This section reviews the various aspects (hepatic injury, mycotoxins, aflatoxin, aflatoxicosis, *E. caryophyllata* (Clove) and its properties, eugenol and sylimarin) that are relevant to the aim.

### 2.1. HEPATIC INJURY

#### 2.1.1. CAUSES OF HEPATIC INJURY AND HEPATOTOXICITY

Liver, the largest organ in the body is essential in keeping the body functioning properly. It removes or neutralizes poisons from the blood, produces immune agents to control infection and removes microbes from the blood. It makes proteins that regulate blood clotting and produces bile to help absorb fats and fat soluble vitamins. Because of these activities it is exposed to a wide variety of insults and is therefore one of the most frequently injured organs of the body. Yet one can not live without a functioning liver (Kirsch *et al.*, 1995).

**MICROBIAL INFECTIONS**

**Viral Hepatitis**

Viral hepatitis is one of the major pathogenic liver diseases and the most common form of pathogen-induced acute liver injury. The viruses on entering the liver cause degenerative changes that leads to inflammatory reactions. The severity of the hepatitis (inflammation of liver) depends on the different types like Type A (infections H), Type B (serum H), Type C and type non-A and non-B viruses. An estimated 300-350 million people (5-6% of the total world population) are chronically infected with hepatitis-B virus (Kaplan, 1999). Around 170 million people (3% of the world’s population) are estimated to be infected with hepatitis-C virus (Alter *et al.*, 1999) and death due to hepatitis-C virus induced cirrhosis is on the rise in USA (Davis *et al.*, 1998).

**Fungal Hepatic Injury**

In man, acute liver failure is observed following ingestion of mushrooms (fungi) like *Amanita phalloides* and *A. verna*. The toxins
present in these mushrooms, namely phalloidin and phalloon are extremely lethal to liver cells (Rensberg, 1977).

**Other Infections**

In addition to viruses and fungi, bacteria and protozoans can infect the liver. The liver is almost inevitably involved to some extent in all blood-borne infections (Kirsch *et al.*, 1995).

**HEPATIC DISORDERS**

**Injury from metabolic disturbances**

In experimental animals, specific dietary deficiencies can produce fatty liver and liver cell necrosis. Similarly in man, protein malnutrition can produce marked fatty changes and there is evidence that malnutrition may considerably exacerbate other forms of injury. Specific enzyme deficiencies may cause various hepatic storage disease, or failure of bile excretion (Glaister, 1986).

Ischaemic injury affects the perivenular zone of the acinus as do many drugs and toxins, for example alcohol and paracetamol. Less commonly, some agents for example, phosphorous produce periportal injury, while yellow fever characteristically affects zone 2 of the acinus, producing a ‘mid-zonal’ pattern of necrosis (Quiroga *et al.*, 1992).

**Hepatic disfunctions**

Hepatic metabolic disorders, and involvement of liver in the extrahepatic disorders, lead to hepatic disfunctions.

**Hepato metabolic disorders**

The problems with metabolic processes in the liver can be either congenital or acquired. Some of the disorders such as Wilson’s disease (inability to excrete copper into bile resulting in the toxic accumulation of copper in the liver and nervous system) and hemochromatosis (iron overload syndrome causing iron deposits causing liver damage) are congenital (Sherlock and Summerfield, 1991).
A-1-antitrypsin deficiency is an inherent disease acquired due to smoking where 10% of adult patients will develop liver disorders (Quiroga et al., 1992).

**Extrahepatic Disorders**

In the extrahepatic disorders liver may be affected by numerous conditions particularly autoimmune disorders in which the immune system attacks the body’s own normal tissues. Eg. rheumatic diseases and inflammatory bowel disease. Systemic infections such as tuberculosis can spread to liver (Kirsch, 1995).

**HEPATOTOXINS**

**Alcoholism**

Alcohol consumption or rather abuse is considered to have a major share in chemically induced liver injury. Alcohol abuse is a leading cause of mortality and morbidity throughout the world and in USA as many as 10% of man and 35% of women suffer from persistent problems due to alcohol abuse. The incidence of liver cirrhosis among alcoholics is about 10-15% (Pequignot and Cyrulnik, 1970) and in the United States, alcohol abuse is the ninth leading cause of death (Grant et al., 1986). In New York city, alcoholic cirrhosis is the third leading cause of death and people between the age of 25-65 years are the most affected ones (Lieber, 1988).

**Therapeutic Drugs**

One of the major side effects of drugs is liver injury. It has been recorded that approximately 3% of all hospital admissions are due to adverse drug reactions and 20-30% of the cases of severe hepatic failure are drug-induced (Ward et al., 1997). Many non-steroidal antiinflammatory drugs like asprin, indomethacin, ibufenac and fluproguazone can induce hepatic necrosis (Lewis, 1984). Antibiotics like tetracycline are also known to cause injury mainly necrosis and steatosis
(Timbrell, 1983). Paracetamol (acetaminophen) is an analgesic, which at high doses is known to induce hepatic injury (Black, 1980).

**Chemical Toxicants of the Environment**

Chemical toxicants in the occupational or non-occupational environment are also a common cause of hepatic injury. Exposure to environmental chemicals that leads to chronic liver disease or primary hepatic malignancies has also been documented. Solvents and degreasing agents, pesticides, polyhalogenated biphenyls, dioxins, dibenzofuranes and vinyl chloride are some of the major chemical hepatotoxicants that have evolved as a result of rapid industrialization. Industrial exposure to vinyl chloride monomer and solvents like 1, 1, 1-trichloro ethane are known to cause liver damage and can lead to abnormal levels of transaminases (Tamburro et al., 1984; Hodgson et al., 1989). Short exposure to industrial chemical like dimethyl formamide can result in local hepatocellular necrosis and steatosis (Redlich et al., 1990). Industrial and occupational liver injury is under diagnosed and the prognosis of the chronically exposed are uncertain. *(Fig. 1)*

**2.1.2. MANIFESTATIONS OF HEPATOTOXICITY AND HEPATIC INJURY**

**Chemically- induced liver injury**

Hepatic toxicity inflicted by xenobiotic or natural chemicals has been recognized for slightly over a century. During the latter part of the 19th century, attempts to understand the toxicology of yellow phosphorous and the haloalkane, anaesthetic chloroform resulted in an appreciation of the hepatotoxic potential of chemicals (Zimmermann, 1978). Interest in the chemically inflicted hepatic toxicity has steadily grown since then and has led to remarkable progress in our understanding of the mechanism governing many toxicological manifestations induced by toxic chemicals.

The susceptibility of the liver to injury by chemical agents appears to be a consequence of the anatomical position of this organ and the
central role it plays in the metabolism and disposition of foreign chemicals. Liver contains by far the highest levels of enzymatic systems capable of biotransforming foreign chemicals.

Number of features predispose the liver to chemically induced tissue injury. Because the liver’s predominant blood supply has first passed through the intestines, it is low in oxygen but highly enriched in nutrients. Likewise it also contains endotoxin (lipopolysaccharide products of intestinal bacteria), metabolic waste products, absorbed chemicals and other cell debris which may all enter the liver via the portal circulation and present a risk of toxicity to the liver as well as other organs, if there is a spill over. Following oral ingestion, toxicants can achieve high concentration in the liver (Koporec et al., 1995).

**Examples of hepatotoxicants which induce tissue injury**

**Mediated by phagocytes and inflammatory mediators**

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<tr>
<th>Neutrophils</th>
<th>Endotoxin</th>
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<td>Alcohol</td>
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<tr>
<td></td>
<td>α-Napthyl-isothiocyanate</td>
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<td>Galactosamine</td>
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<th>Macrophages</th>
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<td>Phenobarbital</td>
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<td>1,2-Dichlorobenzene</td>
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<td>Cadmium</td>
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Toxic chemicals can also be delivered to the liver in the arterial blood that mixes with venous blood in the sinusoids. For example, compounds that are absorbed via inhalation can be delivered directly to the liver, which receives 29% of the resting cardiac output. Once present in the blood of the sinusoidal space, chemicals can be readily extracted from blood into the hepatocyte. This is primarily facilitated through the leaky sinusoids which are the exchange vessels of the liver and which permit plasma to enter the space of Disse (space between hepatocytes and endothelial cells), where it has direct contact with the hepatocytes.
Further more microvilli on the sinusoidal surface of the hepatocyte greatly increase the area available for the absorption of chemicals (Guyton, 1991).

**Metabolic disorders and hepatic damage**

Another feature of the liver that predisposes it to chemically induced liver injury is its ability to biotransform or metabolize chemicals. This important process is catalyzed by numerous enzymes which converts lipophilic compounds into more hydrophilic metabolites. These can be more readily excreted in the urine and the feces. The liver is the most important organ of biotransformation, due largely to its high content and large diversity of enzymes capable of metabolizing foreign as well as endogenous chemicals. These enzymes include UDP-glucuronyl transferase, glutathione-S-transferase, cytochrome P<sub>450</sub>, FAD-containing mono oxygenase as well as others.

