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CELLULAR INTERACTIONS AND METABOLISM OF AFLATOXIN: AN UPDATE

MICHELLE McLEAN and MICHAEL F. DUTTON

Department of Physiology, Faculty of Medicine, University of Natal, Congella, Durban 4013, South Africa

Abstract—The aflatoxins are a group of closely related mycotoxins that are widely distributed in nature. The most important of the group is aflatoxin B_1 (AFB_1), which has a range of biological activities, including acute toxicity, teratogenicity, mutagenicity and carcinogenicity. In order for AFB_1 to exert its effects, it must be converted to its reactive epoxide by the action of the mixed function mono-oxygenase enzyme systems (cytochrome P450-dependent) in the tissues (in particular, the liver) of the affected animal. This epoxide is highly reactive and can form derivatives with several cellular macromolecules, including DNA, RNA and protein. Cytochrome P450 enzymes may additionally catalyse the hydroxylation (to AFQ_1 and AFM_1) and demethylation (to AFP_1) of the parent AFB_1 molecule, resulting in products less toxic than AFB_1. Conjugation of AFB_1 to glutathione (mediated by glutathione *S*-transferase) and its subsequent excretion is regarded as an important detoxification pathway in animals. Resistance to AFB_1 toxicity has been interpreted in terms of levels and activities of these detoxifying pathways. This article reviews the multiple reactions and effects attributed to aflatoxin, with particular reference to the interaction of aflatoxin with nucleic acids and proteins, and the contribution this mycotoxin has in disease development and in the promotion of hepatocellular carcinoma (HCC). The anti-mutagenic properties of several dietary factors are also considered in this article. Undoubtedly, the most important aspect of aflatoxin action is its putative role in the development of human cancer, in particular, HCC. Recently, there has been a renewed interest in this aspect and experimental evidence is rapidly accumulating at the molecular level, indicating aflatoxin as an important consideration in the aetiology of human HCC.

Keywords—Aflatoxin, anti-mutagenic, cytochrome P450, glutathione *S*-transferase, metabolism, mycotoxin.

CONTENTS

Abbreviations—AF, aflatoxin; DAS, diallyl sulphide; DES, diethylstilbestrol; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NLS, nuclear location sequence; PLC, primary liver cancer.

1. INTRODUCTION

1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by fungi in foods and feeds, which, on ingestion, can result in the illness or death of animals, including humans (Hayes, 1981). The diseases caused by these mycotoxins are called mycotoxicoses and generally are classified according to the symptoms resulting from ingestion (Smith and Moss, 1985). The classical mycotoxicosis is ergotism, where, during the 9th and 10th centuries, there were numerous records of gangrenous ergotic outbreaks, with limbs rotting and falling off. The Order of St Anthony was established in the 11th century to provide hospitals for those suffering from this condition, which was known as St Anthony's fire. It was not until about 1850 or so that it became known that the disease was associated with the consumption of cereals, especially rye, contaminated with the plant pathogenic, ergot-producing (then not known) fungus, *Claviceps purpurea* (Smith and Moss, 1985; Berry, 1988).

Through the years, there have been numerous outbreaks of different mycotoxicoses in humans and other animals that have tended to be endemic to certain areas. Examples include yellow rice disease in Japan and sheep facial eczema in New Zealand. In the 1940s and 1950s, certain fungal metabolites were discovered to have useful antibiotic properties, with the unique property of being selectively toxic towards pathogenic bacteria, while remaining relatively harmless to animals (including humans) during treatment. It was also about this time that it was recognised that some diseases could be attributed to certain fungal toxins involuntarily ingested with food. In the Ukraine, the use of over-wintered wheat for bread-making resulted in an epidemic of a fatal disease known as alimentary toxic aleukia. Although Soviet scientists investigated the occurrence, little was known about illnesses associated with fungal toxins, let alone an awareness of the many mycotoxins. For example, Forgacs and Carll (1962) described mycotoxicoses as "neglected diseases", while at about the same time, Garner (1961) wrote that "in only a few instances has it been shown that extracts of fungi are harmful".

It was, however, a singular event that occurred in 1960 in Britain that established the significance of mycotoxicoses at the international level. This was the death of thousands of turkey poults, ducklings and chicks, and so became known as 'Turkey-X' disease (Butler, 1974). Analysis of the feed established the presence of a fungus, *Aspergillus flavus*, and thin layer chromatography identified several compounds that fluoresced under ultraviolet illumination. These compounds were named *aflatoxins*. More recent evidence suggests that the symptoms described in the field cases during the 1960s are not consistent with those known to be produced by the aflatoxins alone. With current knowledge and expertise, the findings would indicate that at least one other mycotoxin known to be produced by *Aspergillus flavus* (amongst other fungi), cyclopiazonic acid, may have been involved (Cole, 1986).

Since then, many more toxic fungal secondary metabolites have been identified, and as technology advances, the list becomes longer. While Huff *et al.* (1988) have reported that at least 200 substances of fungal origin are sufficiently toxic to warrant mycotoxin status, others report much higher figures (Cole and Cox, 1981; Watson, 1985). Watson (1985), on reviewing the literature, considers that 432 fungal compounds can be regarded as toxins, although only about one-quarter of these are toxic to mammals. Currently, hitherto unknown toxic compounds are being isolated and identified continually. Particular reference to the fumonisins (Bezuidenhout *et al.*, 1988) may be made in this regard. The present article utilises the extensive reviews of Kiessling (1986) and Hsieh (1987) as a basis for updating and summarising the more recently published data on aflatoxin B_1 (AFB_1) metabolism.

1.2. Aflatoxins

Although 17 compounds, all designated as aflatoxins, have been isolated, the term usually refers to four metabolites of this group of bis-furocoumarin metabolites produced by *Aspergillus flavus* and/or *Aspergillus parasiticus*. These are named AFB_1, B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2), all of which occur naturally (Fig. 1). The four compounds are distinguished on the colour of their fluorescence under long-wave ultraviolet illumination (B = blue; G = green), with the subscripts relating to their relative chromatographic mobility. AFB_1 is usually found in the highest concentrations, followed by AFG_1, AFB_2 and AFG_2. *Aspergillus flavus* produces only AFB_1 and AFB_2, while *Aspergillus parasiticus* produces these and additional compounds. The order of acute and chronic toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$, reflecting the role played by epoxidation of the 8,9-double bond (Wogan, 1966) and also the greater potency associated with the cyclopentenone ring of the B series, when compared with the six-membered lactone ring of the G series. Aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) are hydroxylated forms of AFB_1 and AFB_2. Aflatoxin B_{2a}

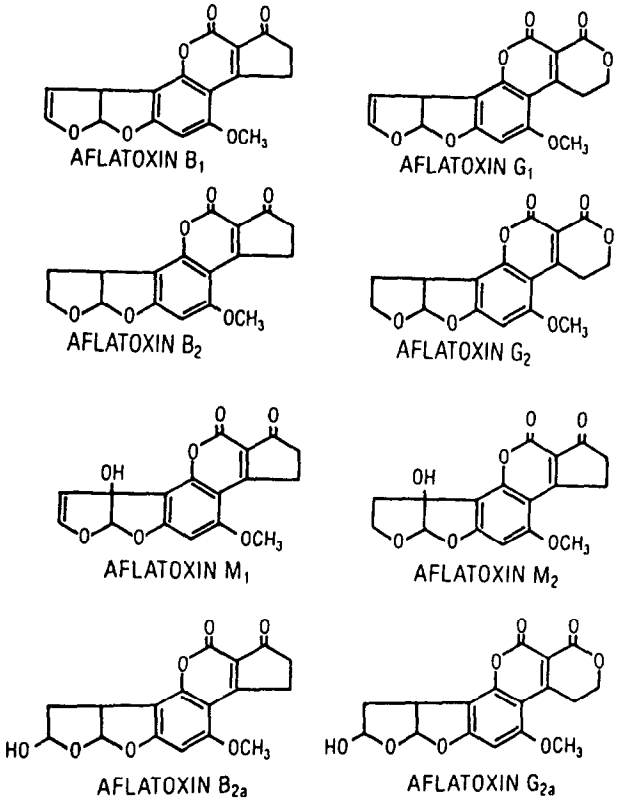

Fig. 1. Structures of aflatoxins B_1, B_2, G_1, G_2, M_1, B_{2a} and G_{2a}.

(AFB_{2a}) and aflatoxin G_{2a} (AFG_{2a}) are 8,9-hydrated products of AFB_1 and AFG_1 (Dutton and Heathcote, 1968). These compounds are relatively non-toxic when compared with AFB_1 and AFG_1.

The aflatoxins are freely soluble in moderately polar solvents (e.g. chloroform and methanol), especially in dimethylsulphoxide, and also have some water solubility. These compounds are very stable at high temperatures, with little or no destruction occurring under ordinary cooking conditions or during pasteurisation. The presence of the lactone ring in their structure makes the aflatoxin molecule susceptible to alkaline hydrolysis. Acid treatments (e.g. propionic acid) are also used frequently for their detoxification.

1.3. Aflatoxicosis

Toxicologically, aflatoxin may be regarded as a quadruple threat — as a potent toxin, a mutagen, a teratogen and a carcinogen (Ueno and Ueno, 1978). The lethal toxicity of AFB_1, however, varies in different animals: from extremely susceptible (sheep, dog, rat) to resistant species (monkey, chicken, mouse). There are no toxicity values for humans, but there is ample epidemiological evidence from case studies in Africa, South East Asia and India to implicate aflatoxins in the incidence of liver cancer and infant mortality (Hsieh, 1986). AFB_1 has also been reported to form adducts with DNA and so may play a role in the development of extrahepatic cancers. In this regard, Dvorackova *et al.* (1981) previously have implicated AFB_1 in the development of lung cancer. In comparing the ability of AFB_1 to bind to bladder and tracheobronchial tissues derived from several animals, Stoner *et al.* (1982) found that extrahepatic binding of AFB_1 to DNA was higher in AFB_1-resistant species than in susceptible species.

Although the epidemiological evidence relating AFB_1 to primary liver cancer (PLC) appears convincing, as yet, it is circumstantial. Stoloff (1989) is of the opinion that a correlation between high levels of aflatoxin in the diet and liver cancer does not prove a causal relationship. In countries where the incidence of liver cancer is high, the hepatitis B virus (HBV) is also common. Since this virus is known to be oncogenic, it is likely that liver carcinomas may arise from contributions of both agents (Hsieh, 1986). A further factor to consider in each case is the general malnutrition that prevails in one form or another in these areas. Lack of certain nutritional factors, e.g. protein or vitamin A, may predispose an individual to the toxic or even carcinogenic effects of AFB_1 (Smith and Moss, 1985; Newberne, 1987; Decoudu *et al.*, 1992). Recently, Prabhu *et al.* (1989) have reported that in rats, a copper deficiency enhanced the conversion of AFB_1 to its reactive metabolite, resulting in greater AFB_1–DNA adduct formation and increasing the risk of liver cancer.

