to proteins and DNA in the same way as AFB_1 (Gabriele and Wild, 1991) it will, therefore, be of interest to conduct further studies to find out the role of multimycotoxicosis in diseases conditions. Gqaleni (1996), has shown that between 7 and 15% cyclopiazonic acid conjugate to a carrier protein. Aflatoxin G_1 has also been shown to conjugate to proteins. It will therefore, be of importance to set up an ELISA so as to validate HPLC results. The two techniques, used together, will ensure a high specificity to eliminate the risk of false positive results (Wild and Montesano, 1990). Moreover, an immunoassay is more sensitive than HPLC with low quantities of albumin (Wild *et al.*, 1990b).

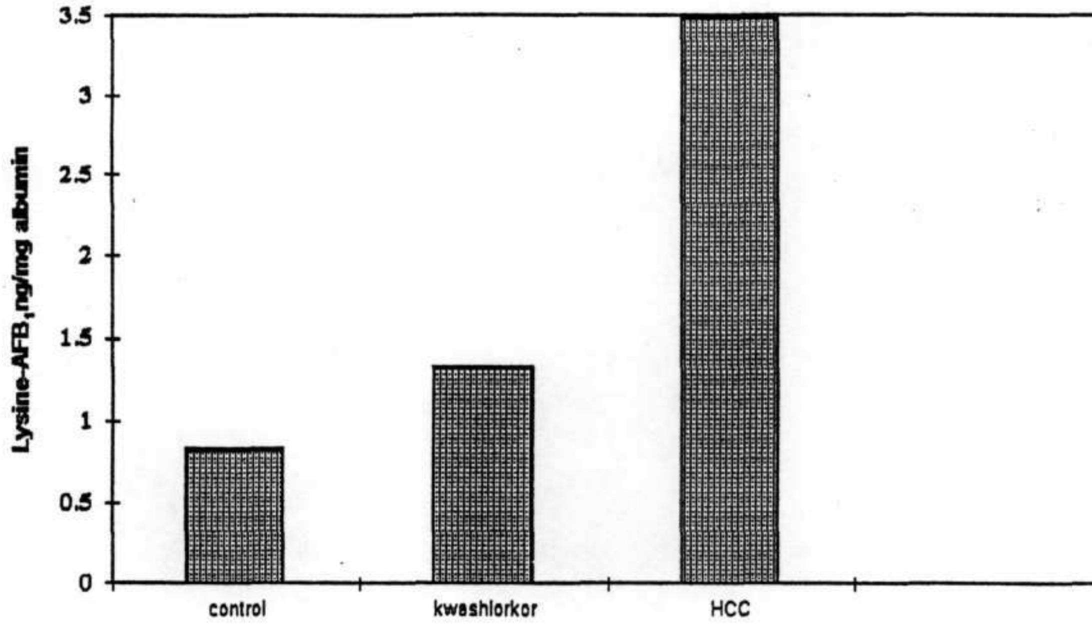


Figure 6.4 A chart comparing the mean level of lysine AFB_1-adduct in control, kwashiorkor and HCC patients.

CHAPTER 7

SUMMARY AND CONCLUSION

Two objectives of this study were to develop a laboratory based method to detect the occurrence of AFB_1-conjugates in humans and to use this to evaluate exposure of human subjects to AFB_1. To achieve this, the following had to be developed:-

* Synthesis of lysyl-AFB_1 adduct for use as a standard.
* Determine the reaction of selected N_α-acetyl amino acids with aflatoxin B_1-epoxide *in vitro*.
* Ion exchange chromatography clean-up of AFB_1-lysine adduct.
* The *in vitro* binding of arginine, histidine and lysine to AFB_1O.
* Thin layer chromatography methods for the detection of AFB_1-macromolecular adducts.
* The use of capillary electrophoresis in the detection of AFB_1-macromolecular adduct detection.
* High performance liquid UV detection of AFB_1-lysine adduct.