Toxic or reactive metabolites can initiate a series of events that ultimately result in liver injury. Such events include initiation of lipid peroxidation, covalent modification of critical cellular molecules, consumption and ultimate depletion of important cellular components, mutations in DNA, and inhibition of protein synthesis (Hinson et al., 1994). At low rates of formation, reactive metabolites can be detoxified by conjugation with endogenous molecules or their damage can be repaired. However, if the rate of utilization of these endogenous molecules exceeds their synthesis, they will ultimately be depleted. At this stage, the hepatic parenchymal cell becomes extremely vulnerable to damage by reactive metabolites of chemicals.

**Manifestations of hepatic disfunctions** (Quiroga et al., 1992)

**Hypoxia**

Owing to their active and complex metabolism, liver cells are readily injured by hypoxia, as in shock, venous congestion or anaemia. However, the dual blood supply to the liver affords some protection against hypoxic injury.
Portal hypertension

This is caused by obstruction to the blood flow through the liver. As a result, veins which provide an anastomosis between the portal and systemic circulation, instead of passing through the liver. This increase the blood level of toxic compounds absorbed from the gut, thus aggravating the effect of hepatocellular failure on the central nervous system and other organs.

Ascites

It is the increased sinusoidal pressure, as with severe inflammation or scarring of the liver leads to the fluid accumulation in the abdomen that becomes more difficult to control with progressive decompenation.

Abnormal excretion

The accumulation of serum bilirubin in the serum, which is normally taken up by the liver and excreted in to bile, resulting in jaundice.

Abnormal clearance

It is the decreased clearance of gut-absorbed proteins and ammonia from the liver which produces hepatic encephalopathy, a poisoning of brain with symptoms ranging from confusion to coma.

Hepatitis

It is the inflammation and damage of normal liver cells most commonly associated with viral and toxic insults. It can be acute fulminant or chronic.

Liver cell necrosis and cirrhosis

Massive hepatic necrosis, which represents more severe degree of confluent necrosis is sequel to viral hepatitis or drug-induced injury. Clinically it results in fulminant acute hepatocellular failure.
In chronic liver disease piecemeal necrosis occurs and is defined as destruction of liver cells at an interface between parenchyma and fibrous tissue, together with a predominantly lymphocytic or plasma cell infiltrate.

Cirrhosis is the end point of many chronic liver diseases, since inflammation and cell death eventually yield to fibrosis or scar formation. Cirrhosis involves irreversible damage to the lobular architecture with diffuse fibrous bands of scar tissue surrounding nodules of regenerating hepatocytes (Sherlode and Summerfield, 1991).

Tumours

Primary tumours of the liver are relatively uncommon in developed countries though is of great frequency in parts of Africa and the far east: they are associated with cirrhosis. The liver is a very common site of metastatic carcinoma, particularly from primary tumours, of the gastrointestinal tract (Mac Sween and Whaley, 1992).

Hepatocellular carcinoma

It is the primary malignant cancer of the liver and is associated with dismal prognosis. Chronic inflammatory disease of the liver may increase the risk of developing cancer. Nodular regeneration in cirrhotic livers may lead to cellular dysplasia (alteration in size, shape and organization of cells), since errors are more likely to be made into more actively dividing cells (Redlich et al., 1990).

Hepatic failure

Hepatocellular failure arises when total liver cell function falls below the minimum required to maintain a physiological state. It results from loss of a large number of liver cells and from impaired function of liver cells, attributable to interference with hepatic blood flow or interference with intracellular metabolic functions. Hepatic failure may be acute, as in massive liver cell necrosis due to hepatitis or drugs, or it may be chronic, for example in cirrhosis. The most important effects
include, change in nitrogen metabolism with a rise in the blood level of toxic nitrogenous compounds, jaundice, defective synthesis of plasma proteins, hormonal and circulatory disturbances, functional renal failure (the hepato renal syndrome), encephalopathy, coagulopathy, shock and sepsis (Kirsch et al., 1995) (Fig. 2).

MORPHOLOGICAL RESPONSES OF THE LIVER TO CHEMICAL INJURY

From a morphological aspect, the cells which comprise the liver can react to toxicant-induced damage in a limited number of ways (Kirsch et al., 1995). Therefore, there are only a few, rather well defined responses to a multitude of toxic chemicals. These responses are categorized as

(i) fatty change (or steatosis), which is a reversible condition
(ii) cell cytotoxicity which in the early stages (cell swelling) is reversible but can progress to irreversible coagulative necrosis
(iii) cholestasis, in which bile flow slows or ceases,
(iv) fibrosis and cirrosis, in which hepatocyte necrosis and deposition of collagen fibres disrupt normal hepatic architecture and function and
(v) the development of liver neoplasia.

POTENTIATION OF CHEMICALLY INDUCED HEPATOTOXICITY

It is well established that subjects exposed to several chemical agents simultaneously can exhibit altered pharmacologic or toxicologic responses. The effect of a second chemical can have a marked influence on the response elicited by a previously administered chemical and vice versa. Many of these have led to the discovery that biotransformation to a more active metabolite is involved in the hepatotoxic response.

Many instances of potentiation of hepatotoxicity have been described. Individuals recovering from an acute ingestion of ethanol are more susceptible to the liver damaging properties of the halogenated hydrocarbons than do individuals not ingesting ethanol.
Cornish and Adefuin (1967) showed that several aliphatic alcohols, such as methanol, ethanol, isopropanol, n-butanol, and tert-butanol exert similar potentiating effect on the acute inhalation toxicity of carbon tetrachloride.

Experimentally, it was shown that diabetes induced in rats by either alloxan or streptozotocin enhances the hepatotoxicity of carbon tetrachloride (Hanosono et al., 1975).

Fulminant hepatitis can be induced in experimental animals by the synergistic action of a small endotoxin dose and an inhibitor of hepatocellular ribonucleic acid synthesis and is hepatotoxic even in the absence of endotoxin. However, an additional injection of endotoxin induces fulminant hepatitis within six hours, even in the NMRI mouse strain which is relatively resistant to D-galactosamine. Corresponding to this action of D-galactosamine, α-amanitin, an inhibitor of ribonucleic acid polymerase II, sensitized the liver to the action of a small dose of endotoxin (Fiume, 1972).

2.2. MYCOTOXIN
2.2.1. Occurrence and nature

Mycotoxins are produced mainly by the mycelial structure of filamentous fungi, or more specifically, the molds. These toxins are produced by saprophytic fungi during storage or by endophytic fungi during plant growth. They are found mainly in post-harvest crops such as cereal grains or forages. Mycotoxins in humans or animals are characterized as food or feed related, non-contagious, non-transferable, non-infectious, and non-traceable to microorganisms other than fungi. Since they are generally lipophilic (except for FB) they tend to accumulate in the fat fraction of plants and animals. Mycotoxins are secondary metabolites that have no biochemical significance in fungal growth and development (Moss, 1991). Toxigenic molds are known to produce one or more of these toxic secondary metabolites. It is well established that not all molds are toxigenic and not all secondary metabolites from molds are toxic. Although there are over 300 mycotoxins that have been isolated
and chemically characterized (Betina, 1984), research has focused on those forms causing significant injuries to humans and their farm or companion animals. These include AF, OT, trichothecenes, ZEN, F, and ergot alkaloids. There have also been recent concerns over other toxins such as citrinin and sterigmatocystin. Factors contributing to the presence or production of mycotoxins in foods or feeds include storage, environmental, and ecological conditions. Often times most factors are beyond human control (D’Mello and McDonald, 1997). Examples of fungal species and mycotoxins of biological and economical significance in agricultural animals are presented in Table 3.

Examples of mycotoxins of greatest public health and agroeconomic significance include aflatoxins (AF), ochratoxins (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids. These toxins account for world wide losses of millions of dollars annually in human health, animal health, and condemned agricultural products (Shane, 1994; Vasanthi and Bhat, 1998). In 1993, the WHO-International Agency for Research on Cancer (WHO-IARC, 1993 a,b) evaluated the carcinogenic potential of AF, OT, trichothecenes, ZEN, and F. Naturally occurring AF were classified as carcinogenic to humans (Group 1) while OT and F were classified as possible carcinogens (Group 2B). Trichothecenes and ZEN, however, were not classified as human carcinogens (Group 3). 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. In general, mycotoxins are categorized by fungal species, structure, and (or) mode of action. It should be noted, however, that a single species of fungi may produce one or several mycotoxins and
individual mycotoxins may be produced by different fungal species. For example, AF produced by several fungal species, have numerous structural variations, and have different modes of action depending on the target animal (Eaton et al., 1994).