2. CELLULAR METABOLISM

2.1. Activation

Activation of AFB_1 is important in any mycotoxicological consideration of the effects of AFB_1 on organisms. AFB_1 in itself is not carcinogenic, but is metabolised by the body to produce an ultimate carcinogenic metabolite (Swenson *et al.*, 1974), AFB_1–8,9-epoxide, formed by oxidation of the 8,9-vinyl ether bond (Fig. 2). Patterson (1973) traced the biotransformation of AFB_1 in susceptible cells by a pathway later modified by Ueno and Ueno (1978). Following transport across the plasma membrane, the AFB_1 molecule is activated by microsomal (smooth/tubular endoplasmic reticulum (ER)-associated) mixed-function mono-oxygenases (requiring cytochrome P450, NADPH and molecular oxygen) to form the highly reactive AFB_1–8,9-epoxide (Swenson *et al.*, 1974). Additionally, the nuclear envelope of rat hepatocytes is also reported to contain all the enzymes necessary for the metabolic activation of AFB_1 (Kasper and Gonzalez, 1982). The AFB_1 epoxide may bind to nuclear DNA, resulting in nuclear damage, or may bind to sex-linked sites on the ER. This binding to the latter may result in ribosomal detachment and polysome degradation. AFB_1 may also be *reversibly* converted by an NADPH-reductase to aflatoxicol. The aflatoxicol thus may act both as a sink and a reservoir for AFB_1 (Patterson, 1973; Hsieh *et al.*, 1977; Wong and Hsieh, 1978). The microsomal mono-oxygenase system is also responsible for transforming the AFB_1 into polar molecules such as AFM_1, aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1). AFM_1, AFP_1 and AFQ_1 can be eliminated by

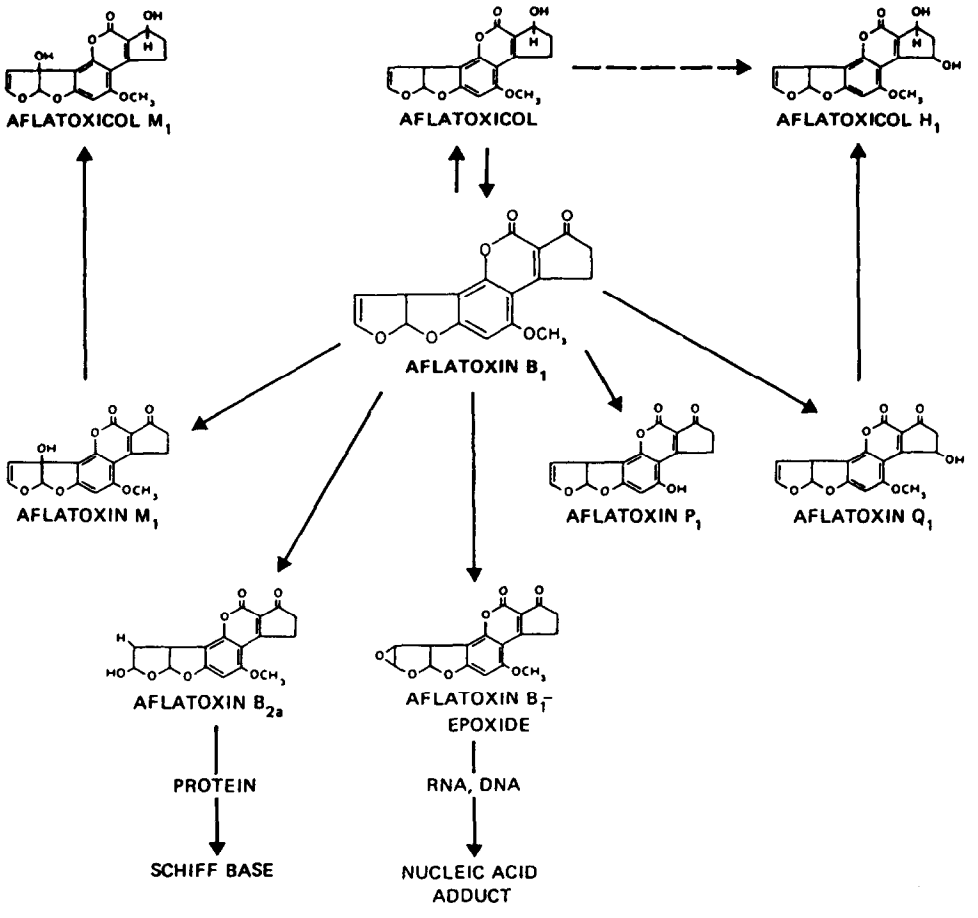

Fig. 2. Metabolism of aflatoxin B_1 (World Health Organization, 1979).

the hepatocytes, but the epoxide binds to nucleic acids and proteins and is thought to be the carcinogenic form of AFB_1 (Swenson *et al.*, 1974). The AFB_1-epoxide may become hydrated to its dihydrodiol (8,9-dihydro-8,9-dihydroxy AFB_1), followed by rearrangement to a putative dialdehyde phenolate intermediate, which is capable of condensing with primary amino acid groups of proteins and other cellular constituents, forming Schiff bases (Neal and Colley, 1979). AFB_{2a}, thought to be a hydrolytic product of the AFB_1 or its conjugates, in the phenolate form, binds to proteins, forming Schiff bases (Fig. 2). This AFB_{2a} may then cause the acute toxic effects of AFB_1 (Hsieh *et al.*, 1977; Hsieh, 1987). The decreased toxicity of AFB_{2a} when administered orally can be explained on the basis of non-absorption in the gut (Thompson *et al.*, 1992).

Amstad *et al.* (1984) have postulated an alternative mode of action for AFB_1 to this direct mechanism of binding to critical intracellular macromolecules. AFB_1 may exert its genotoxic effects by an indirect mechanism: through being membrane-active, via the intermediacy of active oxygen, lipid hydroperoxidases and small aldehydes (Amstad *et al.*, 1984). In that study, sister chromatid exchanges were induced in human lymphocytes at very low levels of covalent AFB_1–DNA adducts, which could not be explained entirely in terms of a direct genotoxic action.

2.2. Detoxification

Detoxification reactions of mycotoxins invariably involve conjugation of the toxin to glucuronic acid, sulphate or glutathione (Hsieh, 1987). The major detoxification reaction of AFB_1 is conjugation of the reactive epoxide to glutathione (mediated by glutathione *S*-transferase, GST) (Degen and Neuman, 1978, 1981). The AFB_1–glutathione conjugate is excreted primarily through the bile

(Hsieh, 1987). The conjugate, however, is reported to have the potential to be hydrolysed by the intestinal microflora, to release the AFB_1 for reabsorption and enterohepatic circulation (Hsieh and Wong, 1982). AFB_1–8,9-epoxide might also be detoxified by the UDP-glucuronyl-transferase, sulphotransferase and possibly the epoxide hydrolase systems (Hayes *et al.*, 1991a).

Most of the other aflatoxins (AFP_1, AFH_1, AFG_1, AFM_1) form glucuronide or sulphate conjugates and can be excreted in the urine (Wong and Hsieh, 1980). AFB_1 (or its epoxide) may be hydroxylated to form AFQ_1 and AFM_1 (Roebuck and Wogan, 1977; Raney *et al.*, 1992b) or demethylated to form AFP_1 (Roebuck and Wogan, 1977). The relative resistance or susceptibility of different animal species may depend then, not only on differences between activation of AFB_1, but also on differing abilities for its conversion to conjugation products that can be excreted (Hsieh *et al.*, 1977; Roebuck and Wogan, 1977). In this regard, Roebuck and Wogan (1977) have reported that resistant species (e.g. monkey, mouse and human) were able to excrete AFQ_1 and AFP_1, while the more susceptible species (e.g. duck and rat) produced aflatoxicol and no AFP_1.

2.3. Interaction with Biomolecules

2.3.1. *Nucleic Acids*

Nucleophilic hetero-atoms (e.g. nitrogen and oxygen) in the organic bases of nucleic acids are susceptible to electrophilic attack by metabolites of mycotoxins, forming covalent adducts. Any alteration in nucleic acid (both DNA and RNA) structure effected by these adducts will impair DNA and RNA template activity, resulting in inhibition of DNA, RNA and ultimately protein synthesis. The possible resultant point mutations may lead to the manufacture of non-functional molecules (Hsieh, 1987). Adduct formation *in vivo* may result, therefore, in transformation of cells, or even cell death, depending on the severity of impairment of template activity (Hsieh, 1987). Ewaskiewicz *et al.* (1991), however, have reported that low doses of AFB_1 may result in transient non-covalent AFB_1–DNA binding, which forms prior to AFB_1 activation and DNA adduct formation.

Both AFB_1–epoxide and, to a lesser extent, its hydration product, the dihydrodiol form of AFB_1, react with nucleic acids. The epoxide specifically makes an electrophilic attack on the N^7 position of guanine of DNA and RNA (Croy and Wogan, 1981a,b; Croy *et al.*, 1978; Essigmann *et al.*, 1980; Benasutti *et al.*, 1988), while the dihydrodiol forms a Schiff base with amino groups of the bases (Hsieh, 1987). The dihydrodiol is highly reactive and binds to proteins at the site of its formation (Neal and Colley, 1979). While AFB_1–N^7-guanine (*trans*-2,3-dihydro-2-(N^7-guanyl)-3-hydroxy AFB_1) is the major adduct formed, other metabolites of AFB_1 have the ability to form adducts with DNA, in particular, AFM_1–N^7-guanine and AFP_1–N^7-guanine (Essigmann *et al.*, 1982). The structure of the epoxide formed may be an important consideration in the affinity of the molecule for DNA. For example, the cyclopentenone ring fused to the lactone ring of the coumarin allows intercalation with DNA, while the less planar delta-lactone ring of aflatoxins G_1 and G_2 reduces DNA binding affinity by approximately one order of magnitude (Raney *et al.*, 1990).

The N^7-guanyl adduct is unstable and may either undergo spontaneous, non-enzymatic depurination or be stabilised by the opening of the imidazole ring to yield pyrimidyl adducts [2,3-dihydro-2-(N^5-formyl-2,3,6-triamino-4-oxopyrimidine-N^5-yl)-3-hydroxy AFB_1 (AFB_1 FAPY) and 8,9-dihydro-8-(2-amino-6-formamide-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB_1 (AFB_1 III)] within 24 hr of AFB_1 exposure (Hayes *et al.*, 1991a). The pyrimidyl adducts are not lost spontaneously, but appear to be removed catalytically by DNA repair enzymes. The presence of the N^7-guanyl adduct in the urine of exposed individuals arises as a result of this spontaneous depurination. Depurination at guanine residues could lead to a GC → TA conversion during replication, while the formamidopyrimidine derivatives are repair-resistant and thus, relatively persistent, resulting in mutations if present at the time of DNA replication (Croy and Wogan, 1981b).

2.3.2. *Proteins*

Besides being important structural and functional cellular components, proteins also act as cellular receptors, having nucleophilic nitrogen, oxygen and sulphur hetero-atoms in their functional groups

(Hsieh, 1987). The structure and activities of proteins may be altered by non-specific-irreversible-covalent (conformational change resulting in denaturation or blocking of binding sites) and specific-reversible-non-covalent (competitive binding) binding with mycotoxins. Proteins that bind mycotoxins reversibly may act as reservoirs of the toxin, prolonging toxin exposure, or they may serve as carriers in the transport of reactive metabolites (Ch'ih and Devlin, 1984; Hsieh, 1987).

There is evidence that some AFB_1 molecules become cytoplasmically bound to molecules destined for the nucleus (Ch'ih and Devlin, 1984; Ch'ih *et al.*, 1993). The former researchers have proposed the presence of a cytoplasmic binding protein(s). On entering the cell, AFB_1 is translocated (non-covalently bound) to microsomes (Ewaskiewicz *et al.*, 1991) for activation, facilitated by this cytoplasmic binding protein. The majority of the epoxide is detoxified and is removed rapidly from the cell as water-soluble, polar metabolites (Ch'ih and Devlin, 1984). A portion of the activated AFB_1 is translocated to various subcellular sites where covalent binding occurs, first to cellular macromolecules (e.g. rER) and then later in the nucleus, and finally in mitochondria (Ch'ih and Devlin, 1984). More recently, several cellular proteins (e.g. pyruvate kinase > albumin > carbonic anhydrase > pancreatic RNase > histones) were found to bind AFB_1, while the presence of a nuclear location sequence (NLS) (as is found in histones) markedly increased nuclear translocation and activation of the AFB_1 in the nucleus (Ch'ih *et al.*, 1993). Such findings exemplify the opportunistic nature of AFB_1.