Having achieved the above, the method was applied to detect AFB_1-lysine adducts in humans. All the samples that were analysed were found to have detectable level of AFB_1-lysine adduct. It is evident, from this study, that some patients that attend at KEH have at some stage of their life history, been exposed to aflatoxins. Since some diseases are now thought to be multifactorial, e.g., kwashiorkor and liver cancer, it is possible that aflatoxicosis also plays a significant role in some of them. Most of the studies that are conducted, concentrate on the effects of individual mycotoxins, neglecting the multimycotoxicosis effect.

Future Prospects

Aflatoxicosis is not the only contributing factor to disease conditions of humans in South Africa. There are several other predominant mycotoxins, like *Fusarium* mycotoxins (Rava *et al.*, 1996; Rava, 1996), which are of health concern. It is, therefore, important that more biomarkers be developed for such mycotoxins as fumonisins. In this way, a clear picture of multimycotoxicosis may be obtained.

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APPENDIX Ia: DETECTION REAGENTS

Spray reagents and solvents

Tolidine spray reagent: For nitrogen containing compounds which can be converted into chloramines. O-tolidine (60 mg) are dissolved in 30 ml acetic acid, the solution diluted to 500 ml with distilled water and 1 g potassium iodide then added. N.B. o-tolidine is **carcinogenic!**

Ninhydrin: For amino acids. Ninhydrin gives colours with the free amino-terminal groups of peptides; the epsilon-NH_2 group of lysine residues produce a weak colour which appears slowly. Peptides with blocked N-termini (e.g., N_α-acetyl peptides) are not detected with ninhydrin (except for a slow reaction with any epsilon-NH_2 groups of lysine which may be present) and chlorination method is used for the detection of such peptides.

Ninhydrin (0.2g) was in 100ml of 95% ethanol/acetic acid (20:1, v/v). The thin layer was dipped into or sprayed with this solution and dried at 60°C for 30 min. Colour development may alternatively take place at room temperature overnight.

Ninhydrin-cadmium acetate chromogenic reagent: Cadmium acetate (0.5g) was dissolved in 50ml water to which 10ml glacial acetic acid had been added. Acetone was then added until the total volume is 500ml. Portions of that solution were taken before use and sufficient solid ninhydrin added until the final concentration is 0.2% (w/v).

Dipping solutions for epoxides

Dipping solution I: Depending on the amount that was needed, 9g 4-(4-nitrobenzyl)pyridine (NBP) and 90mg butylhydroxytoluene (BHT) (this is 2,6 Di-tert-butyl-4-methylphenol) were dissolved in 90ml chloroform.

Dipping solution II: Depending on the amount that was needed, 24ml tetraethylene pentamine was dissolved in 60mg butylhydroxytoluene in 36ml dichloromethane.

APPENDIX Ib: MEDIA

Yeast extract sucrose (YES) medium

The following ingredients were dissolved in a litre of distilled water and dispensed into media bottles and autoclaved at 115°C for 15 minutes:

Yeast extract	20.0g
Sucrose	100.0g
Agar	0.1g

Potato dexrose agar (PDA)

Potato dexrose agar (39g) was suspended in 1L of distilled water and boiled until it dissolved. The medium was then sterilized at 121°C for 15 minutes in the autoclave.

APPENDIX 1c: UV VISIBLE SPECTRA OF ADDUCTS

N_α-acetyl-lysine-AFG_1 in 0.1M sodium phosphate

pH	λ(ε)
7.4	225(19 644) 255(19 721) 346(14 741) 413(27 783)
6.0	225(19 695) 255(18 844) 346(15 364) 413(23 124)
5.0	218(20 365) 345(15 905)
4.0	216(21 242) 326(16 447)

Isoberic points: 246(18 215); 282(11 046) and 379(12405).