2.2.2. Toxic action

The health hazards of mycotoxins to humans (Peraica et al., 1999) or animals (D'Mello and MacDonald, 1997) have been reviewed extensively in recent years. Factors affecting the magnitude of toxicity of humans or animals consuming mycotoxin contaminated foods or feeds, respectively, include species, mechanisms and modes of action, metabolism, and defense mechanisms. In early studies on AF, species specific acute toxicities were reported. The LD_{50} were 0.4, 1, and 500 mg kg for ducklings, rats, and sheep, respectively (Wogan, 1966). Mechanisms and modes of mycotoxin action are only beginning to shed light on the interspecies and sometimes individual variations in toxic end-points. For example, AF are known to bind DNA and induce mutagenic and carcinogenic effects in rats (Croy et al., 1978; Bennett et al., 1981; Foster et al., 1983; Muench et al., 1983). However, thymic depression, decreased T-cell function, and cellular immunity are the modes of AF action in bovines (Paul et al., 1977), ovines (Fernandez et al., 2000), and porcines (Pang and Pan, 1994). Metabolism and defense mechanisms are important factors in understanding mycotoxin toxicity in specific species or individual animals. Specificity of such mechanisms are well demonstrated in the significant difference between ruminants and non-ruminants in handling mycotoxins. Ruminants have generally been more resistant to the adverse effects of mycotoxins (Wogan, 1966; Helferich et al., 1986). In vitro studies have shown the ability of the rumen microbiota to degrade mycotoxins (Ribelin et al., 1978; Kiessling et al., 1984; Swanson et al., 1987). Understanding the metabolic pathways of mycotoxins in ruminants and non-ruminants could enable researchers and public health officials to gain insight on how to assess the associated risks of mycotoxin exposure in various species.
2.2.3. Economic impact of mycotoxins

There are multiple criteria for assessing the economic impact of mycotoxins on humans and on animal agriculture. Considerations include loss of human and animal life, health care and veterinary care costs, loss of livestock production, loss of forage crops and feeds, regulatory costs, and research cost focusing on relieving the impact and severity of the mycotoxin problem. Formulas for worldwide economic impact have been difficult to develop and, therefore, most reports on economic impact are on a single aspect of mycotoxin exposure or contamination. The worldwide contamination of foods and feeds with mycotoxins is a significant problem. Studies have shown extensive mycotoxin contamination in both enveloping and developed countries. In a recent review (Fink-Gremmels, 1999), it was estimated that 25% of the world’s crops may be contaminated with mycotoxins. Surveillance studies (Placinta et al., 1999) showed that world-wide contamination of cereal grains and other feeds with Fusarium mycotoxins is a global concern. In Yugoslavia, studies on mycotoxigenic fungi in raw milk have indicated that 91% of the samples tested were contaminated (Skrinjar et al., 1995). In the US, a study was conducted in seven Midwestern states in 1988–1989 and found mycotoxins in 19.5% of corn samples assayed prior to any induced environmental stress and 24.7% of the samples following stress induction (Russell et al., 1991). Shane (1994) estimated the 1980 losses due to AF in corn of eight Southeastern states at 97 million dollars with additional 100 million dollars in production losses at hog farms feeding the contaminated corn. India is a prime example of a country in which the economy is affected heavily by mycotoxins. In a study in the Bihar region from 1985 to 1987 (Ranjan and Sinha, 1991), nearly 51% of the 387 samples tested were contaminated with molds. Of the 139 samples containing AF, 133 had levels above 20 g/kg. In another study (Phillips et al., 1996), levels as high as 3700 g/kg of AF were reported in groundnut meal used for dairy cattle. Researchers also found 21 of 28 dairy feed samples from farms in and around Luhiana and Pun-jab to be contaminated with AFB1 at levels ranging from 50 to 400 g/kg.
(Dhand *et al.*, 1998). It was estimated that 10 million dollars were lost in India’s export within a decade due to groundnut contamination with mycotoxins (Vasanthi and Bhat, 1998).

### 2.3. AFLATOXIN

Aflatoxins are toxins produced by the fungi *Aspergillus* on foods and feeds. They are probably the best known and most intensively researched mycotoxins in the world. Aflatoxins have been associated with various diseases, such as aflatoxicosis, in livestock, domestic animals and humans throughout the world. The occurrence of aflatoxins is influenced by certain environmental factors; hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during preharvest, storage, and/or processing periods. Aflatoxins have received greater attention than any other mycotoxin because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans. As it is realized that absolute safety is never achieved, many countries have attempted to limit exposure to aflatoxin by imposing regulatory limits on commodities intended for use as food and feed. Although 17 compounds, all designated as aflatoxins, have been isolated, the term usually refers to four metabolites of this group of bisfurocoumarin metabolites produced by *A. flavus* and/or *A. 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. 3a](#)).

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$_2$. *A. flavus* produces only AFB$_1$, and AFB$_2$, while *A. 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 M₁ (AFM₁) and aflatoxin M₂ (AFM₂) are hydroxylated forms of AFB₁ and AFB₂. Aflatoxin B₂a (AFB₂a) and aflatoxin G₂a (AFG₂a) are 8, 9-hydrated products of AFB₁ and AFG₁, (Wogan, 1966.). These compounds are relatively non-toxic when compared with AFB₁ and AFG₁. The aflatoxins are freely soluble in moderately polar solvents (e.g. chloroform and methanol), especially in dimethyl-sulphoxide, 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 pasteurization. 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.

2.3.1. AFLATOXINS AND HUMAN HEALTH

Evidence of acute aflatoxicosis in human has been reported from many parts of the world, namely the Third World Countries, like Taiwan, Uganda, India, and many others. The syndrome is characterized by vomiting, abdominal pain, pulmonary edema, convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidneys, and heart.

Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal development in crops and commodities, and lack of regulatory systems for aflatoxin monitoring and control.

Because aflatoxins, especially aflatoxin B₁, are potent carcinogen in some animals, there is interest in the effects of long-term exposure to low levels of these important mycotoxins on humans. In 1988, the IARC placed aflatoxin B₁ on the list of human carcinogens. This is supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and Liver Cell Cancer (LCC). Additionally, the expression of aflatoxin-related diseases in humans may be influenced by factors such as age, sex,
nutritional status, and/or concurrent exposure to other causative agents such as viral hepatitis (HBV) or parasite infestation.

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 AFB₁, 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). AFB₁ 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 AFB₁ in the development of lung cancer. In comparing the ability of AFB₁ to bind to bladder and tracheobronchial tissues derived from several animals, Stoner et al., (1982) found that extrahepatic binding of AFB₁ to DNA was higher in AFB₁-resistant species than in susceptible species.

Although the epidemiological evidence relating AFB₁ 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 AFB₁ (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 AFB₁ to its reactive metabolite, resulting in greater AFB₁-DNA adducts formation and increasing the risk of liver cancer.
Acute AF exposures have been associated with epidemics of acute toxic hepatitis in areas of China and Africa with death rates ranging from 10 to 60% (Bhat and Krishnamachari, 1977). Studies on an individual attempting suicide by ingesting purified AF have demonstrated that single doses are not as effective in humans as long-term doses (Willis et al., 1980). Toxicity symptoms in a young woman, who attempted suicide with AF in the amounts of 5.5 mg over 2 days and 35 mg over 2 weeks (6 months from the initial dose), included transient nonpruritic macular rash, nausea, and headache. The woman recovered completely and had no significant signs of liver injuries when examined 14 years later (Willis et al., 1980). Results of this case and others suggested that extended sub acute doses, as seen in dietary exposures in certain countries, may be required for inducing the lethal acute toxic effects (Willis et al., 1980; Peraica et al., 1999).

The largest risk of AF to humans is usually the result of chronic dietary exposure. Such dietary AF exposures have been associated with human hepatocellular carcinomas, which may be compounded by hepatitis B virus. Approximately 250 000 deaths are caused by hepatocellular carcinomas in China and Sub-Saharan Africa annually (Groopman et al., 1992) and are attributed to risk factors such as high daily intake (1.4 g) of AF (Wild et al., 1992) and high incidence of hepatitis B (Kensler et al., 1991; Wild et al., 1992). Aflatoxins have been found in tissues of children suffering from Kwashiorkor and Reye’s syndrome and were thought to be a contributing factor to these diseases (Becroft and Webster, 1972). Reye’s syndrome, which is characterized by encephalopathy and visceral deterioration, results in liver and kidney enlargement and cerebral edema (Becroft and Webster, 1972; Blunden et al., 1991). Another potential human carcinogen is OTA which was implicated as the causative agent in epithelial tumors of the upper urinary tract in the Balkan regions (Krogh, 1978).
2.3.2. AFLATOXINS AND ANIMAL HEALTH

Aflatoxicosis is primarily a hepatic disease. The susceptibility of individual animals to aflatoxins varies considerably depending on species, age, sex and nutrition. In fact, aflatoxins cause, liver damage, decreased milk and egg production, recurrent infection as a result of immunity suppression (e.g. Salmonelalosis), in addition to embryo toxicity in animals consuming low dietary concentrations. While the young of a species are most susceptible, all ages are affected but in different degrees for different species. Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproductivity, reduced feed utilization and efficiency, anemia, and jaundice. Nursing animals may be affected as a result of the conversion of aflatoxin B\(_1\) to the metabolite aflatoxin M\(_1\) excreted in milk of dairy cattle.