Mycotoxin binding to functional proteins may inhibit protein activity, particularly in the case of enzymes. If biosynthesis of the protein is unaffected, then effects on the protein can be reversed, as soon as the non-functional proteins are replaced by *de novo* synthesis. Proteins involved in biosynthetic pathways, neurotransmission, hormone functions, membrane transport and immune mechanisms are critical factors when considering the biochemical and physiological effects of mycotoxins. In addition, binding to molecules distal from the active site or to inert proteins may represent a detoxification and sequestering mechanism and as such, may act as a toxin sink (Hsieh, 1987).

2.4. Inhibition of ATP Generation

At acute mycotoxin exposure levels, inhibition of cellular energy production is a major metabolic effect (Hsieh, 1987). In this regard, AFB_1, AFG_1 and AFM_1 inhibit oxygen uptake in tissue homogenates (Smith and Moss, 1985). The aflatoxins act on the electron transport system, with AFB_1, AFG_1 and AFM_1 inhibiting the electron transport chain between cytochromes b and c or c_1 (Site II) in rat liver mitochondria (Doherty and Campbell, 1972, 1973). AFB_1 is also known to act at the cytochrome oxidase level (Kiessling, 1986; Betina, 1989). It would appear, however, that the biochemical effects of AFB_1 on liver mitochondria do not require metabolic activation (Hsieh, 1987), although Niranjan and Avadhani (1980) have reported the presence of a cytochrome P450 type of mono-oxygenase system in rat liver mitochondria that is capable of generating electrophilic reactive metabolites that could covalently modify mitochondrial DNA, RNA and proteins.

Uncoupling of oxidative phosphorylation results in depletion of cellular ATP. As a result, sodium and potassium gradients within the cell are affected and mitochondria swell (Hsieh, 1987). AFM_1 has been found to uncouple *in vitro* oxidative phosphorylation and inhibit electron transport (Pai *et al.*, 1975). AFB_1 is a similar uncoupler, but is more effective as an electron transport inhibitor, also inhibiting ATPase activity (Hsieh, 1987). AFB_1 may also inhibit rat liver oligomycin-sensitive Mg^{2+} ATPase (i.e. ATP synthase) of the inner mitochondrial membrane (Hayes, 1978).

2.5. Immunocytochemical Localisation of Aflatoxin B_1

Recent immunological advances have made it possible to obtain from commercial sources a wide range of antibodies directed against many naturally occurring compounds. By means of a primary antibody and a secondary antibody to which was attached a 5-nm colloidal gold probe (all available commercially; Sigma Immunochemicals, St Louis, MO), an indirect immunocytochemical technique was designed to detect AFB_1 within the cells of plant tissues. This indirect immunocytochemical technique involved the use of a primary antibody (anti-AFB_1), directed against an antigen (AFB_1).

The immunological reaction was visualised electron microscopically by means of a secondary antibody (raised against the primary antibody) to which was attached a 5-nm gold probe. Ultrathin sections of tissues were then exposed to the above immunological reagents. The use of appropriate controls (first level [method] and second level [adsorption]) confirmed the validity of the positive results obtained. Following a continuous supply of AFB_1 in the medium, AFB_1 could be immuno-located within the nucleus (specifically associated with the nucleoplasm rather than within the nucleolus), the vacuoles and the cytoplasm of the stem cells of regenerating tobacco plantlets (*Nicotiana tabacum*) and root tips of excised, germinating embryos of maize (*Zea mays*) cultured *in vitro* (Fig. 3). Occasional gold particles were associated with the cell walls and with organelles (mitochondria and plastids). The results of this immunocytochemical investigation confirm the published evidence for animals, that AFB_1 acts directly on the nucleic acids, particularly the DNA (Meneghini and Schumacher, 1977). It is probable that several of the measured decreases (e.g. RNA and protein syntheses) following AFB_1 exposure will then be secondary manifestations resulting from AFB_1-DNA binding.

3. EFFECT ON MACROMOLECULAR BIOSYNTHESIS

3.1. DNA

Inhibition of macromolecular biosynthesis is a major metabolic effect of mycotoxins and may lead to failure to replace essential molecules, particularly functional proteins, possibly resulting in cell death (Hsieh, 1987). Mast cell stimulation, as a result of tissue damage, could cause inflammation, leakage of body fluids and subsequent haemorrhage (Hsieh, 1987), although AFB_1 itself does not appear to stimulate histamine release from these cells (Bent *et al.*, 1993). If the animal survives, cell regeneration may promote the expression of existing DNA lesions and, hence, the possible development of the tumorous condition (Hsieh, 1987).

One of the first measurable effects of AFB_1 on cells and tissues is inhibition of DNA synthesis. In the liver, this inhibition occurs at toxin concentrations, which apparently are not inhibitory to RNA or protein synthesis (Meneghini and Schumacher, 1977), suggesting interference of DNA synthesis to be a primary biochemical effect. It would appear that AFB_1 blocked the initiation step in DNA replication rather than the elongation process. Inhibition may result from covalent binding of AFB_1 to DNA and proteins, leading to modification of DNA template activity and/or inactivation of certain enzymes in DNA synthesis (Hsieh, 1987). Covalent binding of AFB_1 to membrane proteins may also reduce uptake of thymidine and other precursor nucleotides necessary for DNA synthesis (Kunimoto *et al.*, 1974).

3.2. RNA

Synthesis of rat liver RNA is inhibited rapidly by AFB_1 (Sporn *et al.*, 1966; Lafarge and Frayssinet, 1970; Yu, 1977, 1981), especially nucleolar RNA synthesis, related to formation of rRNA (18S and 28S) and rRNA precursors (32S and 45S) (Roy, 1968; Yu, 1977). This inhibition is due primarily to reduction of DNA template activity and inhibition of RNA polymerase II, an enzyme largely responsible for mRNA synthesis (Gelboin *et al.*, 1966; Pong and Wogan, 1970; Saunders *et al.*, 1972; Yu, 1977), and from impairment of nucleotide transport (Kunimoto *et al.*, 1974; Akinrimisi *et al.*, 1974). On the other hand, RNA polymerase I activity was largely unaffected by AFB_1 administration (Yu, 1977). Yu (1983) found that after activation *in vitro* and *in vivo*, AFB_1 binds preferentially to the physiologically active regions of the nucleolar chromatin of rat liver cells, possibly explaining the measured decreases in RNA synthesis. In a previous report, Yu (1981) had suggested that AFB_1 may interfere with RNA chain elongation. Additionally, chromosomal proteins may play a role in the binding of AFB_1 to DNA, since removal of these proteins resulted in a substantial loss of this specific binding (Yu, 1983). Contrary to this, however, Ch'ih *et al.* (1993) have found that several extranuclear proteins (e.g. albumin, pyruvate kinase) could bind AFB_1 more effectively than could histone proteins.

AFB_1 disrupts post-transcriptional processing of nuclear RNA for the manufacture of rRNA from nucleolar RNA precursors (Harley *et al.*, 1969), interfering with cleavage of the 45S RNA (into 18S and 28S rRNA) in rat liver (Hsieh, 1987). Transfer RNA processing is interrupted similarly, resulting

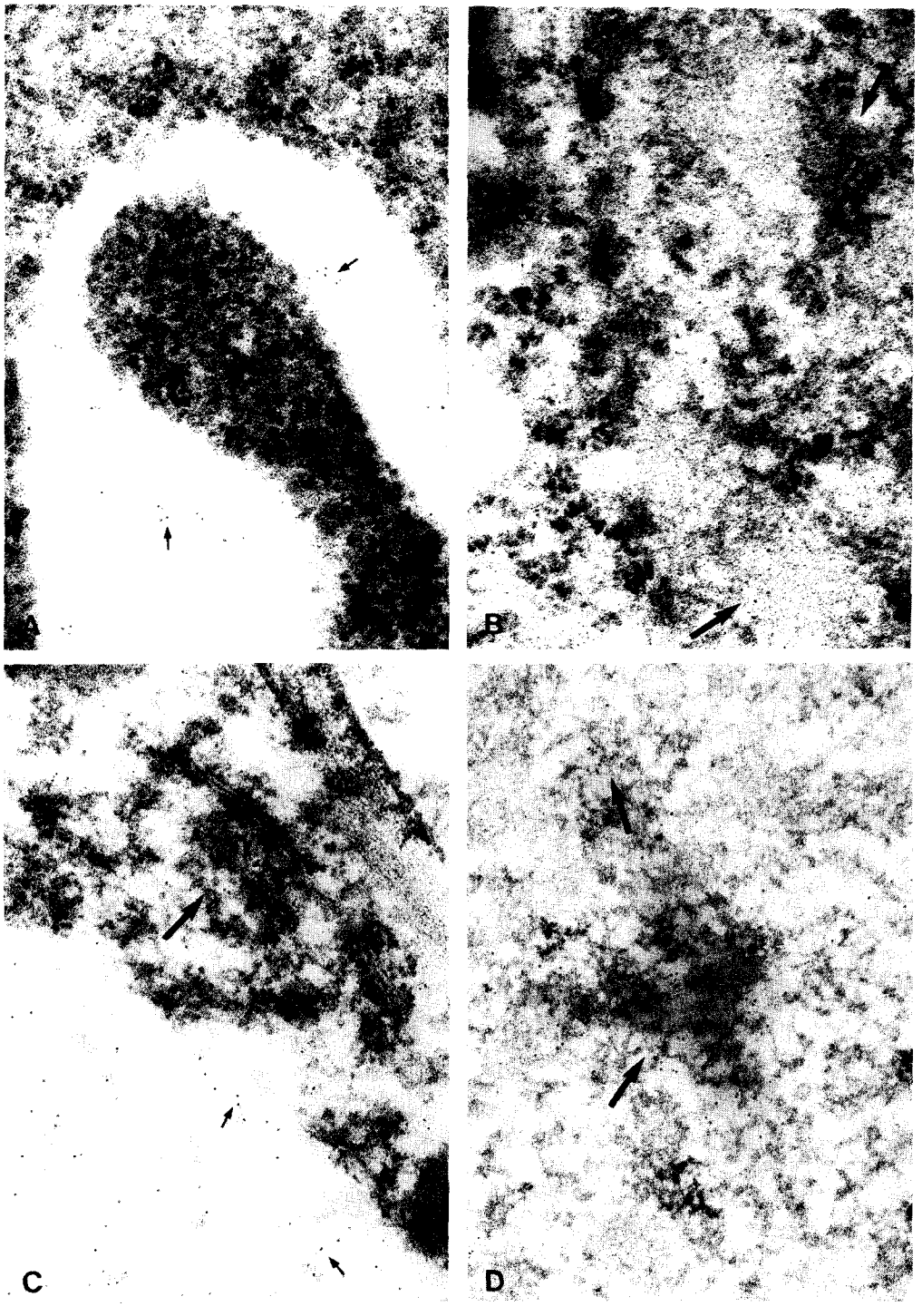

Fig. 3. Immunocytochemical localisation of aflatoxin B_1 in plant cells (magnification = 75,000 ×). **A** and **B**: Gold particles within the vacuole (**A**) and nucleus (**B**) of root tip cells of excised, germinating embryos of *Zea mays*. **C** and **D**: Localisation of aflatoxin B_1 within the cytoplasm and vacuole (**C**) and nucleus (**D**) of stem cells of regenerating plantlets of *Nicotiana tabacum*.

in elevated cytoplasmic levels of the 5S precursor of tRNA (Hsieh, 1987). In rats treated with AFB_1, Irvin and Wogan (1984) found that rDNA regions of liver DNA were preferentially accessible to AFB_1 modification, which may be explained in terms of the diffuse conformation within the transcribing gene.