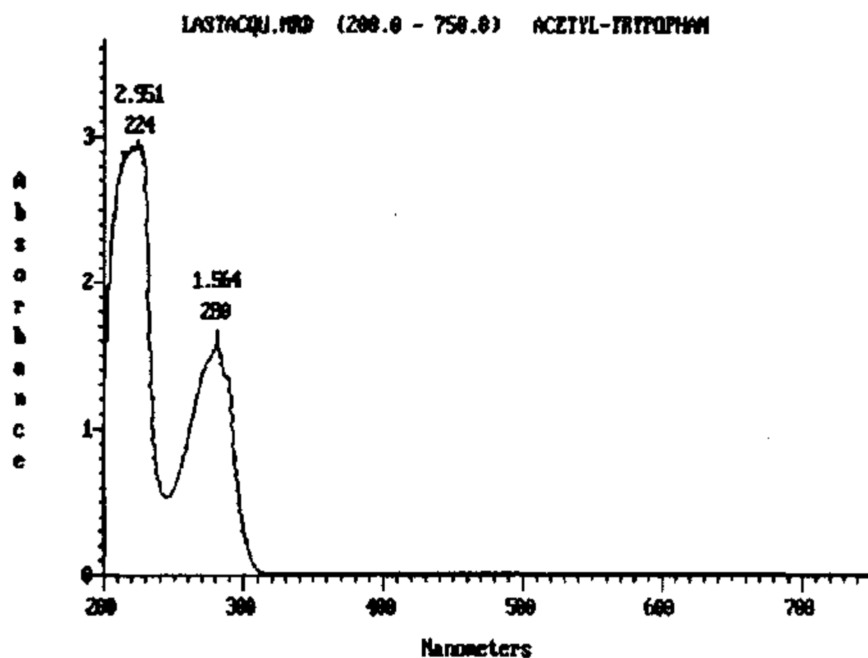
Lysine-AFB_1 in 0.05M sodium phosphate

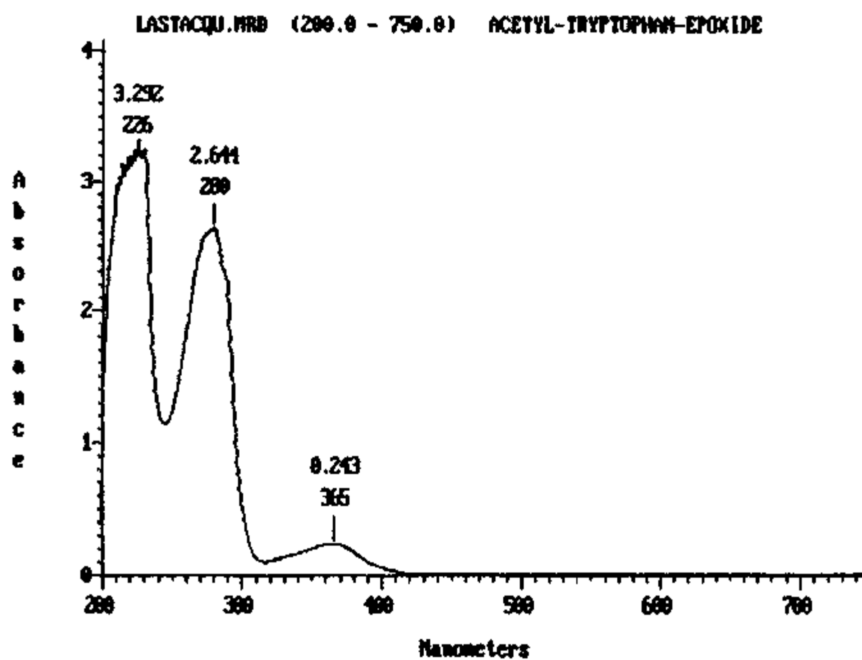
pH	λ(ε)
7.0	232(15 200) 258(16 300) 346(15 500) 399(25 400)
4.0	217(15 900) 248(16 300) 310(19 600)