The induction of cancer by aflatoxins has been extensively studies. Aflatoxin B\(_1\), aflatoxin M\(_1\), and aflatoxin G\(_1\) have been shown to cause various types of cancer in different animal species. However, only aflatoxin B\(_1\) is considered by the International Agency for Research on cancer (IARC) as having produced sufficient evidence of carcinogenicity in experimental animals to be identified as a carcinogen.

Early studies on the effects of acute aflatoxicosis indicated various toxicities in different animal species (Wogan, 1966). *(Table 4)*

**Poultry**

Early investigations (1960) of the sudden death of 100,000 turkey poults consuming groundnuts in England linked AF (from *A. flavus*) to acute hepatic necrosis and hyperplasia of the bile ducts of the intoxicated birds (Newberne and Butler, 1969). Chickens have been shown to bruise and hemorrhage from AF (Tung *et al.*, 1971). Injection of AFB\(_1\) alone or in combination with OTA showed impaired development and increased mortality in chicken embryos (Edrington *et al.*, 1995). In India, high levels of AF (ranging from 0.2 to 1 mg/kg) in combination with other mycotoxins (i.e. OTA and/or trichothecenes) in poultry feed have resulted in diseases such as hepatitis, salmonellosis, coccidiosis, and infectious
bursal disease (Jand et al., 1995). The negative effects of aflalotoxins on chicken performance have been demonstrated in numerous studies. For example, feeding a high level (3.5 mg/kg of feed) of an AF mixture (i.e. 79% AFB₁, 16% AFG₁, 4% AFB₂, and 1% AFG₂) to broilers reduced their body weight and increased their liver and kidney weights (Smith et al., 1992).

The negative impact of AF on the immune response was investigated in vitro (NeldonOrtiz and Qureshi, 1992). In this study, chicken peritoneal macrophages were exposed to various levels of AFB₁ alone (i.e. 5, 10, and 20 g/ml of culture medium) or with microsomal mixed function oxidase (AFB₁ levels were 0.01, 0.1, 0.5, 1, and 5 g/ml) that activates AFB₁ to its toxic form (i.e. AFB₁-epoxide). The exposure to AFB₁ alone resulted in a dose-dependent reduction in macrophage adherence potential and an increase in cell damage. The activated form of AFB₁ (i.e. incubated with mixed function oxidase) also induced similar responses and caused morphological alterations and reduced phagocytosis.

**Pigs**

Swine are among the most sensitive species to mycotoxins. For example, Southern and Clawson (1979) have demonstrated that total AF at 385 g/kg of feed was close to the maximum tolerance level for finishing pigs. In their study, feeding diets containing various levels (i.e. 0.02, 0.385, 0.75, and 1.48 mg/ kg of feed) of AF (mostly AFB₁ with small amounts of AFG and AFB₂) reduced average daily gain linearly at 0.385 mg/kg of feed or higher while feed efficiency was decreased only at the highest level (i.e. 1.48 mg/kg of feed). Liver weights (as a percentage of body weight) were increased by feeding AF at 0.385 mg/kg of feed or higher, but hepatocellular lesions were only found in pigs receiving the highest AF level (i.e. 1.48 mg/kg of feed).

Progression of aflatoxicosis also was evaluated in barrows given graded levels (1, 2, 3, or 4 mg/kg of feed) of AF (Harvey et al., 1988). Body weight gains were decreased in a linear fashion (from 7 kg for the
control group to 4, 3, 2.5, and 0.2 kg for barrows receiving 1, 2, 3, or 4 mg of AF/kg of feed, respectively) over a 4-week feeding period. In a study by Huff et al. (1988), swine response to AF (2 mg/kg of feed), OTA (2 mg/kg of feed), or both was evaluated. Compared with the control group, body weight gains were reduced by 26, 24, and 52% for animals consuming diets containing AF, OTA, or both, respectively.

The swine immune response to AF has been inconsistent. Miller et al. (1978) reported decreased lymphocyte blastogenic response to mitogens, reduced macrophage migration, and depressed delayed hypersensitivity when AF was fed at 0.4 to 0.8 mg/kg of feed for 10 week. Other studies, however, have shown that swine humoral immune response was not altered by feeding mixed AF at levels ranging from 0.4 to 0.8 mg/kg of feed (Miller et al., 1981) to acutely toxic levels as high as 500 mg/kg of feed (Panangala et al., 1986). In vitro studies have confirmed that the immunosuppression caused by AF (140 or 280 g/kg of feed) only occurs at the cellular and not the humoral level (van Heugten et al., 1994). Other in vitro studies (Pang and Pan, 1994) have shown inhibition of DNA synthesis in porcine lymphocytes when AFB_1 was added to the medium at various levels (0.1–10 000 mg/ml of medium).

**Horses**

The history of mycotoxicosis and poisoning in equine has been reviewed by Asquith (1991). In a case study, mature horses consuming AFB_1-contaminated feed (58.4 g/kg) were jaundiced and anorexic before death (Greene and Oehme, 1976). Post-mortem examinations revealed enlarged liver, kidney damage, and lesions of bile-duct hyperplasia. In other cases (Asquith and Edds, 1981), equine aflatoxicosis has been characterized by depression, lameness, and death. Postmortem examinations revealed subcutaneous and enteric hemorrhage, enlarged kidneys, enlarged necrotic livers, and hepatic, nephritic, and myocardial lesions. Studies with ponies have shown damage in the skeletal muscles and heart along with liver dysfunction when acute lethal doses of AFB_1 were administered (Asquith and Edds, 1981). Post-mortem examination
of horses consuming corn contaminated with a mixture of AF (AFB₁, AFB₂, and AFM₁ at 114, 10, and 6 g/kg, respectively) revealed severe hepatic lesions (Vesonder et al., 1991).

**Dogs and cats**

The effects of mycotoxins on companion animals are severe and can lead to death. As early as 1952, a case of hepatitis in dogs was directly linked to consumption of moldy food (Devegowda and Castaldo, 2000). Following the discovery of AF (Asao et al., 1963), the agent responsible for the 1952 case was identified as AFB₁ (Newberne et al., 1966) and the symptoms of aflatoxicoses in dogs were elucidated (Newberne et al., 1966; Ketterer et al., 1975). In the case study by Ketterer et al. (1975), three dogs on a farm in Queensland became ill (severe depression, anorexia, and weakness) and died at different times within a month following consumption of a commercial dog food mixed with AF-contaminated bread. The vomitus specimens from one dog contained high levels of AF (100 μg/g of AFB₁ and 40 μg/g of AFG₁). In addition to hepatitis and sudden death in dogs, symptoms of acute aflatoxicoses in both dogs and cats include vomiting, depression, polydipsia, and polyuria. Death usually occurs in 3 days with LD₅₀ levels ranging from 0.5 to 1.0 mg/kg in dogs and 0.3 to 0.6 mg/kg in cats depending on the age of the animal (Newberne et al., 1966).

Necropsy observations revealed enlarged livers, disseminated intravascular coagulation, and internal hemorrhaging. In sub-acute aflatoxicosis (at 0.5–1 mg/kg of pet food over 2–3 week), dogs and cats become lethargic, anorexic, and jaundiced (Newberne et al., 1966). This can be followed by disseminated intravascular coagulation and death. Such impaired blood clotting has been reported in other animals such as rats, rabbits, goats, and chickens (Bababunmi et al., 1997). In chronic aflatoxicoses (at 0.05–0.3 mg/kg of pet food over 6–8 week), dogs and cats had clinical signs similar to those for the sub acute phase but jaundice was the predominant manifestation. Histopathology of animals with chronic aflatoxicoses revealed shrunken livers with extensive
fibrosis (Newberne et al., 1966; Ketterer et al., 1975). The development of accurate screening methods for AF in recent years has led to a vast improvement in the exclusion of AFB₁ from pet food. As a result, there have been only two isolated cases of aflatoxicosis in dogs in the US since 1980 (Rumbeiha, 2000). Deoxynivalenol is a major health concern for companion animals and it contaminates pet food via corn even after processing (Scott, 1984).

In another study (Hughes et al., 1999), the effects of dietary DON on dogs and cats were investigated. Food refusals were noted when DON levels exceeded 4.5 mg/kg of dog food and 7.7 mg/kg of cat food. This observation suggested a higher sensitivity in dogs than in cats. It has been shown that food consumption was significantly reduced at DON levels of 4.5 g/kg of dog food and of 7.5 g/kg of cat food. In the same study (Hughes et al., 1999), vomiting was noted at different times in dogs and cats (depending on the mycotoxin dose) during the 14 day of DON feeding.