Alterations in nuclear and nucleolar morphology are some of the most prominent effects of aflatoxin (and several other mycotoxins) in treated animal cells (Terao and Ueno, 1978). Ultrastructural morphological changes to the nucleolus that frequently have been reported include a gradual redistribution of nucleolar components (macrosegregation), resulting in segregation of granular and fibrillar components, fragmentation and the development of ring-shaped nucleoli. These observations may be visible manifestations of the measured alterations in nucleolar RNA synthesis (Roy, 1968; Yu, 1977) or the apparent accessibility of rDNA regions by AFB_1 (Irvin and Wogan, 1984).

3.3. Protein

Inhibition of protein synthesis by mycotoxins such as AFB_1, may arise directly from inactivation of biosynthetic enzymes, or indirectly by alteration of DNA template activity, or inhibition of RNA synthesis and maturation, translation, and/or interference with amino acid transport (Hsieh, 1987). Ultrastructurally, degranulation (detachment of ribosomes) from ER frequently has been reported in AFB_1-treated cells (Terao and Ueno, 1978). Such observations may arise as a result of disruptive changes, including direct damage to the ER membranes, interference with the ribosome binding sites on the membrane, interference with the ribosomal cycle, inhibition of the release of newly synthesised proteins and a suppression of mRNA synthesis (Terao and Ueno, 1978). As a result of this dissociation of ribosomes, ER-mediated protein synthesis is likely to be disrupted. In this regard, Viviers and Schabort (1985) found AFB_1-induced alterations in the phosphoprotein patterns in soluble and insoluble rat liver fractions, possibly by changes in amount and properties of specific proteins, the substrate proteins themselves and specific phosphatases. It is possible that *de novo* protein synthesis may also be affected.

3.4. Carbohydrate and Lipid Metabolism

Several animal species, when administered AFB_1, exhibit reduced hepatic glycogen levels and elevated serum glucose levels (Kiessling, 1986). These may arise from either an inhibition of glycogenic enzymes (e.g. glycogen synthase), an inhibition of glyconeogenesis, a decrease in glucose transport into hepatocytes or an increase in the activity of enzymes metabolising glycogen precursors (e.g. glucose 6-phosphate dehydrogenase) (Kiessling, 1986; Hsieh, 1987).

AFB_1 is known to cause lipid accumulation in the liver (Hamilton, 1975). This is generally believed to arise as a result of impaired lipid transport rather than increased lipid biosynthesis. Chou and Marth (1975) have reported an increase in hepatic lipid levels in mink injected with AFB_1, although there was no observable difference in [^{14}C]acetate uptake. Based on their findings, Chou and Marth (1975) have suggested that such hepatic lipid increases result from reduced oxidation of fats or increased lipid synthesis. In this regard, damage to mitochondria [which is frequently observed in AFB_1-treated cells (Terao and Ueno, 1978)] may result in decreased oxidation by these organelles, with a concomitant accumulation of lipids in the liver. The possibility that AFB_1 (or its metabolites) may alter the mobility of lipids is not overlooked by these workers.

This alteration in lipid transport or synthesis occurs at dietary toxin concentrations that do not affect growth rate or RNA synthesis (Hsieh, 1987). In chickens, AFB_1 not only affected lipid synthesis and transport, but appeared to influence lipid absorption and degradation (Tung *et al.*, 1972). Thus, impaired triacylglyceride transport (in chickens, at least) is a primary lesion and is not a secondary effect resulting from impaired nucleic acid metabolism (Tung *et al.*, 1972).

4. SPECIFIC EFFECTS

4.1. Immune Response

Several review articles on impairment of the immune response by mycotoxins in several experimental animal species have been published (Pier, 1973, 1986, 1992; Richard *et al.*, 1978; Pier *et al.*, 1980, 1986; Pier and McLoughlin, 1985; Pestka and Bondy, 1990, 1994; Sharma, 1993). Generally, an inhibition of protein synthesis could result in an alteration of serum protein concentrations, leading to suppression of non-specific, humoral substances. Subacute doses of AFB_1 in guinea pigs resulted in complement deficiency (Thurston *et al.*, 1972), delayed interferon production in turkeys (Pier, 1973; Pier and McLoughlin, 1985) and delayed lymphokine activity (Pier *et al.*, 1977). At higher doses, AFB_1 lowered levels of immunoglobulin G and immunoglobulin A in chicks (Giambrone *et al.*, 1978), leading to an impairment of acquired immunity. Recently, Pier (1992) has reported that mycotoxins may reduce the efficacy of acquired immunity during vaccination. Pier *et al.* (1986) found that *in vitro* exposure of B-lymphocytes to AFB_1 (and T-2 toxin) caused suppression of the lymphogenic response. Exposure of 18-day-old chick embryos to AFB_1 was found to induce dose-related increases in sister chromatid exchanges in T-lymphocytes (2-fold increase) and B-lymphocytes (6- to 8-fold increase). AFB_1 also reduced the mitotic index of B-cells and reduced the progression of B-lymphocytes, and to a lesser extent, T-lymphocytes, through successive rounds of replication (Potchinsky and Bloom, 1993). In human lymphocytes, low doses of AFB_1 were found to cause mitotic aberrations in a dose-dependent manner (Amstad *et al.*, 1984).

AFB_1 affects the cell-mediated immune response, causing a reduction in the response of T-lymphocytes to phytohaemagglutinin, thymic involution and failure to develop immunity following vaccination in turkeys (Pier *et al.*, 1972) and in chickens (Giambrone *et al.*, 1978). Experimentally, AFB_1 has been found to reduce antibody production, inhibit the phagocytic ability of macrophages, reduce complement, decrease T-cell number and function and cause thymic aplasia (Richard *et al.*, 1978; Pier, 1986; Reddy *et al.*, 1987).

Haemopoiesis also appears to be affected by AFB_1. Cukrová *et al.* (1991) found that a dose of AFB_1 as low as 0.5 $\mu g\ mL^{-1}$ exerted a strong suppression of myelopoiesis in bone marrow cultures. Recently, exposure of rats to aflatoxin resulted in an initial suppression of granulocyte and monocyte colony-forming units in the bone marrow (Cukrová *et al.*, 1992a,b), possibly as a result of an inhibition of mRNA transcription.

Impairment of the efficiency of the mononuclear phagocytic system has been observed. AFB_1 was found to suppress the activity of Kupffer cells in the liver (Mohapatra and Roberts, 1985), while others have reported an inability of bovine macrophages to produce interleukin 1, when presented with *Listeria monocytogenes* and other bacteria, following pretreatment of the animals with 10 $\mu g\ mL^{-1}$ AFB_1 (Kurtz and Czuprynski, 1992). *In vitro* exposure of chicken peritoneal macrophages to AFB_1 resulted in a dose-dependent increase in cellular damage and a decrease in macrophage adherence ability (Neldon-Ortiz and Qureshi, 1992). If mixed-function oxidases were added to this culture system, in addition to these observations, reduced phagocytic ability of macrophages was detected at much lower AFB_1 concentrations. It is likely that on addition of mixed-function oxidases, AFB_1 was more readily metabolised to its reactive metabolite, resulting in exacerbated cellular damage.

Secondary mycotoxin-related diseases may result from impairment of the immune system. Animals showed increased susceptibility to candidiasis, coccidiosis, salmonellosis and general immunologic deficiency. Thus, mycotoxins could predispose livestock to infectious diseases, resulting in decreased productivity (Pestka and Bondy, 1990), and possibly mortality as a result of complications.

4.2. Hormonal Effects

Steroid hormones regulate cellular functions by specifically and non-covalently binding to cytoplasmic receptor proteins and membranes in target cells. Following activation, the hormone–receptor complexes are transported to the nucleus and there induce selective gene transcription (mRNA) by binding to chromatin acceptor sites (Guyton, 1987). AFB_1 is known to bind covalently to DNA (specifically at the guanine residues), thereby possibly decreasing nuclear acceptor sites for hormone receptor complexes, with a consequent reduction in the activity of the

hormone. AFB_1 is known to reduce, in a dose-dependent manner, the nuclear acceptor sites for the glucocorticoid–cytosol receptor complex in rat liver (Wogan and Friedman, 1968). The formation of the hormone–receptor complex appeared unaffected (Hsieh, 1987). An interesting discussion involving a possible AFB_1–steroid hormone–ER–lysosomal enzyme pathway in the development of tumorous cells was presented by Money-Kyrle (1977).

AFM_1 is known to compete with oestradiol for the uterine cytosol receptor site at concentrations at which AFB_1, AFB_2 and AFG_1 were ineffective (Kyrein, 1974). AFB_1 inhibits the binding of polysomes to ER, thereby inhibiting protein synthesis. Incubation with corticosterone (but not hydrocorticosterone) reduced the effect of AFB_1, presumably by competing for the polysome-binding sites on the ER membrane (Williams and Rabin, 1969). *In vitro*, sex-linked binding sites of smooth or degranulated microsomes from rat livers were completely inhibited by AFB_1 (i.e. selective binding of testosterone by female liver microsomes and of oestradiol by male liver microsomes) (Blyth *et al.*, 1971; Sunshine *et al.*, 1971; Kiessling, 1986; Hsieh, 1987).

4.3. Mutagenic and Teratogenic Effects

AFB_1 (or more correctly, its epoxide) is the most potent mutagen of the aflatoxins, and there is a strong correlation between the ability of aflatoxins to be mutagenic and carcinogenic (Smith and Moss, 1985). AFB_1 causes chromosomal aberrations (chromosomal fragments, with occasional bridges, chromatid bridges and chromatid breakages) and DNA breakage in plant and animal cells (World Health Organization, 1979; Smith and Moss, 1985). It also produces gene mutations in bacterial test systems (Ames' test), where activation by rat or human microsomal preparations is essential (Wong and Hsieh, 1976).

Several mycotoxins, including AFB_1 are teratogenic (Hayes, 1981; Smith and Moss, 1985). Mycotoxins, which are potent inhibitors of protein synthesis, might be expected to cause impairment of development of primordia and differentiation in the foetus.

4.4. Carcinogenic Effects

4.4.1. *Initiators And Promoters*

Transformation of cells to the tumorous state is a two-step process: initiation and promotion (Hsieh, 1987). In the initiation step, the biochemical lesions produced in RNA, and particularly DNA, become 'fixed' features following cell division (Hsieh, 1987). Rapidly dividing cells are more at risk from mutation than are quiescent cells, since during DNA replication, adducts are converted to mutations and the time required for DNA repair may be insufficient (Hayes *et al.*, 1991a). Altered cells are potentially cancerous, but must undergo promotion. Under favourable conditions, promotion will occur, and transformed cells may become malignant, proliferating independently of normal cellular regulatory mechanisms (Hsieh, 1987).

Carcinogenic chemicals may be classified as initiators, promoters or both. The latter category (initiator and promoter), which includes AFB_1, AFG_1, AFM_1, sterigmatocystin, versicolorin, luteoskyrin and rugulosin, are referred to as complete carcinogens. Ochratoxin A, zearalenone and the trichothecenes are generally regarded as promoters (Hsieh, 1987). The importance of co-contamination of food by more than one mycotoxin-producing fungus, therefore, cannot be overemphasised, e.g. fumonisins and AFB_1 (Ueno *et al.*, 1993).

AFB_1 has been reported to bind to DNA in a selective, non-random manner in rats: i.e. it binds specifically to hepatic mitochondrial DNA (Niranjan *et al.*, 1982), nuclear ribosomal RNA gene sequences of liver DNA (Irvin and Wogan, 1984) and transcriptionally active regions of liver nucleolar chromatin (Yu, 1983). This binding is related to the accessibility of these areas of DNA to the toxin. Such areas generally lack histones (Yu, 1983), while the rDNA regions maintain a diffuse conformation due to high transcriptional activity (Irwin and Wogan, 1984). Recently, Ch'ih *et al.* (1993) investigated the *in vitro* binding ability of AFB_1 to various proteins. Binding ability of AFB_1 to histones was comparatively low. Additionally, nuclear translocation and activation of AFB_1 and AFB_1–protein conjugates were assessed using rat liver nuclei. Proteins containing a NLS, e.g. histones and albumin–NLS, facilitated AFB_1 translocation into the nucleus, where activation and adduct formation occurred (Ch'ih *et al.*, 1993).