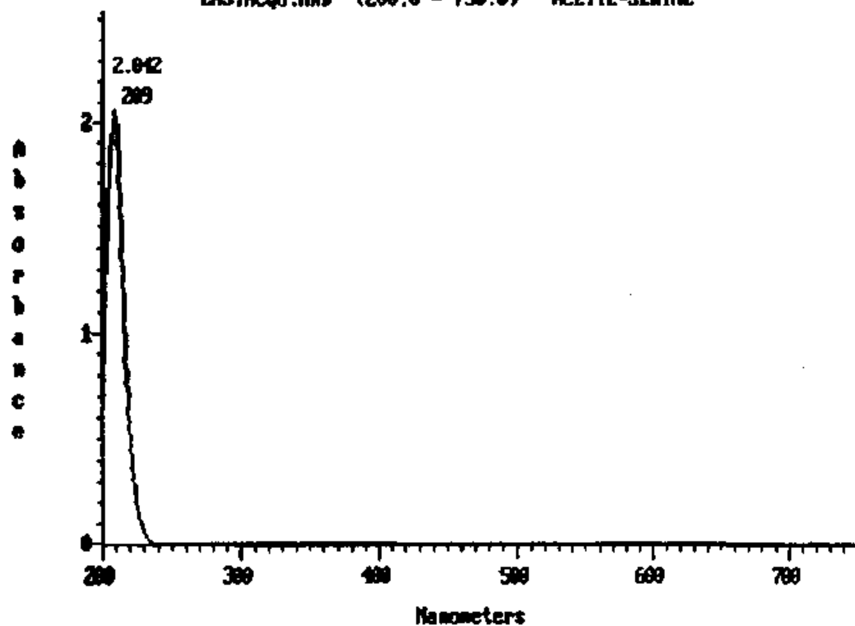
Isoberic points: 287 and 334.