**Rats and mice**

Rats have been used extensively for decades as a model for human mycotoxicoses especially with regard to the carcinogenic potential of AF. This model system, however, has been a subject for debate due to the differences in the detoxification mechanisms between rats and humans as shown by cytosolic conjugation of AFB₁ in vitro (Raney et al., 1992). In early studies, the LD₅₀ for AFB₁ were established in rats at various levels such as 0.5–7 mg/kg (Butler, 1964; Wogan and Newberne, 1967) and 6–18 mg/kg (Patterson, 1973) depending on the method of administration (i.v. or oral, respectively). Necropsy results revealed hepatic damage (i.e. lesions, necrosis, and biliary proliferation) similar to that of other species. In another study (Newberne and Butler, 1969), oral administration of AFB₁ at 5 mg/kg for 9 week in rats has resulted in 100% hepatocellular carcinomas. A recent study (Stetinova et al., 1998), has suggested secondary effects of AFB₁ on the gastrointestinal tract due to changes in the liver detoxification mechanisms and possible reductions in nutrient
uptake. In contrast to rats, mice are generally resistant to the hepatocarcinogenic effects of AFB$_1$. This may explain the high level of glutathione S-transferase (GST) activity in mice challenged with AFB$_1$ (Quinn et al., 1990). Contrary to the hepatocellular carcinomas commonly found in rat studies with AFB$_1$ (Butler, 1964; Wogan and Newberne, 1967; Patterson, 1973), mice given AFB$_1$ by intraperitoneal injection at 0.02 mg/kg of body weight for 12 injections over 3 weeks (average 5.6 mg/kg body weight) have expressed pulmonary tumors (Weider et al., 1968). Other mycotoxins such as FB$_1$ also have been implicated in hepatic tumor formation in rats (Gelderblom and Snyman, 1991). In this study, 25 rats were given AFB$_1$ at 50 mg/kg of feed in a 26-month experiment. Similar to AFB$_1$, the liver was the primary target for AFB$_1$ toxicity.

### 2.3.3. CELLULAR METABOLISM OF AFLATOXIN

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. 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 P$_{450}$, 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 AFB₁ (Patterson, 1973; Hsieh et al., 1977; Wong and Hsieh, 1978). The microsomal mono-oxygenase system is also responsible for transforming the AFB₁ into polar molecules such as AFM₁, aflatoxin P₁ (AFP₁) and aflatoxin Q₁ (AFQ₁). AFM₁, AFP₁ and AFQ₁ can be eliminated by the hepatocytes, but the epoxide binds to nucleic acids and proteins and is thought to be the carcinogenic form of AFB₁ (Swenson et al., 1974). The AFB₁-epoxide may become hydrated to its dihydrodiol (8,9-dihydro-8,9-dihydroxy AFB₁), 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). AFB₂α, thought to be a hydrolytic product of the AFB₁ or its conjugates, in the phenolate form, binds to proteins, forming Schiff bases (Fig. 3b). This AFB₂α may then cause the acute toxic effects of AFB₁ (Hsieh et al., 1977; Hsieh, 1987). The decreased toxicity of AFB₂α 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 AFB₁ to this direct mechanism of binding to critical intracellular macromolecules. AFB₁ may exert its genotoxic effects by an indirect mechanism: through being membrane-active, via the intermediary 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 AFB₁-DNA adducts, which could not be explained entirely in terms of a direct genotoxic action.

2.3.4. Interaction with Biomolecules and their synthesis

DNA synthesis

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 hemorrhage (Hsieh, 1987) although AFB₁ 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 AFB₁ 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 AFB₁ blocked the initiation step in DNA replication rather than the elongation process. Inhibition may result from covalent binding of AFB₁ 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 AFB₁ to membrane proteins may also reduce uptake of thymidine and other precursor nucleotides necessary for DNA synthesis (Kunimoto et al., 1974).

RNA Biosynthesis

Synthesis of rat liver RNA is inhibited rapidly by AFB₁ (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 AFB₁ administration (Yu, 1977). Yu (1983) found that after activation in vitro and in vivo, AFB₁ 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 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).

**Interactions with 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 AFB₁ may result in transient non-covalent AFB₁-DNA binding, which forms prior to AFB₁ activation and DNA adduct formation.

Both AFB₁ epoxide and, to a lesser extent, its hydration product, the dihydrodiol form of AFB₁ react with nucleic acids. The epoxide specifically makes an electrophilic attack on the N⁷ position of guanine of DNA and RNA (Croy et al., 1978; Essigmann et al., 1980; Croy and Wogan, 1981a,b and 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 AFB₁-N⁷-guanine (trans-2, 3-dihydro-2{(N⁷-guanyl)-3-hydroxy AFB₁}) is the major adduct formed, other metabolites of AFB₁ have the ability to form adducts with DNA, in particular, AFM₁-N⁷-guanine and AFP₁-N⁷-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 G₁ and G₂ reduces DNA binding affinity by approximately one order of magnitude (Raney et al., 1990).

The N⁷-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-(N⁵-formyl-2,3,6-triamino-4-oxopyrimidine-N⁵-yl)-3-hydroxy AFB₁ (AFB₁ FAPY) and 8,9-dihydro-8-(2-amino-6-formamide-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB₁ (AFB₁ III)] within 24 hr of AFB₁ 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).

**Interaction with 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
AFB₁ in the nucleus (Ch’ih et al., 1993). Such findings exemplify the opportunistic nature of AFB₁.

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).

**Interaction with Carbohydrate**

Several animal species, when administered AFB₁, 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).

**Interaction with Lipid**

AFB₁ is known to cause lipid accumulation in the liver (Hsieh, 1987). 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 AFB₁, although there was no observable difference in [¹⁴C] 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 AFB₁-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 AFB₁ (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, AFB₁ 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).

**Inhibition of ATP Generation**

At acute mycotoxin exposure levels, inhibition of cellular energy production is a major metabolic effect (Hsieh, 1987). In this regard, AFB₁, AFG₁ and AFM₁ inhibit oxygen uptake in tissue homogenates (Smith and Moss, 1985). The aflatoxins act on the electron transport system, with AFB₁, AFG₁ and AFM₁ inhibiting the electron transport chain between cytochromes b and c or c₁ (Site II) in rat liver mitochondria (Doherty and Campbell, 1972, 1973). AFB₁ is also known to act at the cytochrome oxidase level (Kiessling, 1986; Betina, 1989). It would appear, however, that the biochemical effects of AFB₁ 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). AFM₁ has been found to uncouple *in vitro* oxidative phosphorylation and inhibit electron transport (Pai et al., 1975). AFB₁ is a similar uncoupler, but is more effective as an electron transport inhibitor, also inhibiting ATPase activity (Hsieh, 1987). AFB₁ may also inhibit rat liver oligomycin-sensitive Mg²⁺
ATPase (i.e. ATP synthase) of the inner mitochondrial membrane (Hayes, 1978).

**Immunocytochemical Localization of Aflatoxin B₁**

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 AFB₁ within the cells of plant tissues. This indirect immunocytochemical technique involved the use of a primary antibody (anti-AFB₁, directed against an antigen (AFB₁)).

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. Ultra thin 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 AFB₁ in the medium, AFB₁ 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. 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 AFB₁, 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 AFB₁, exposure will then be secondary manifestations resulting from AFB₁-DNA binding.
2.4.5. SPECIFIC EFFECTS

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 McLaughlin, 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, delayed interferon production in turkeys (Pier, 1973; Pier and McLaughlin, 1985) and delayed lymphokine activity (Pier et al., 1977). At higher doses, AFB\(_1\) lowered levels of immunoglobulins G and 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\textsubscript{1}. Cukrova et al. (1991) found that a dose of AFB\textsubscript{1} as low as 0.5 μg/mL 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 (Cukrova 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\textsubscript{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 \textit{Listeria monocytogenes} and other bacteria, following pretreatment of the animals with 10 μg/ml AFB\textsubscript{1} (Kurtz and Czuprynski, 1992). \textit{In vitro} exposure of chicken peritoneal macrophages to AFB\textsubscript{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\textsubscript{1} concentrations. It is likely that on addition of mixed function oxidases, AFB\textsubscript{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.

**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\textsubscript{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).

**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, 1978; 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.

**Carcinogenic Effects**

**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 favorable 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 (Irvin and Wogan,
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).