AFB_1 is reported to be capable of covalently binding to mitochondrial DNA with a 3- to 4-fold greater affinity than for nuclear DNA (Niranjan *et al.*, 1982). Lesions in mitochondrial DNA are persistent, perhaps reflecting a lack of *appropriate* excision repair mechanisms in this organelle. As a result, mitochondrial transcription and translation may be persistently inhibited by these lesions, contributing to neoplastic transformation of the cell (Hsieh, 1987).

Methylation of DNA may be inhibited by covalent binding of AFB_1 to DNA, thereby altering gene expression and cellular differentiation. Then, oncogenes may be activated, precipitating oncogenic transformation of mammalian cells by producing heritable transcriptional mutations in these genes (Wilson and Jones, 1983).

4.4.2. *The* Ras *Oncogenes and Hepatocellular Carcinoma*

Ras proto-oncogene activation by several carcinogens in tumour development has been well documented (Reynolds *et al.*, 1987; Balmain and Brown, 1988). More recently, AFB_1 has been demonstrated to activate the Ki-*ras* gene in rat liver. In this regard, in the final stages of AFB_1-induced rat liver hepatocellular carcinoma (HCC), two activating mutations in the codon 12 region of Ki-*ras* genes (GGT → GAT (McMahon *et al.*, 1987) and GGT → TGT (Sinha *et al.*, 1988)) have been identified. Soman and Wogan (1993) have confirmed the Ki-*ras* codon 12 GGT → GAT mutation in rat liver, suggesting the involvement of this genetic mutation in the development of AFB_1-induced HCC in rats. There is, however, no evidence that *ras* gene mutations occur in human HCC (Bailey and Williams, 1993).

4.4.3. *The* p*53 Gene and Hepatocellular Carcinoma*

Recently, evidence has been accumulating regarding the development of human HCC, with respect to aflatoxin, the *p*53 tumour-suppressor gene, and more specifically, codon 249 of this gene. Hsu *et al.* (1991) have found in Chinese patients a striking mutational specificity in the third base position of codon 249 of the *p*53 gene, resulting primarily in a G → T substitution. In Southern African and Asian patients, this transversion was detected at codon 249 in about 50% of the analysed HCC tumours (Hsu *et al.*, 1991; Bressac *et al.*, 1991). In non-human primates, however, no mutations at codon 249 were detected in AFB_1-induced tumours (Fujimoto *et al.*, 1992). The data for rats suggest that AFB_1 alone is not sufficient to account for the specificity of the *p*53 mutations in HCC. While Lilleberg *et al.* (1992) are of the opinion that alteration of the *p*53 suppressor gene is involved in HCC induction in rats, the results of Hulla *et al.* (1993) regarding the specificity of the *p*53 mutation, however, suggest that AFB_1 is not responsible for these lesions.

Hsieh *et al.** attempted to assess the correlation between mutations at codon 249 and the level of AFB_1–DNA adducts in the liver tissue of HCC patients from a high AFB_1 risk area (Taiwan) and a low AFB_1 risk area (Japan). The AGG → AGT transversion was found in 21% of Taiwanese patients and none of the Japanese patients. AFB_1–DNA adducts, however, were found in tumorous and non-tumorous tissues from both groups of patients. Furthermore, AFB_1–DNA adducts were found in 50% of patients lacking the *p*53 mutation. It is the opinion of Hsieh *et al.* that adducts reflect recent AFB_1 exposure and so may not be a reliable index of earlier AFB_1 exposure that may have precipitated the induction of HCC.

In Japan, HCC is the third leading cause of cancer-related deaths (Nose *et al.*, 1993). Nose *et al.* (1993) have detected *p*53 gene alterations in only 30% of HCC patients, and invariably only in advanced cases, suggesting that *p*53 gene alteration may be a late event in tumorigenesis of HCC in Japan. Additionally, in Japan, hepatocarcinogenesis is often associated with a persistent HBV (or hepatitis C virus) infection, rather than with aflatoxin exposure (Nose *et al.*, 1993).

Chen *et al.* (1992) have reported that in Taiwanese patients, 70% of the HCC smears assessed were positive for AFB_1–DNA adducts. The findings relating the involvement of both HBV and AFB_1 in HCC development are controversial (Santella *et al.*, 1993). The results of these recent investigations serve to reinforce the idea of a multifactorial aetiology for the development of HCC. In attempting

*Hsieh, D. P. H., Atkinson, D. N. and Zhao, M.-S. (1992) Aflatoxin-DNA adducts and *p*53 gene alterations in human liver tumors. In: *Proceedings of the VIII International Symposium on Mycotoxins and Phycotoxins*, November 1992, Mexico City, Mexico, p. 36.

to determine the agent(s) involved in regional development of liver cancer, many factors need to be considered: physiological and ethnic differences; additional microbial agents (other than aflatoxigenic fungi) and local contaminants of foods and feeds. Wogan (1992)* has suggested that a possible synergistic response may exist between chemical and viral agents in the environment and has considered the possibility of other mycotoxins acting as mutagenic and carcinogenic agents. Of these mycotoxins, the fumonisins and sterigmatocystin are the most likely candidates. While these mycotoxins have been found to be relatively potent liver carcinogens in experimental animals, little is known about human exposure (Wogan, 1992). It is the opinion of Fujimoto *et al.* (1992) that the development of the mutation in codon 249 of the *p*53 gene in human HCC is likely to involve environmental carcinogens other than AFB_1, or that the HBV hepatitis is a prerequisite for AFB_1-induced G → T transversion in the codon. It is also probable, as is suggested by Puisieux *et al.* (1991), that the *p*53 mutational hotspots identified in different tumours are selected targets for specific environmental carcinogens.

In a study on Gambian children, Wild *et al.* (1992) found that the majority of individuals (75–100%) had AFB_1-albumin adducts. Children who were positive for HBV surface antigens had higher adduct levels than children with markers of past infection or who had never been infected with the virus. There were highly significant differences between three major ethnic groups, necessitating consideration of other physiological factors, such as polymorphism in cytochrome P450 and GST. In this regard, Hollstein *et al.* (1993) found an AGG → AGT transversion at codon 249 and an ATC → AAC transversion at codon 254 in 15 Taiwanese HCC patients. All but one patient were negative for AFB_1–liver adducts and AFB_1–serum albumin adducts. On genotyping patients for GST, it was found that 12 of the 15 patients possessed the null genotype.

Ozturk *et al.* (1991) have provided some of the best evidence relating AFB_1 ingestion with HCC development. They noted a specific mutation in the *p*53 tumour-suppressor gene in hepatoma tissue from patients at high risk of AFB_1 exposure. In their study, in four countries where AFB_1 intake was high, 22% of tumour samples had the characteristic mutation at codon 249 of the *p*53 gene, in comparison with less than 1% in tumours from patients from countries where the risk of AFB_1 intake was low. HBV infection was commensurately high in countries where AFB_1 contamination of food was prevalent. Ozturk *et al.* (1991) then compared similar incidents of HCC and HBV in a high AFB_1 intake area (Mozambique) with a low AFB_1 intake area (Transkei). In Mozambique, 53% of patients exhibited this mutation, while only 8% of the patients had this mutation in the Transkei. Considering that both groups had similar HBV exposure levels (approximately 11%), it was suggested that HBV was not responsible for the difference in incidence of codon 249 mutations. Those findings substantiate earlier reports of van Rensburg *et al.* (1985) that the estimated daily intake of AFB in Mozambique was approximately four times that of Transkeians. The ratio of HCC incidence in these two areas was similar, suggesting an aetiological role for AFB_1 as a procarcinogen in the development of liver cancer. However, Kolars (1992) still is of the opinion that HBV (or other agents of chronic liver disease endemic to particular areas) may be a prerequisite for AFB_1-mediated HCC. In this regard, Hsing *et al.* (1991) found that in 65 counties in China, HCC mortality rates were significantly linked to the prevalence of HBV surface antigen positivity. Incidence was higher where there was elevated levels of blood cholesterol, greater liquor consumption and a diet high in rapeseed oil and mouldy corn. No significant correlation was found between mortality and the levels of AFB_1 in urine. It is the opinion of Hsing *et al.* (1991) that HBV infection contributes to the substantial variation in liver cancer mortality in China, but they recognise the importance of dietary and environmental factors.

Patel *et al.* (1992) assessed patients from the United Kingdom, other countries of low AFB_1 intake and countries of high intake for *p*53 gene mutation at codon 249. The incidence of the mutation was low in all samples, and it is their opinion that other environmental factors need to be considered regarding the aetiology of human HCC. More recent work, however, has demonstrated the presence

*Wogan, G. N. (1992) Experimental and epidemiological evidence associating aflatoxin exposure, liver cancer risk and the involvement of oncogenes and tumor suppressor genes in liver carcinogenesis in humans. In: *Proceedings of the VIII International IUPAC Symposium on Mycotoxins and Phycotoxins*, November 1992, Mexico City, Mexico, p. 60.

of AFB_1–DNA adducts in a range of tissues taken from autopsy specimens in the United Kingdom (Harrison *et al.*, 1993).

Indirect evidence for AFB_1 involvement in HCC comes from Chongming Island, a high risk region for HCC near Shanghai. Since the 1960s, there has been a marked decrease in the use of maize, a commodity invariably high in aflatoxins. Concomitantly, a subsequent regression in the local incidence of liver cancer has been recorded (Ross *et al.*, 1992). In another study, Yu (1992) measured AFB_1 intake and AFM_1 excretion in 81 households in 10 villages in the Chinese province of Guangxi and found a positive correlation between PLC mortality and AFB_1 intake from maize and peanut oil, but interestingly not from rice. Groopman *et al.* (1992b), in the Guangxi Autonomous Region, when analysing total AFB_1-N^7-guanine excretion in urine plotted against total AFB_1 exposure, found a correlation of 0.8, suggesting that measuring excreted AFB_1 is a good indication of the level of AFB_1 consumption and adduct formation.

Yap *et al.* (1993), on reviewing the incidence of HCC, HBV and AFB_1 intake, concluded that both HBV and AFB_1 are risk factors and, in fact, may have a cumulative effect on HCC development. Yap *et al.* (1993) comment further that while HBV increases the likelihood of HCC, it is not essential for the development of HCC. Similarly, Zhang and co-workers (Zhang *et al.*, 1991), investigating the presence of AFB_1–DNA adducts and of HBV surface antigens in Taiwanese HCC patients, concluded that both AFB_1 and HBV may be involved in HCC development in Taiwan. In another study, Wu-Williams *et al.* (1992) have utilised the data generated for HBV, AFB_1 and HCC incidence in southern Guangxi, China, to generate models evaluating the relative importance of AFB_1 and HBV in the development of HCC. While purely additive models fitted the data poorly, multiplicative relative risk and interactive excess risk models provided satisfactory descriptions of that data and the data for the United States, a low risk area.

Recently, Wild and co-workers (Wild *et al.*, 1993), in assessing the numerous investigations involving HCC, HBV and aflatoxin, have concluded that despite the plausibility of an interaction between these two aetiological agents in HCC development, strong evidence supporting an interactive mechanism has not been elucidated.

In an extensive review of the research involving AFB_1–DNA adduct studies, Choy (1993) has commented that from both ingestion and injection studies, the dose–response of DNA adduct formation (mainly in rats) is linear, with no apparent threshold value. Based on these assessments, Choy (1993) has warned that extrapolation of this data to humans should be viewed critically, since human AFB_1–DNA adduct data are incomplete, although Groopman *et al.* (1993) concluded from their study that the presence of AFB_1–guanine adducts in urine is a good non-invasive marker for exposure to AFB_1 and the risk of genetic damage. Additional investigations undoubtedly will improve the risk assessment for humans with respect to AFB_1, and perhaps elucidate the individual contribution of AFB_1 and HBV to the development of HCC.