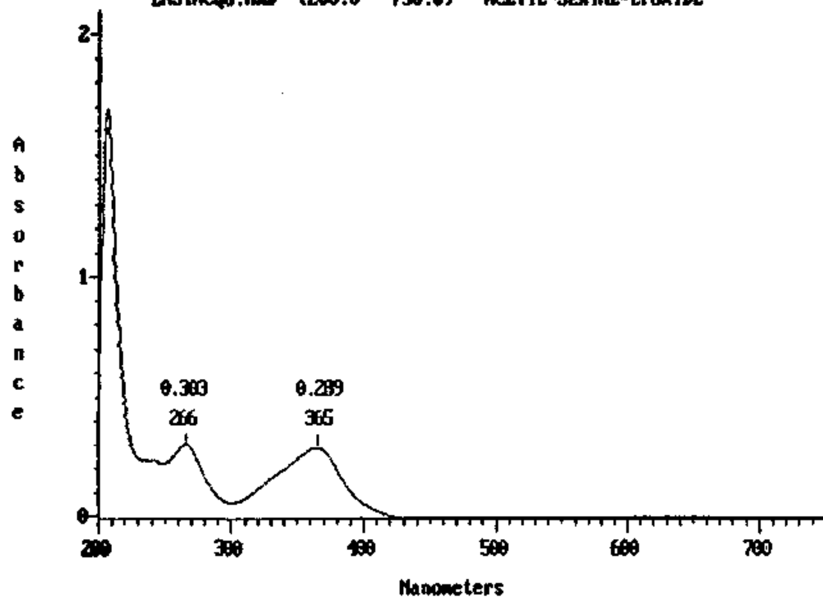
APPENDIX II

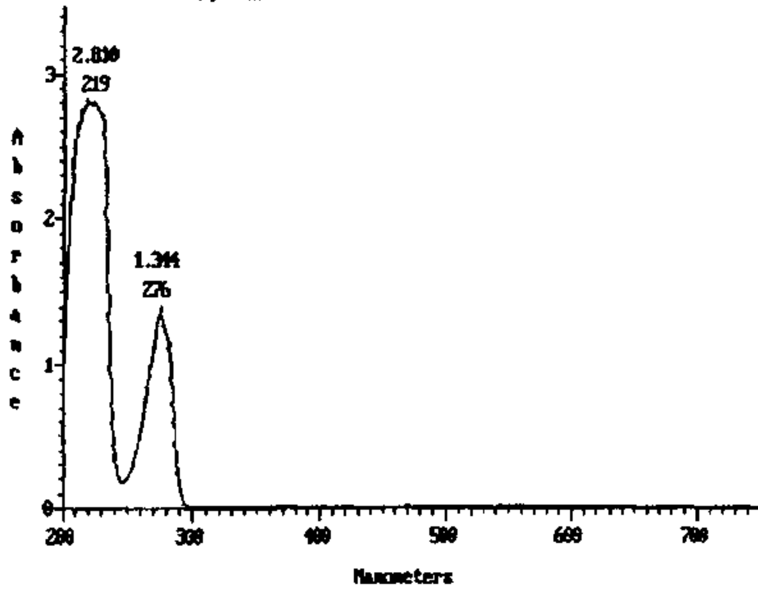
SPECTROPHOTOMETRIC SCANS OF N_α-ACETYL AMINO ACIDS AND THEIR EPOXIDE DERIVATIVES