**The Ras Oncogenes and Hepatocellular Carcinoma**

*Ras* protooncogene 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 Kiras 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 Kiras genes (GGT—GAT (McMahon *et al.*, 1987) and GGT --- TGT (Sinha *et al.*, 1988)) have been identified. Soman and Wogan (1993) have confirmed the Kiras 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).
The p53 Gene and Hepatocellular Carcinoma (HCC)

G—T substitution in codon 249

Recently, evidence has been accumulating regarding the development of human HCC, with respect to aflatoxin, the p53 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 p53 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 analyzed HCC tumours (Hsu et al., 1991; Bressac et al., 1991). In non-human primates, however, no mutations at codon 249 were detected in AFB1-induced tumours (Fujimoto et al., 1992). The data for rats suggest that AFB1 alone is not sufficient to account for the specificity of the p53 mutations in HCC. While Lilleberg et al. (1992) are of the opinion that alteration of the p53 suppressor gene is involved in HCC induction in rats, the results of Hulla et al. (1993) regarding the specificity of the p53 mutation; however, suggest that AFB1 is not responsible for these lesions.

Hsieh et al. (1992) attempted to assess the correlation between mutations at codon 249 and the level of AFB1-DNA adducts in the liver tissue of HCC patients from a high AFB1 risk area (Taiwan) and a low AFB1 risk area (Japan). The AGG --- AGT transversion was found in 21% of Taiwanese patients and none of the Japanese patients. AFB1-DNA adducts, however, were found in tumorous and non-tumorous tissues from both groups of patients. Furthermore, AFB1-DNA adducts were found in 50% of patients lacking the p53 mutation. It is the opinion of Hsieh et al., (1992) that adducts reflects recent AFB1 exposure and so may not be a reliable index of earlier AFB1 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 p53 gene alterations in only 30% of HCC patients, and invariably only in advanced cases, suggesting that p53 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).

**Multifactorial etiology**

Chen et al. (1992) have reported that in Taiwanese patients, 70% of the HCC smears assessed were positive for AFB₁-DNA adducts. The findings relating the involvement of both HBV and AFB₁ in HCC development are controversial (Santella et al., 1993). The results of these recent investigations serve to reinforce the idea of a multifactorial etiology for the development of HCC.

In attempting 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 p53 gene in human HCC is likely to involve environmental carcinogens other than AFB₁, or that the HBV hepatitis is a prerequisite for AFB₁-induced G --- T transversion in the codon. It is also probable, as is suggested by Puisieux et al. (1991) that the p53 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 AFB₁-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 P<sub>450</sub> and GST. In this regard, Hollstein <em>et al.</em> (1993) found an AGG --- ACT 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<sub>1</sub>-liver adducts and AFB<sub>1</sub>-serum albumin adducts. On genotyping patients for GST, it was found that 12 of the 15 patients possessed the null genotype.

**AFB<sub>1</sub> ingestion and HCC development in different countries**

Ozturk <em>et al.</em> (1991) have provided some of the best evidence relating AFB<sub>1</sub> ingestion with HCC development. They noted a specific mutation in the p53 tumour-suppressor gene in hepatoma tissue from patients at high risk of AFB<sub>1</sub> exposure. In their study, in four countries where AFB<sub>1</sub> intake was high, 22% of tumour samples had the characteristic mutation at codon 249 of the p53 gene, in comparison with less than 1% in tumours from patients from countries where the risk of AFB<sub>1</sub> intake was low.

HBV infection was commensurately high in countries where AFB<sub>1</sub> contamination of food was prevalent. Ozturk <em>et al.</em> (1991) then compared similar incidents of HCC and HBV in a high AFB<sub>1</sub> intake area (Mozambique) with a low AFB<sub>1</sub> 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 1%), 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 <em>et al.</em> (1985) that the estimated daily intake of AFB<sub>1</sub> in Mozambique was approximately four times that of Transkeians. The ratio of HCC incidence in these two areas was similar, suggesting an etiological role for AFB<sub>1</sub> 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<sub>1</sub>-mediated HCC. In this regard, Hsing <em>et al.</em> (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 an 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 AFB₁ 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.

Pate1 et al. (1992) assessed patients from the United Kingdom, other countries of low AFB₁ intake and countries of high intake for p53 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 of AFB₁-DNA adducts in a range of tissues taken from autopsy specimens in the United Kingdom (Harrison et al., 1993).

Indirect evidence for AFB₁ 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 AFB₁ intake and AFM, excretion in 81 households in 10 villages in the Chinese province of Guangxi and found a positive correlation between PLC mortality and AFB, intake from maize and peanut oil, but interestingly not from rice. Groopman et al. (1992b), in the Guangxi Autonomous Region, when analyzing total AFB₁-N⁷-guanine excretion in urine plotted against total AFB₁ exposure, found a correlation of 0.8, suggesting that measuring excreted AFB₁ is a good indication of the level of AFB₁ consumption and adduct formation.
Cumulative effect of HBV and AFB₁ on HCC development

Yap et al. (1993) on reviewing the incidence of HCC, HBV and AFB₁ intake, concluded that both HBV and AFB₁ 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 AFB₁-DNA adducts and of HBV surface antigens in Taiwanese HCC patients, concluded that both AFB₁ 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, AFB₁ and HCC incidence in southern Guangxi, China, to generate models evaluating the relative importance of AFB₁ 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 etiological agents in HCC development, strong evidence supporting an interactive mechanism has not been elucidated. In an extensive review of the research involving AFB₁-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 AFB₁-DNA adduct data are incomplete, although Groopman et al. (1993) concluded from their study that the presence of AFB₁-guanine adducts in urine is a good non-invasive marker for exposure to AFB₁ and the risk of genetic damage. Additional investigations undoubtedly will improve the risk assessment for humans with respect to AFB₁ and perhaps elucidate the individual contribution of AFB₁ and HBV to the development of HCC.
Cytochrome P$_{450}$, 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 P$_{450}$ 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 P$_{450}$ supergene family of isoenzymes. Different cytochrome P$_{450}$ 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 P$_{450}$ IA2, P$_{450}$IIA3, P$_{450}$IIIA4 and P$_{450}$IIB [decreasing order] (Aoyama et al., 1990) and cytochrome P$_{450}$IA enzymes metabolise the detoxification of AFB$_1$ to AFM$_1$ (Koser et al., 1988). Other cytochrome P$_{450}$ isoenzymes are responsible for the conversion of AFB$_1$ to other less toxic metabolites: AFQ$_1$ (in humans, by P$_{450}$IIIA (Forrester et al., 1990)) and AFP$_1$ (Hayes et al., 1991a).

At least 10-fold differences in cytochrome P$_{450}$IIIA and P$_{450}$IA expression have been observed between individuals (Watkins, 1990). In humans, it would appear then that the cytochrome P$_{450}$IIIA family is responsible for both AFB$_1$ epoxidation and the formation of AFQ$_1$ (Forrester et al., 1990). The ability of a cytochrome P$_{450}$ 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 P$_{450}$ IA2 (Forrester et al., 1990) while in rats, the P$_{450}$ cytochrome appears to be involved (Koser et al., 1988).
Bioactivation of AFB\textsubscript{1}

The ability of tissue to bioactivate AFB\textsubscript{1} is an important consideration in understanding the ability of AFB\textsubscript{1} to induce toxic, mutagenic or carcinogenic transformation in cells. In this regard, Imaoka and co-workers (Imaoka \textit{et al.}, 1992) have investigated the genotoxic and mutagenic activation of AFB\textsubscript{1} on \textit{Salmonella typhimurium} by rat hepatic, renal and pulmonary microsomal fractions and purified cytochrome P\textsubscript{450} enzymes.

Hepatic microsomes displayed the greatest mutagenic activation, while renal microsomes had the lowest activity. Additionally, cytochrome P\textsubscript{450}IIC2 (a major hepatic cytochrome P\textsubscript{450} in male rats) had the highest activating ability, while renal forms of P\textsubscript{450} (e.g. cytochromes P\textsubscript{450} IVA2 and P\textsubscript{450} 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\textsubscript{1} is dependent on the different classes of cytochrome P\textsubscript{450}s present in that tissue.

Various hepatic cell populations (hepatocytes, Kupffer and endothelial cells) have been found to differ in their AFB\textsubscript{1}-bio-activating ability (Schlemper \textit{et al.}, 1991). Ten-fold higher AFB\textsubscript{1} concentrations were required by non-parenchymal (Kupffer and endothelial) cells to obtain a similar number of \textit{Salmonella typhimurium} TA98 revertants (as compared with parenchymal cells). In freshly isolated cells, AFB\textsubscript{1} was found to bind (although differentially) to DNA in both parenchymal and non-parenchymal cells in a dose-dependent manner (Schlemper \textit{et al.}. 1991).

Metabolic activation of AFB\textsubscript{1} was studied using human cell lines that expressed individual cytochrome P\textsubscript{450}s (Crespi \textit{et al.}, 1991). Cells expressing cytochrome P\textsubscript{450}IA2 were the most sensitive (at 10 ng/mL) to the toxic and mutagenic effects of AFB\textsubscript{1}. Cells expressing cytochrome P\textsubscript{450}IIA4 were 5- to 10-fold less sensitive than those expressing P\textsubscript{450}IA2. The least sensitive cells expressed cytochrome P\textsubscript{450}IIA6, while cells resistant to 1 \(\mu\)g/ml AFB\textsubscript{1} expressed no cytochromes (Crespi \textit{et al.}, 1991).