4.4.4. *Cytochrome P450, Glutathione* S-*transferase and Hepatocellular Carcinoma*

In the recent literature, efforts at understanding AFB_1 toxicity and the enigma of HCC development appear to concentrate on the GST enzymes (resulting in AFB_1 detoxification) and, to a lesser extent, on the cytochrome P450 bio-activation (and in some instances, detoxification) isoenzymes. Many of these studies are still in their early stages, but the general opinion is that a protective effect is afforded by GSTs in different tissues, as measured by decreases in AFB_1–DNA adduct formation with increased GST activity (Hayes *et al.*, 1991a; Coulombe, 1993).

Metabolism of AFB_1 involves oxidative reactions by members of the cytochrome P450 supergene family of isoenzymes. Different cytochrome P450 isoenzymes can result in AFB_1 metabolites of varying carcinogenic potential; for example, in humans, the formation of DNA–AFB_1 adducts depends on activation by cytochromes P450IA2, P450IIA3, P450IIIA4 and P450IIB [decreasing order] (Aoyama *et al.*, 1990) and cytochrome P450IA enzymes metabolise the detoxification of AFB_1 to AFM_1 (Koser *et al.*, 1988). Other cytochrome P450 isoenzymes are responsible for the conversion of AFB_1 to other less toxic metabolites: AFQ_1 (in humans, by P450IIIA (Forrester *et al.*, 1990)) and AFP_1 (Hayes *et al.*, 1991a).

At least 10-fold differences in cytochrome P450IIIA and P450IA expression have been observed between individuals (Watkins, 1990). In humans, it would appear then that the cytochrome P450IIIA

family is responsible for both AFB_1 epoxidation and the formation of AFQ_1 (Forrester *et al.*, 1990). The ability of a cytochrome P450 to catalyse both the activation and the detoxification of AFB_1 has been reported elsewhere (Guengerich *et al.*, 1992). AFM_1 production by human hepatic microsomes from different individuals correlates with the level of P450IA2 (Forrester *et al.*, 1990), while in rats, the $P_3$450 cytochrome appears to be involved (Koser *et al.*, 1988).

The ability of tissue to bio-activate AFB_1 is an important consideration in understanding the ability of AFB_1 to induce toxic, mutagenic or carcinogenic transformation in cells. In this regard, Imaoka and co-workers (Imaoka *et al.*, 1992) have investigated the genotoxic and mutagenic activation of AFB_1 on *Salmonella typhimurium* by rat hepatic, renal and pulmonary microsomal fractions and purified cytochrome P450 enzymes. Hepatic microsomes displayed the greatest mutagenic activation, while renal microsomes had the lowest activity. Additionally, cytochrome P450IIC2 (a major hepatic cytochrome P450 in male rats) had the highest activating ability, while renal forms of P450 (e.g. cytochromes P450IVA2 and P450 K-4) exhibited the lowest activities. It would appear that the greater ability of hepatic microsomes (as compared with pulmonary and renal equivalents) to bio-activate AFB_1 is dependent on the different classes of cytochrome P450s present in that tissue.

Various hepatic cell populations (hepatocytes, Kupffer and endothelial cells) have been found to differ in their AFB_1-bio-activating ability (Schlemper *et al.*, 1991). Ten-fold higher AFB_1 concentrations were required by non-parenchymal (Kupffer and endothelial) cells to obtain a similar number of *Salmonella typhimurium* TA98 revertants (as compared with parenchymal cells). In freshly isolated cells, AFB_1 was found to bind (although differentially) to DNA in both parenchymal and non-parenchymal cells in a dose-dependent manner (Schlemper *et al.*, 1991).

Metabolic activation of AFB_1 was studied using human cell lines that expressed individual cytochrome P450s (Crespi *et al.*, 1991). Cells expressing cytochrome P450IA2 were the most sensitive (at 10 ng mL^{-1}) to the toxic and mutagenic effects of AFB_1. Cells expressing cytochrome P450IIIA4 were 5- to 10-fold less sensitive than those expressing P450IA2. The least sensitive cells expressed cytochrome P450IIIA6, while cells resistant to 1 μg mL^{-1} AFB_1 expressed no cytochromes (Crespi *et al.*, 1991).

The ability of an organism (or a tissue) to form the AFB_1-epoxide might explain the sensitivity of trout and quail to AFB_1. A cytochrome P450 isolated from the livers of β-naphthoflavone-treated rainbow trout had a 15-fold greater ability to form AFB_1–8,9-epoxide than did either the phenobarbital-induced or β-naphthoflavone-inducible rat liver cytochrome P450s (Williams and Buhler, 1983). Similarly, Neal *et al.* (1986) found a cytochrome P450 with a particularly high epoxidation ability in quail liver microsomes.

In vitro epoxidation of AFB_1 was determined using liver microsomes from rats of different ages (as measured by adduct formation with calf thymus DNA). Newborn rats were capable of minimal AFB_1–DNA binding when compared with adults. Levels of the AFB_1–glutathione conjugate were similarly low in neonatal rats (Behroozikha *et al.*, 1992). These findings suggest that the immature liver is less efficient than the mature organ at activating and detoxifying foreign chemicals.

Kitamura *et al.* (1992) were able to transfect MCF-7 breast cancer cells with a plasmid containing cytochrome P450IIIA7 complementary DNA, obtaining three cell lines. These transgenic cell lines showed 8- to 10-fold higher sensitivity to AFB_1 than did the parental MCF-7 cells. These results would suggest that expression of this class of cytochrome P450 promoted the formation of reactive AFB_1 metabolites.

Expression of cytochrome P450 enzymes can be influenced by exogenous agents (Nebert *et al.*, 1991), e.g. cytochrome P450IIIA enzymes are inducible by glucocorticoids and rifampin (Watkins, 1990), and cytochrome P450IA enzymes can be induced by polycyclic hydrocarbons in cigarette smoke and by dietary 'green plant' flavones (Nebert *et al.*, 1991). In this regard, environmental agents might influence the susceptibility to AFB_1-mediated hepatocarcinogenesis by altering the expression of individual cytochrome P450 enzymes that either activate or detoxify AFB_1 (Kolars, 1992). In this regard, the observations of Lin *et al.* (1991) suggest that smoking might have a protective effect on individuals at risk of developing HCC. In that study, in a Fujian province considered to be a high AFB_1 intake area, the risk of hepatoma was significantly increased in non-smokers. One interpretation by Lin *et al.* (1991) is that smoking could impart protection, as cytochrome P450IA enzymes may be induced, thereby possibly promoting metabolism of AFB_1 to AFM_1 (essentially a detoxification reaction), rather than activation to more reactive metabolites by other cytochrome

P450 enzymes. Contrary to this, however, cytochrome P450IA2 has been shown elsewhere to be the most important cytochrome P450 isoenzyme promoting AFB_1 binding to DNA in humans (Aoyama *et al.*, 1990). Interestingly, Raney *et al.* (1992b) have postulated (based on experimental evidence) cytochrome P450IIIA4 to be the dominant enzyme in human liver microsomes involved in both the oxidation of AFB_1 to its epoxide (activation) and hydroxylation of AFB_1 to AFQ_1 (detoxification).

While it would appear that cytochrome P450 enzymes may be important considerations in explaining the relative susceptibilities of different animal species to AFB_1 (and many other noxious substances), the evidence implicating the protective effects of GST enzymes against AFB_1–DNA adduct formation in tissues is equally compelling. Although much of the work is either *in vitro* or utilises rats as experimental animals, the importance of AFB_1–glutathione conjugation as a significant detoxification mechanism cannot be ignored. The GSTs comprise a supergene family of enzymes that have been subdivided into 5 classes: α, μ, π, τ and microsomal (Hayes *et al.*, 1991a). However, little is known about the specific GSTs responsible for detoxifying AFB_1. In rats, the α-GSTs appear to have the greatest ability to metabolise 8,9-epoxides (Coles *et al.*, 1985).

It is well documented that animal species have differing susceptibilities to the mutagenic or carcinogenic effects of AFB_1 (Wong and Hsieh, 1976, 1980; Hsieh *et al.*, 1977; Roebuck and Wogan, 1977). In comparing AFB_1 toxicity in mice and rats, it is generally accepted that mice fall into the 'resistant' category, while rats are highly 'susceptible'. It was predicted that this difference was likely to depend (among other factors) on the differing abilities to detoxify AFB_1 (Hsieh *et al.*, 1977; Wong and Hsieh, 1980). When the proteins of complementary DNAs of rat GST Yc_1 and of mouse GST Yc were expressed from a prokaryotic expression vector in *Escherichia coli*, mouse isoenzyme activity towards AFB_1–8,9-epoxide had a 50-fold higher conjugating activity than did the equivalent isoenzyme of the rat (Beutler *et al.*, 1992). Beutler and colleagues are of the opinion that the α class GST Yc isoenzymes in mouse liver protect these animals from the hepatotoxic effects of AFB_1, perhaps explaining the differing (and marked) susceptibilities of these two animal species to AFB_1.

In a series of mouse whole body autoradiographic studies, a group of Swedish researchers have interesting results regarding the extrahepatic tissue localisation of AFB_1 (Larsson *et al.*, 1992; Larsson and Tjälve, 1992). Pretreatment of adult mice with a glutathione-depleting agent resulted in accumulation of tissue-bound label (AFB_1) in the nasal olfactory and respiratory mucosae, as well as the mucosae of the nasopharynx, trachea and oesophagus (which was not observed in non-pretreated mice) (Larsson and Tjälve, 1992). The authors of the latter study are of the opinion that glutathione is normally responsible for scavenging AFB_1 in these tissues, thereby preventing AFB_1–DNA adduct formation. In additional studies, AFB_1–DNA adduct formation was also located in several extrahepatic sites in rainbow trout (uveal melanin, vitreous humor, kidneys, olfactory rosettes and pyloric caecae) (Larsson *et al.*, 1992). Furthermore, in autoradiographic studies in 1- and 5-day-old mice, a marked localisation of [^{3}H]-AFB_1 was found in the nasal olfactory mucosa. *In vitro* incubation of nasal olfactory mucosa with AFB_1 demonstrated marked binding in this tissue. If, however, glutathione was added to the incubation medium, this binding was reduced. Autoradiography of [^{3}H]-AFB_1 in pregnant mice showed labelling of the foetal olfactory mucosa (at day 18 but not at day 14) (Larsson and Tjälve, 1992). It would appear then that *in vivo* accumulation of AFB_1 in extrahepatic tissues of infant mice may be related to low GST activity in the tissues of these animals, or alternatively, to the development of AFB_1 bio-activating enzymes (cytochrome P450s).

More recently, in whole body autoradiography of [^{3}H]-AFB_1 in marmoset monkeys, AFB_1 was localised in several extrahepatic sites, including the nasal olfactory (quantitatively the greatest binding) and respiratory mucosae, the mucosae of the nasopharyngeal duct, pharynx, larynx, trachea and oesophagus and the melanin of the eyes and hair follicles (Larsson and Tjälve, 1993). In addition, in *in vitro* micro-autoradiography, AFB_1 could be detected in the epithelial lining of several areas of the respiratory and alimentary tracts and the liver. If a cytochrome P450 inhibitor was added to the incubation medium, this binding was no longer apparent. Interestingly, in this study, the grey matter of the brain exhibited a greater binding capacity than did the white matter. The possible interaction between the binding of AFB_1 to melanin, photo-activation of AFB_1 upon UV exposure, and the development of skin tumours in albino mice has been discussed, the possible relevance of which may previously have been overlooked (Larsson and Tjälve, 1993).