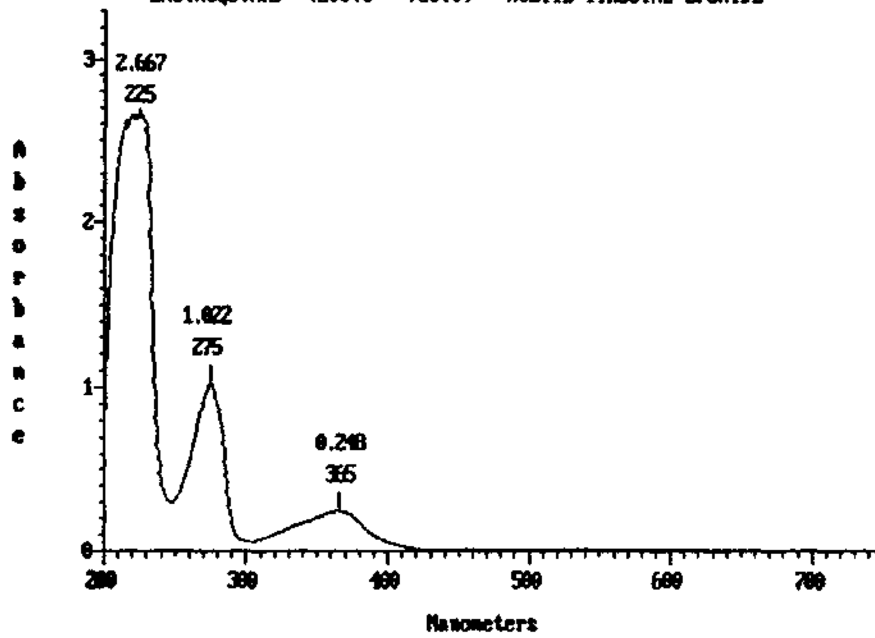


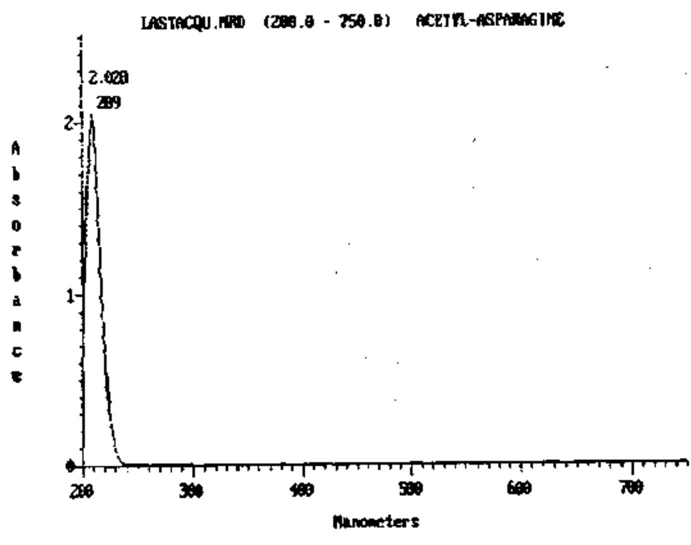



LASTACQU.MRD (200.0 - 750.0) ACETYL-SERINE
2.042
209
Absorbance
Nanometers

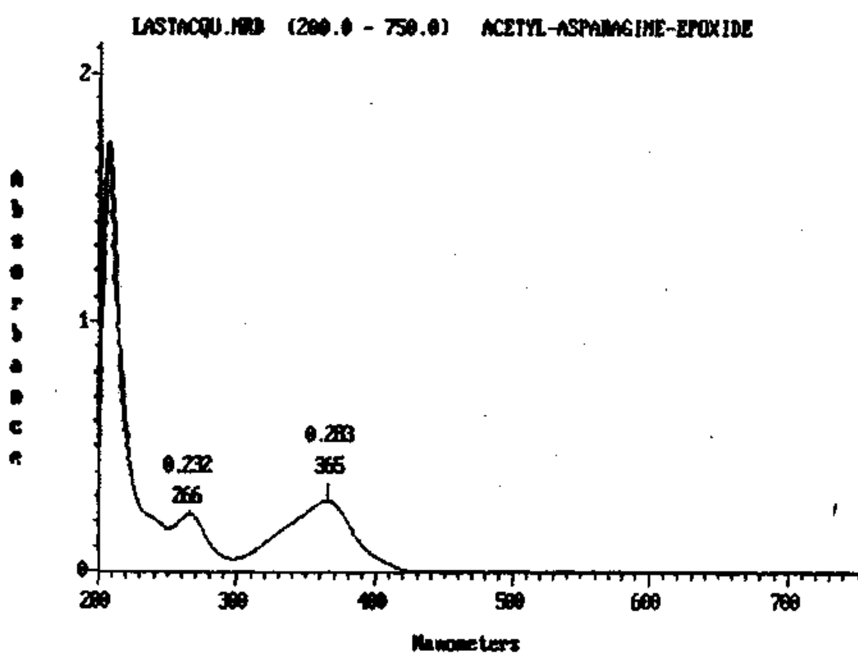

LASTACQU.MRD (200.0 - 750.0) ACETYL-SERINE-EPOXIDE
0.303
266
0.289
365
Absorbance
Nanometers


LASTACQU.MRD (200.0 - 750.0) ACETYL-TYROSINE
2.810
219
1.344
276
Absorbance
0
1
2
3
200
300
400
500
600
700
Nanometers


LASTACQU.MRD (200.0 - 750.0) ACETYL-TYROSINE-EPOXIDE
2.667
225
1.022
275
0.248
365
Absorbance
0
1
2
3
200
300
400
500
600
700
Nanometers



LASTACQU.MRD (200.0 - 750.0) ACETYL-ASPARAGINE
2.028
209
Absorbance
Nanometers



LASTACQU.MRD (200.0 - 750.0) ACETYL-ASPARAGINE-EPOXIDE
0.232
266
0.283
365
Absorbance
Nanometers

PAPERS AND POSTERS PRESENTED AT SCIENTIFIC MEETINGS

Papers

1. Reaction of Selected N_α -Acetyl Amino Acids With Aflatoxin B_1 - 8,9 Epoxide. South African Biochemistry and Molecular Biology / Biotechnology, Fourteenth Conference (1997) Rhodes University, Grahamstown.

2. The Detection and Measurement of Aflatoxin B_1 Conjugates in Humans in Natal South Africa. 9th IUPAC Symposium on Mycotoxins and Phycotoxins (1996) Rome, Italy.

3. Synthesis of Aflatoxin B_1 - Lysine Adduct for Use as a Biomarker for Toxicity. Regional Biochemistry Symposium (1995) University of Zululand, Empangeni.

Poster

1. Synthesis of Aflatoxin B_1 - Lysine Adduct for Use as a Biomarker for Toxicity. South African Biochemistry and Molecular Biology / Biotechnology, Fourteenth Conference (1997) Rhodes University, Grahamstown.