Tjalve \textit{et al.} (1992) have found that microsomal preparations of bovine olfactory mucosa have a greater affinity than liver microsomes to
induce covalent binding of AFB₁ to calf thymus DNA and microsomal proteins. Addition of glutathione to these preparations decreased AFB₁-DNA binding. When cytosolic fractions of mouse liver (where AFB₁ resistance may be related to high hepatic GST activity) were added to the olfactory mucosal incubation medium, the decrease in AFB₁-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 AFB₁ bio-activating ability of bovine olfactory mucosa (i.e. P₄₅₀ involvement) and perhaps lower levels of GST activity (Tjalve et al., 1992).

**Formation of AFB₁ epoxide**

The ability of an organism (or a tissue) to form the AFB₁-epoxide might explain the sensitivity of trout and quail to AFB₁. A cytochrome P₄₅₀ isolated from the livers of β-naphthoflavone-treated rainbow trout had a 15-fold greater ability to form AFB₁-8,9-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 AFB₁ 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 AFB₁-DNA binding when compared with adults. Levels of the AFB₁-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 AFB₁ than did the parental MCF-7 cells.
These results would suggest that expression of this class of cytochrome P\textsubscript{450} promoted the formation of reactive AFB\textsubscript{1} metabolites.

**GST and AFB\textsubscript{1}-DNA aduct**

Several other researchers have shown GST enzymes to be important protective agents against AFB\textsubscript{1}-DNA adduct formation, e.g. Mandel et al. (1992) using low protein diets in 3-week-old rat weanlings and Liu et al. (1999) in comparing the ability of human liver fractions and lymphocytes to deal with aflatoxin and other foreign chemicals. Liu et al. (1999) found a highly significant correlation ($r=0.88$) between AFB\textsubscript{1}-DNA adduct concentrations and GST \textmu class activity. Tsuji et al. (1992) in comparing species and sex differences in AFB\textsubscript{1}-induced GST placental forms, concluded that glutathione and GST play an important role in modulating hepatic AFB\textsubscript{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\textsubscript{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\textsubscript{1}, DES-pretreated animals showed a 35% decrease in AFB\textsubscript{1}-DNA adduct formation and a 2-fold increase in the levels of \textalpha-GST. Results suggest that neonatal DES treatment resulted in long-term protective increases in basal \textalpha-GST levels, causing lower levels of DNA adduction following adult exposure to AFB\textsubscript{1} (Zanger et al., 1992). More specifically, Gopalan et al. (1992) found that in \textit{in vitro} rat studies, the different classes of \textalpha-GSTs induced were dependent on the foreign chemical used. For example, the highest catalytic activity with microsome-mediated AFB\textsubscript{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).

**Impact of exogenous agents on the expression of cytochrome P$_{450}$ Enzyme**

Expression of cytochrome P$_{450}$ enzymes can be influenced by exogenous agents (Nebert et al., 1991) e.g. cytochrome P$_{450}$1IIA enzymes are inducible by glucocorticoids and rifampin (Watkins, 1990) and cytochrome P$_{450}$IA enzymes can be induced by polycyclic hydrocarbons in cigarette smoke and by dietary ‘green plant’ flavones (Nebert et al., 1991). In this regard, environmental agents might influence the susceptibility to AFB$_1$-mediated hepatocarcinogenesis by altering the expression of individual cytochrome P$_{450}$ 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 P$_{450}$IA 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 P$_{450}$ enzymes. Contrary to this, however, cytochrome P$_{450}$IA2 has been shown elsewhere to be the most important cytochrome P$_{450}$ 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 P$_{450}$IIIA4 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).
2.3.6. Factors affecting production, contamination of foods and feeds, and toxicity of mycotoxins

A main difficulty in assessing the risk of mycotoxins to human and animal health is the multiplicity of factors affecting the production or presence of mycotoxins in foods or feeds. Mere isolation and confirmation of mycotoxigenic fungal species in foods or feeds does not indicate the presence of mycotoxins. Upon development of accurate and sensitive techniques for qualitative and quantitative analysis of mycotoxins, researchers have found that various factors operate interdependently to affect fungal colonization and/or production of the mycotoxins. D’Mello and MacDonald (1997) categorized the factors as physical, chemical, and biological. Physical factors include the environmental conditions conducive to fungal colonization and mycotoxin production such as temperature, relative humidity, and insect infestation. Chemical factors include the use of fungicides and/or fertilizers. Stresses such as drought, an increase in temperature, and an increase in relative humidity may selectively alter colonization and metabolism of mycotogenic fungi and thus alter mycotoxin production (Russell et al., 1991). These researchers also indicated that unseasonable conditions may render crops and forages susceptible to mycotoxin production. Cool and damp springtime weather favour the germination of the sclerotia and thus ergot alkaloid formation in fescue and ryegrass (Cheeke, 1998a).

The biological factors are based on the interactions between the colonizing toxigenic fungal species and substrate. While some plant species are more susceptible to colonization, environmental conditions may increase the vulnerability of other more resistant plant species. The biological factors have been further sub-categorized (Moss, 1991) into intrinsic factors including fungal species, strain specificity, strain variation, and instability of toxigenic properties. Such intrinsic factors underscore the difficulty of risk assessment of mycotoxin exposure based on mold contamination. Species and strain specificity are well described by the numerous mycotoxins produced by two or more fungi. A strain variation refers to a specific culture identity for the same species fungal
isolate and how these strains produce mycotoxins in a variable fashion. Finally, the toxigenic properties may vary over time and as the mycoecology changes toxins may be reduced. Several studies have shown that optimal conditions for fungal growth are not necessarily optimum for toxin production. For example, different strains of *A. flavus* have been shown to produce AF at different rates when cultured under similar conditions (Hesseltine *et al*., 1970).

*A. flavus* have shown optimum temperatures for colonization to range from 25 to 35 °C with the substrate water activity being 0.90 (Smith and Moss, 1985). In a more recent study (Gqaleni *et al*., 1997), however, AF production by *A. flavus* was optimal at 30 °C and substrate of 0.996. In the same study, AF production was affected by substrate source, incubation time, and the presence of other mycotoxins produced by *A. flavus* (Gqaleni *et al*., 1997).

### 2.3.7. Detoxification of AFB₁ in different organisms

While it would appear that cytochrome P₄₅₀ enzymes may be important considerations in explaining the relative susceptibilities of different animal species to AFB₁ (and many other noxious substances), the evidence implicating the protective effects of GST enzymes against AFB₁-DNA adduct formation in tissues is equally compelling. Although much of the work is either *in vitro* or utilizes rats as experimental animals, the importance of AFB₁-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 AFB₁. In rats, the α-GSTs appear to have the greatest ability to metabolize 8, 9-epoxides (Cole and Cox, 1981).

It is well documented that animal species have differing susceptibilities to the mutagenic or carcinogenic effects of AFB₁ (Wong and Hsieh, 1976, 1980; Hsieh *et al*., 1977; Roebuck and Wogan, 1977). In comparing AFB₁ 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 a 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 Tjalve, 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 (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 labeling 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\textsubscript{1} bio-activating enzymes (cytochrome P\textsubscript{450}s).

More recently, in whole body autoradiography of [\textsuperscript{3}H]-AFB\textsubscript{1} in marmoset monkeys, AFB\textsubscript{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 Tjitalve, 1993). In addition, in \textit{in vitro} microautoradiography, AFB\textsubscript{1} could be detected in the epithelial lining of several areas of the respiratory and alimentary tracts and the liver. If a cytochrome P\textsubscript{450} 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\textsubscript{1} to melanin, photo-activation of AFB\textsubscript{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 Tjalve, 1993).

**Prevention of aflatoxin related disease**

Interventions to reduce aflatoxin-related disease can be considered in terms of those which are applicable at the individual level or those applicable at the community level. The community level approach can be further divided into pre-harvest or post-harvest measures to reduce aflatoxin contamination. Both these areas represent primary prevention with the aim of avoiding exposure to aflatoxins. At the individual level one can consider primary prevention related to a change in diet to avoid foods known to be frequently contaminated with aflatoxins. Alternatively, because primary prevention measures are unlikely to be comprehensive, it is possible to consider secondary prevention for people at risk of high exposure. These individuals are targeted with chemo-preventive agents to
reduce the toxicity of aflatoxins once ingested. The agents involved may be natural dietary constituents or drugs.