Tjälve *et al.* (1992) have found that microsomal preparations of bovine olfactory mucosa have a greater affinity than liver microsomes to induce covalent binding of AFB_1 to calf thymus DNA and microsomal proteins. Addition of glutathione to these preparations decreased AFB_1–DNA binding. When cytosolic fractions of mouse liver (where AFB_1 resistance may be related to high hepatic GST activity) were added to the olfactory mucosal incubation medium, the decrease in AFB_1–DNA binding was more pronounced. The nasal olfactory mucosal tumours, which are found in relatively high frequencies in cattle in developing countries (many animals exhibiting signs of severe aflatoxicosis), might be explained in terms of the high AFB_1 bio-activating ability of bovine olfactory mucosa (i.e. P450 involvement) and perhaps lower levels of GST activity (Tjälve *et al.*, 1992).

Several other researchers have shown GST enzymes to be important protective agents against AFB_1–DNA adduct formation, e.g. Mandel *et al.* (1992) using low protein diets in 3-week-old rat weanlings and Liu *et al.* (1991) in comparing the ability of human liver fractions and lymphocytes to deal with aflatoxin and other foreign chemicals. Liu *et al.* (1991) found a highly significant correlation ($r = 0.88$) between AFB_1–DNA adduct concentrations and GST μ class activity. Tsuji *et al.* (1992), in comparing species and sex differences in AFB_1-induced GST placental forms, concluded that glutathione and GST play an important role in modulating hepatic AFB_1–DNA adducts.

Interestingly, human liver cytosolic fractions conjugated epoxide isomers to glutathione to a lesser extent than did similar cytosolic preparations from rats or mice (Raney *et al.*, 1992a). Moss and Neal (1985) previously had reported that human hepatic GSTs do not play an important role in protecting against AFB_1. The information, however, is too scant for any conclusions to be drawn regarding the physiological importance of GSTs in detoxification in humans.

When neonatal rats were exposed (first, third and fifth day) to diethylstilbestrol (DES) [previously used as an anabolic compound with oestrogenic properties], and then at 5 months of age treated with a single dose of AFB_1, DES-pretreated animals showed a 35% decrease in AFB_1–DNA adduct formation and a 2-fold increase in the levels of α-GST. Results suggest that neonatal DES treatment resulted in long-term protective increases in basal α-GST levels, causing lower levels of DNA adduction following adult exposure to AFB_1 (Zanger *et al.*, 1992). More specifically, Gopalan *et al.* (1992) found that in *in vitro* rat studies, the different classes of α-GSTs induced were dependent on the foreign chemical used. For example, the highest catalytic activity with microsome-mediated AFB_1–epoxide conjugation was observed with GST 3-3, while for synthetic AFB_1–epoxide conjugation, GST 4-4 appeared to be important. Thus, in rats, α-GST 3-3 may play an important role in inactivation of AFB_1-epoxide generated *in vivo* (Gopalan *et al.*, 1992).

4.5. Aflatoxin B_1 Transport and Repair of Aflatoxin–DNA Adducts

Transport of foreign chemicals out of cells involves two possible families of efflux pumps: the P-glycoprotein pump (specific for hydrophobic compounds) and the glutathione *S*-conjugate carrier (specific for drug–glutathione conjugates), both of which may play a role in eliminating AFB_1 from the cell (Hayes *et al.*, 1991a). The involvement of these two pumps in AFB_1 toxicity has generally not been researched. In one of the few articles pertaining to this line of work, Burt and Thorgeirsson (1988) have shown that AFB_1 induces the mRNA coding for the P-glycoprotein in mouse liver, thus implicating this pump in transport of the toxin.

Little information is available concerning removal of covalently bound AFB_1 from mammalian cells. The major adduct formed is the chemically unstable AFB_1–N^7-guanyl adduct, which is lost spontaneously (when mutations are likely to arise) from DNA to yield apurinic sites. The other two adducts (AFB_1 FAPY and AFB_1 III) are not lost spontaneously and may be catalytically removed by DNA repair enzymes (Hayes *et al.*, 1991a). In transformed xeroderma pigmentosum cells, Waters *et al.* (1992) found that up to 40% of AFB_1-induced genetic lesions were repaired after 6 hr, indicating that DNA repair may be important following AFB_1 exposure. Ball *et al.* (1990) have shown significant interspecies differences in repair capacity (AFB_1–DNA adduct removal) in cultured tracheal epithelium. This variation may be a factor accounting for the difference in the susceptibility of species to cancer of the respiratory tract. Leadon *et al.* (1981) have reported that the AFB_1–N^7-guanine adduct is removed spontaneously and enzymatically in fibroblasts, probably by nucleotide excision

repair mechanisms. The AFB_1–N^7-guanine adduct, however, may be converted to non-repairable, persistent AFB_1–formamidopyrimidine lesions.

Following a single dosing with AFB_1, maximum liver DNA adduct levels were measured after 2 hr. By 24 hr, 88% of the AFB_1–DNA adducts had been removed (Wogan *et al.*, 1980). Newberne and Wogan (1968) have postulated that in Fischer rats, the rapid removal of DNA adducts may be related to the requirement for multiple exposure to AFB_1 for the induction of tumours.

Thus, susceptibility and resistance to the toxic and carcinogenic effects of AFB_1 may depend on several factors: expression of bio-activating cytochrome P450s, detoxifying cytochrome P450s, GST activity, effective removal of AFB_1 detoxification products from the cell, and the ability for excision of AFB_1 adducts from DNA, and finally, repair of damage to nucleic acid (Hayes *et al.*, 1991a; Coulombe, 1993). While there may be volumes of relevant literature, there is still a need for research into particular aspects (toxicological, physiological and biochemical) of AFB_1 toxicity.

4.6. Anti-mutagenic Substances

If the GST detoxification mechanism is important in affording protection (resistance) against AFB_1–DNA adduct formation, then this resistance might be inducible and acquired (Hayes *et al.*, 1991a). Any factor that stimulates GST activity would have the potential to enhance intrinsic resistance mechanisms (Hayes *et al.*, 1991b). In this regard, several natural food extracts and, interestingly, anti-schistosomal drugs (e.g. oltipraz, a dithiolethione) have been found to enhance GST activity, and hence, AFB_1–glutathione conjugation. In male rats fed a diet supplemented with 0.03% 1,2-dithiole-3-thione and 250 μg AFB_1 kg^{-1} day^{-1} (0–4 days; 7–11 days), there was a 76% diminution in hepatic adducts and a 62 and 66% decrease in urinary AFB_1–N^7-guanine and serum AFB_1–albumin adducts, respectively (Groopman *et al.*, 1992a). Similarly, Kensler *et al.* (1992) have reported that male rats fed 1,2-dithiole-3-thione (0.001–0.003%) all showed signs of elevated hepatic GST activities. Four- to 6-fold increases in the specific activities of aflatoxin–glutathione conjugation were recorded in cytosolic preparations of livers of animals fed 1,2-dithiole-3-thione. Several extrahepatic organs also showed elevated GST activity (Kensler *et al.*, 1992). Roebuck *et al.* (1991) claim that oltipraz (0.075%) offers complete protection against AFB_1-induced hepatocellular neoplasms, reducing mortality and hepatic AFB_1–DNA adducts in rats 3 months after dosing. This anti-schistosomal drug thus appears to be a general inducer of detoxification enzymes and might warrant use as a protective agent.

Bolton *et al.* (1993), based on the results of rat trials, have suggested that oltipraz may exert considerable activity against the cytotoxic and auto-promoting action of repeated exposure to AFB_1. It is suggested that oltipraz be utilised in intervention trials in human populations frequently consuming AFB_1-contaminated foods, and more particularly, in areas with high incidences of liver cancer (Bolton *et al.*, 1993).

There is a wealth of information concerning the anti-mutagenic properties offered by several plant products (Wattenberg, 1985; Brockman *et al.*, 1992; Ueno, 1993). Pretreatment of male rats with geniposide (as a penta-acetyl derivative), an extract of *Gardenia fructus*, resulted in consistent elevation of the activities of GST and γ-glutamylcysteine synthase (Wang *et al.*, 1992). Wang's group is of the opinion that defence mechanisms of rat hepatic tissue may incorporate the enhanced GST activity and induction of γ-glutamylcysteine synthase for glutathione biosynthesis. Geniposide was found to inhibit the damage to DNA caused by AFB_1 in C3H10T1/2 cells (Tseng *et al.*, 1992). Additionally, the inhibitory effect of AFB_1 on DNA synthesis was also overcome. In another study, geniposide was capable of inhibiting the growth and development of C-6 glioma cells in rats (Wang *et al.*, 1993).

The suppressive effects of another plant product, crocetin (a carotenoid), on hepatocellular lesions induced by AFB_1 were investigated in male rats fed AFB_1 and crocetin for 10 days. Thirty-five weeks later, the group that had received AFB_1 and crocetin exhibited a 40% reduction in liver lesions as compared with the AFB_1-alone group (Wang *et al.*, 1991). The crocetin-only group exhibited no lesions. The protective effects of crocetin may result from elevated GST activity and decreased formation of hepatic AFB_1–DNA adducts (Wang *et al.*, 1991).

GST-inducing activity has been documented for active compounds in lemongrass oil (from *Cymbopogon citratus*) and galanga root oil (from *Alpinia galanga*) (Zheng *et al.*, 1993). In female A/J

mice, D-limonene (from lemongrass oil) resulted in a 2.4- to 3-fold increase in GST activity in liver and small and large intestinal mucosae, while geraniol (in lemongrass oil) induced a 2.5-fold increase in intestinal mucosae only. The *trans*-cinnamate (from galanga root oil) induced increased GST activity in the liver and intestines (Zheng *et al.*, 1993). Limonene is a component of several essential oils and is often used for flavourings and colourings and so, these oils may be chemo-protective agents that can be used as dietary supplements.

Recently, grapefruit juice has been demonstrated in *in vitro* experiments with human liver microsomes to have anti-carcinogenic properties against AFB_1 (Guengerich and Kim, 1990). Naringenin, an aglycone of the flavonoid naringin, most abundant in grapefruit and related citrus, was found to inhibit the activation of AFB_1.

A number of plant phenolic compounds have been tested for their ability to modulate microsome-mediated activation of AFB_1 and influence covalent adduct formation of activated metabolites with DNA. Bhattacharya and Firozi (1988) report that polyhydroxyl flavonols, e.g. robinetin, quercetin, fisetin and morin, were active in this regard. In *Salmonella typhimurium*, kaempferol and rutin were very active in inhibiting AFB_1 mutagenicity by up to 50% (Francis *et al.*, 1989). Catechin, a phenolic flavonoid, effected a dose-dependent reduction (up to 40%) in AFB_1–DNA binding (Prasanna *et al.*, 1987). Observations from this last study indicate that mixed-function oxidases (cytochrome P450-dependent), essential for the metabolic activation of these carcinogens, exhibit differing sensitivities to the various inhibitors of their activity.

Other food additives, such as tumeric, garlic and asafoetida, have been shown to contain anti-oxidants, which scavenge free radicals and inhibit chemically-induced carcinogenesis (Unnikrishnan and Kuttan, 1990). Soni *et al.* (1993) found that AFB_1-induced fatty acid changes, necrosis and biliary hyperplasia were inhibited by daily doses of tumeric, curcumin and ellagic acids (all containing anti-oxidants). Garlic and asafoetida inhibited necrosis and degeneration of the tissue, but biliary hyperplasia persisted (Soni *et al.*, 1993).

Garlic extracts have long been recognised to possess anti-mutagenic properties. Tadi *et al.* (1991) tested the effects of two garlic extracts (ajoene and diallyl sulphide (DAS)) on the metabolism and binding of AFB_1 to DNA. These two organo-sulphur extracts inhibited AFB_1 binding to calf thymus DNA and reduced adduct formation. GST levels appeared unaffected by ajoene and DAS, suggesting that AFB_1 metabolism and DNA binding is influenced by inhibition of Phase 1 (activation) enzymes (cytochrome P450s). Additionally, DAS selectively induced cytochrome P450IIB1/2 [reported elsewhere to activate AFB_1 (Aoyama *et al.*, 1990)] in rat liver, primarily due to transcriptional activation, while the levels of cytochrome P450IIE1 were unaffected (Pan *et al.*, 1993). Furthermore, while levels of cytochrome P450IIB1/2 mRNA were elevated in liver, stomach and duodenal tissue, levels in lung and nasal mucosae remained unchanged. This serves to reinforce the idea of tissue selective activation/inactivation by foreign chemicals.

Extracts of four Chinese medicinal plants traditionally used in the treatment of lung, liver and rectal tumours (*Oldenlandia diffusa* (Rubiaceae); *Scutellaria barbata* (Labiatae); *Astragalus membranaceus* (Leguminosae); *Ligustrum lucidum* (Oleaceae)), produced concentration-dependent inhibition of histidine-independent revertant (His^+) *Salmonella typhimurium* colonies induced by AFB_1 (Wong *et al.*, 1992). Additionally, extracts of three of these plants inhibited AFB_1–DNA binding and reduced the formation of organo-soluble metabolites of AFB_1. In some instances, when used in combination, the effects were additive. Similarly, Chulasiri *et al.* (1992) have found two flavonoids (hispidulin and hortensin) from *Millingtonia hortensis* (Bignoniaceae) to have anti-mutagenic properties against AFB_1 in *Salmonella typhimurium* without being cytotoxic to the bacterial cells. Three water- and ethanol-soluble extracts of a Chinese green tea exhibited inhibitory effects on the development of precancerous enzyme-altered hepatocellular foci in rats (Qin, 1991). To date, only a single extract of green tea [(−)-epigallocatechin] has been identified (Ueno, 1993). Ruan and co-workers (Ruan, 1992; Ruan *et al.*, 1992) reported that eight natural foods (sesame, chestnut, dad-lily, laver, red Chinese date, bamboo shoot, kelp and green tea), regularly sold in markets in China, exhibited anti-mutagenic properties, with the more stable anti-mutagenicity being shown by dad-lily and green tea. Interestingly, the mutagenicity of *Salmonella typhimurium* induced by *Aspergillus versicolor* was inhibited by dad-lily, bamboo shoots and green tea, while, red Chinese date inhibited the mutagenicity induced by *Aspergillus ochraceus*. In addition to *Aspergillus flavus*, these two *Aspergillus* species are frequently found associated with maize in that area (Ruan *et al.*, 1992).

Brockman *et al.* (1992) have provided an extensive summary regarding the antimutagenic properties of the retinoids (vitamin A and its derivatives) and the carotenoid, β-carotene. With respect to the anti-mutagenicity against AFB_1, most dietary antimutagens were active against AFB_1. In the light of these findings, Brockman *et al.* (1992) have questioned the current view that AFB_1 is a potent mutagen and carcinogen *in vivo*, particularly in animals and humans on a well-balanced diet.

Recently, chlorophyllin, a non-toxic derivative of chlorophyll, has been shown to inhibit AFB_1–DNA adduct formation in a dose–responsive manner in *in vivo* animal models, suggesting it as a promising candidate for chemo-prevention (Dashwood *et al.*, 1991).

Wattenberg (1985) has divided the chemo-preventative agents (largely dietary), into three categories, depending on the level at which they are effective at preventing tumour development (Fig. 4). Thus, chemo-protectants are classified as compounds that *prevent* formation of carcinogens, as *blocking* agents, preventing the carcinogen from reacting with cellular molecules or as *suppressing* agents that inhibit the neoplastic change in cells following binding of the carcinogen to cellular components. The phenols appear to be effective at all three levels (Wattenberg, 1985).

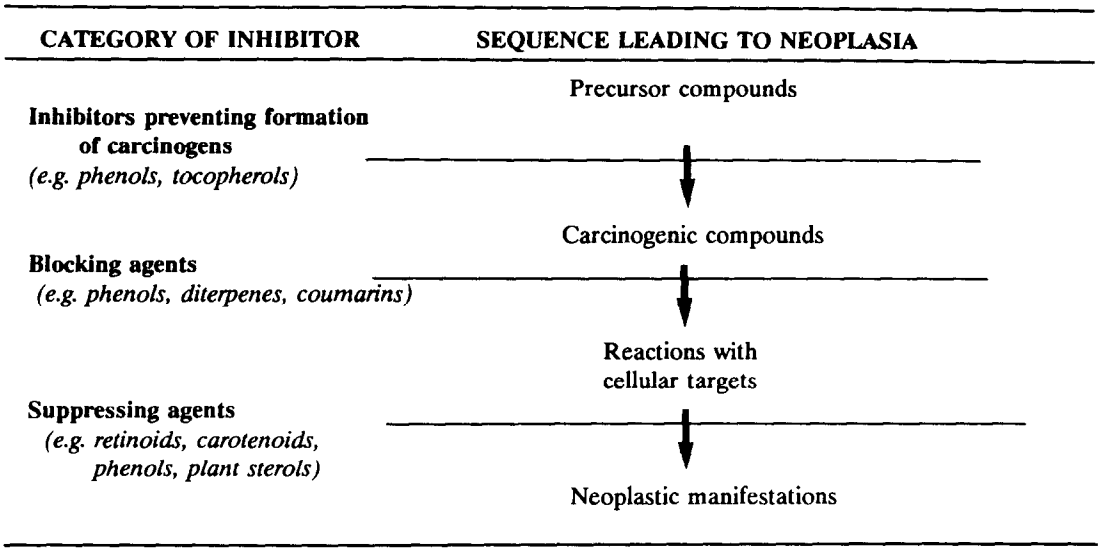

Fig. 4. Categories of chemo-protective agents, based on the stage at which they exert their protective effects. Adapted from Wattenberg (1985).

5. DIET AND CANCER

Dietary compounds may inactivate transcription of cytochrome genes, the products of which may be responsible for epoxide formation of several xenobiotic chemicals. Free radicals have long been thought to be involved in tumour development (Harman, 1962; Mavelli and Rotilio, 1984; Troll and Wiesner, 1985). Considering that a number of potential mutagens and carcinogens are diet-related, it would be impossible to eliminate them completely. Since the generation of free radicals appears to be more or less a universal factor contributing towards tumour development, any compound that can scavenge the oxygen and hydroperoxide radicals, thereby preventing their peroxidation of critical cellular components, would be beneficial.

Many other dietary factors (e.g. selenium, vitamin E, sulphur-containing amino acids, copper and zinc) are known to affect the anti-oxidative ability of an organism (Huang and Fwu, 1993). Protein deficiency has been presumed to impair the anti-oxidative defense system (Chow, 1988). Recently, Huang and Fwu (1993) have demonstrated that the degree of protein deficiency in rats affects the extent of depression of the activities of the anti-oxidative enzymes (e.g. glutathione peroxidase, superoxide dismutase) and hence, the degree of tissue lipid peroxidation. An AFB_1-containing diet supplemented with methionine was found to attenuate the AFB_1-induced increase in hepatic glutathione in domestic fowl (Beers *et al.*, 1992).

Dietary lipids have been demonstrated to modulate the metabolism and toxicity of xenobiotics by regulating the activities of Phase 1 (activation) and Phase 2 (detoxification) metabolism (Yang *et al.*, 1993), with cytochrome P450 enzymes being differentially activated (Yoo *et al.*, 1992).

Bailey and Williams (1993) have provided a review on food-related carcinogens and anti-carcinogens, discussing the concept of free radical scavengers (particularly in the diet) as chemo-protectants against tumour development. Ueno (1993) has also reviewed the concept of diet/toxin interactions and has emphasised the protective role afforded by free radical scavengers.

6. STRUCTURALLY RELATED MYCOTOXINS

Sterigmatocystin is structurally similar to AFB_1 with respect to dimensions and configuration of the bisdihydrofuran moiety, resulting in the possibility of metabolic activation at the same site, the $C_8=C_9$ double bond. Sterigmatocystin, therefore, may operate metabolically in the same fashion as AFB_1. Sterigmatocystin–AFB_1 adducts have been reported (Reddy *et al.*, 1985; Gopalakrishnan *et al.*, 1992), and sterigmatocystin was found to be cytotoxic and mutagenic to cultured Chinese hamster cells (Noda *et al.*, 1981).

7. OTHER MYCOTOXINS

Jelinek *et al.* (1989) has drawn attention to the significant concentrations of a large number of mycotoxins produced by *Fusarium*, *Penicillium* and *Aspergillus* species found naturally contaminating foods and feeds. Only a few of the mycotoxins (such as AFB_1, ochratoxin A, citrinin and to a lesser extent, T-2 toxin, and the ergot alkaloids) have been investigated in sufficient depth such that a risk assessment can be attempted. Newberne (1993), however, has reported than several mycotoxins (including the fumonisins, a number of trichothecenes and ochratoxin) are now under review by the WHO–IARC and several national public health agencies. It is reported, however, that AFB_1 is currently the only mycotoxin that is regulated by the FDA (Coulombe, 1993).

Ruan (1991) considers the possibility of co-mutagenic effects of more than one fungal metabolite in the incidence of liver cancer in Fusui county, China. The geographical variation in incidence of PLC indicates involvement of specific factors, whether these be genetic or environmental epidemiological factors. The evidence involving AFB_1 and HBV is equally provocative and both factors must be considered in any risk assessment.

The fumonisins, which are now being reported as frequent contaminants of crops (Sydenham *et al.*, 1993; Ueno *et al.*, 1993), have received specific attention as causal agents in certain pathological conditions in humans and animals. In this regard, fumonisin B_1 has been epidemiologically associated with human oesophageal cancer in some regions of the Transkei, South Africa (Sydenham *et al.*, 1990), but as with many diseases of suspected mycotoxin aetiology, the evidence is only circumstantial. The pathologies associated with fumonisin B_1 are interesting, since, unlike AFB_1, which is known to be hepatotoxic to many animals, fumonisin mycotoxicoses are manifested in different organs, depending on the animal involved. Fumonisin B_1 results in equine leukoencephalomalacia (Kellerman *et al.*, 1990; Wilson *et al.*, 1992), liver cancer in rats (Gelderblom *et al.*, 1991) and pulmonary oedema in swine (Colvin and Harrison, 1992). Methods of isolation and purification of these mycotoxins have been developed only recently and so, the full extent of the potency of these mycotoxins has not yet been characterised completely. Mention should also be made of the possibility (or likelihood) of the co-contamination of foods and feeds by more than one mycotoxin, making diagnosis of the disease condition difficult. The area of multiple mycotoxin exposure, particularly relating to humans, warrants intensive investigation (Bach and McLean, 1993).

8. CONCLUSIONS

Since the discovery of the aflatoxins in the early 1960s, the study of mycotoxins has passed from a fringe area of vague inter-relationship with nutritionally derived disease to a large-scale

multidisciplinary research effort investigating many fungal metabolites. The research endeavours are ongoing and not least with respect to the studies on AFB_1, the most commonly occurring of the aflatoxins.

One of the most significant discoveries with respect to AFB_1 was its carcinogenic properties when Butler (1964) showed that of all the naturally occurring carcinogens, the rat exhibited the greatest susceptibility to AFB_1. Not surprisingly, the impact of this discovery and the accumulating evidence has led to the implication of AFB_1 in the development of human HCC. It may be that, in the final analysis, aflatoxin together with other environmental agents, such as the HBV and other mycotoxins, will provide a model that explains the origins and mechanisms of cancers that are dependent on an interaction between environmental and genetic factors.

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