**Community level intervention**

**Pre-harvest crop management.**

Pre-harvest would be the most effective point of control because this is the point at which the crop is infected by the fungus. The infestation of crops by *Aspergillus* most readily occurs under conditions of stress involving drought, high temperatures, insect induced injury or other processes which lead to damage of the crop (Hill *et al.*, 1983). For example, in the case of groundnuts drought stress in the 4 to 6 weeks prior to harvest is reported to lead to a decrease in moisture content and an increase in soil temperature which permits the *A. flavus* in the soil to infect the groundnut kernels (Keenan and Savage, 1994). A number of cultural practices pre-harvest may limit fungal infection and aflatoxin contamination (Lisker and Lillego, 1991). Irrigation can be an effective measure but in sub Saharan Africa is often unavailable or not cost-effective. Similarly, fungicides and pesticides may not be an attractive option given the limited cost effectiveness for subsistence farmers and the limited success in their application. As insect damage and wounding is often correlated with aflatoxin contamination of, for example maize, insecticides could also be considered. However, in some instances fungicides and pesticides may actually result in higher levels of mycotoxin contamination and this should be a parameter considered in assessing the effectiveness of these compounds (D'Mello *et al.*, 1998) in addition to the economic and environmental acceptability.

One method suggested to control aflatoxin contamination is the introduction of nonaflatoxigenic strains of *A. flavus* to compete with the aflatoxin producing strains (Bhatnagar *et al.*, 1993, Cotty *et al.*, 1994, and Dorner *et al.*, 1999). For example, the application of nonaflatoxigenic strains in maize plots led to reduced aflatoxin levels in years when weather conditions favoured contamination (Brown *et al.*, 1999 and Dorner *et al.*, 1999). It was noted that different *Aspergillus* strains may be
important for airborne crops such as maize and soilborne crops such as groundnuts. Inoculation of non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* into soil in which peanuts were grown showed reductions in aflatoxin contamination between 74.3% and 99.9% (Dorner *et al.*, 1998). The use of these strains would need careful validation in the environment for which they are intended and there is concern that the strains could revert to aflatoxin producers by anastomosis from toxigenic strains in the field. It worth noting that certain *Aspergillus* strains which are non-aflatoxigenic can produce other toxins, e.g., cyclopiazonic acid the production of which is not always correlated with that of the aflatoxins themselves (Horn and Dorner, 1999). Finally, there is also the problem of food spoilage by the fungus which would not be solved by non-aflatoxigenic strains.

Genetic engineering may offer novel ways of tackling the problems of pre-harvest contamination by mycotoxins. As has been argued recently, the use of biotechnology to counter famine and poverty in Africa is a valid one as long as potential problems of exploitation and adverse environmental impact are countered (Wambugu, 1999). The author cited research underway to develop maize resistant to maize streak virus. A feature of this work is that it is performed in Africa with field trials conducted locally to test safety and efficiency in tropical conditions. Given the widespread fungal and mycotoxin contamination of maize and groundnuts a similar approach to address these latter problems has great potential.

Cloning of the genes involved in the biosynthesis of aflatoxins represents a pathway to engineering non-aflatoxigenic strains of *Aspergillus* (Bhatnagar, *et al.*, 1994). In addition, the genes coding for enzymes, which influence the ability of the aflatoxigenic fungus to colonize the host plant, are also being identified and could be used to create effective biocompetitive, non-aflatoxigenic strains (Bhatnagar *et al.*, 1995). Rather than focus on the *Aspergillus*, an alternative approach has been to select for varieties of cereal grains and oilseeds resistant to aflatoxin. For maize and groundnuts there is known variation in
resistance among genotypes (Brown et al., 1999 and Mehan et al., 1986). Resistance in this sense may involve plant resistance to fungal infection or limitation of aflatoxin biosynthesis once infection has occurred. Initial efforts went into identifying naturally resistant crop varieties but only partial resistance to aflatoxin contamination is observed (Payne, 1992). Nevertheless, these studies provided insights into understanding both the factors, which make the plant resistant to infection and the biosynthetic pathway for aflatoxins, thus providing a basis to potentially interfere with these processes by genetic engineering of resistant plants (Bhatnagar et al., 1995; Ehrlich et al., 1998 and Brown et al., 1999). Some plants, e.g., groundnuts, produce antifungal compounds such as phytoalexins which can provide protection against the invading fungi (Keenan and Savage, 1994). Recently, kernel proteins of maize which contribute to resistance to aflatoxin production have been identified having an ability either to inhibit fungal growth per se or toxin formation (Huang et al., 1997; Chen et al., 1998 and Guo et al., 1998). These observations may permit identification of the genes involved and offer opportunities to insert these genes into the crop to provide resistant genotypes.

**Post-harvest crop management**

Prevention and control methods have been prescribed for mitigating mycotoxin contamination of feeds (Harris, 1997). These methods require that feed handlers and grain mill operators keep grain bins clean and store grain at less than 14% moisture. Feed ingredients must be dry, oxygen free, fermented or treated with mold-growth inhibitors. With regard to silage crops, harvesting at the appropriate moisture content and both packing and sealing the silo (to exclude oxygen and allow for desirable anaerobic fermentation) are essential for reducing mycotoxin contamination potential.

Aflatoxins can accumulate during food storage, particularly under hot, humid conditions where there is additional risk of rodent and insect damage. Food spoilage and contamination with mycotoxins can be significant problems in circumstances of traditional storage at the local
Considerable effort has been put into removing aflatoxins from foods post-harvest by 1) physical methods such as thermal inactivation, irradiation, sorting etc., 2) chemical means, including solvent extraction, 3) adsorption for example using minerals, e.g., phyllosilicate clays, 4) a variety of chemical degradations e.g., acids, alkalis, aldehydes, oxidising agents, ammoniation and sodium bisulphite and 5) by biological decontamination (Karlovsky, 1999 and Phillips et al., 1994). The majority of these methods is aimed at commercial crops and are appropriate only for feeds as they alter the organoleptic properties of the foods making them unfit for human consumption.

The current review focuses only on methods which could be applicable at the local farm level and these ideally comprise a package of measures designed to inhibit further fungal growth and aflatoxin production in storage. The growth of *Aspergillus* post-harvest is influenced most critically by temperature, moisture content and storage time; at the level of subsistence farmers groundnuts may be stored upwards of 6 months and maize for longer periods. Insect infestation can increase both moisture content and temperature, act to spread spores more widely in the crop and cause physical damage which further promotes fungal infection (Franzolin et al., 1999). The main approaches to contain contamination levels are those concerned with management of the crop in the field immediately after harvest, improved drying and storage to limit moisture content, and the possible use of pesticides and biological pest control. Many of the points discussed below have been highlighted more generally in relation to reducing losses of crops at the small farm level (Crompton et al., 1993).

Extended time of leaving the harvested crop in the field prior to storage increases the risk of unseasonal rain damage and promotion of fungal growth prior to storage. If the crop is left in the field for some time then it should be raised on platforms rather than left on the ground. In the case of groundnuts stacking should be with the pods exposed to the air to improve ventilation.
Manual sorting of obviously damaged kernels or pods can reduce the quantity of aflatoxins (Dickens and Whitaker, 1975). Although there is not a direct relationship between aflatoxin level and visible fungal contamination of groundnuts there is a substantial reduction in contamination achieved by this sorting approach. Sun drying is most widely used method prior to storage and this can be effective in reducing moisture content and may lead to destruction of a proportion of the aflatoxins present (Shantha et al., 1986). In the case of groundnuts sun drying should be performed on a cloth rather than directly on the earth to avoid humidity from the ground and to permit rapid gathering in case of unseasonal rainfall.

Once in storage it is advisable to keep the crop from contact with the earth either by raising on wooden pallets or a concrete floor and ensuring adequate ventilation in the storage facility to prevent an increase in moisture content. Measures to control insect and rodent damage should also be employed.

**Intervention at the individual level**

**Dietary change**

The majority of aflatoxin in high exposure countries is from groundnuts and maize where these form the dietary staples of the populations concerned. One option therefore is to avoid consuming these foods so frequently by developing a more varied diet. It is reported that through mass health education programmes in parts of the People’s Republic of China Qidong, Haimen and Fusui individuals have changed from a maize-based to a rice-based diet to reduce aflatoxin exposure even though rice is a more expensive commodity (Yu, 1995). Rice in these areas has been shown to be less frequently contaminated with aflatoxins than has maize (Yu, 1995) and interestingly we observed some years ago that aflatoxin–albumin adduct levels were lower in villages in Fusui having a rice compared to maize based diet (Wild C.P., Chen J. and Montesano R, unpublished data). However, for many communities in developing countries a change in diet is simply not feasible.
Cooking processes can reduce aflatoxins to a limited extent but these effects are variable and can produce other toxic metabolites (Phillips et al., 1994). It would be of interest to investigate in more detail the effects of traditional food preparation techniques on aflatoxin levels (Njapau et al., 1998). Nevertheless, some aflatoxin will be ingested even in situations where community level interventions are implemented.

Chemoprevention

Chemical treatment and processing are anthropogenic factors that may decrease mycotoxin contamination of foods or feeds. Wet and dry milling processes as well as heat in the cooking process have been shown to reduce AF in foods (Scott, 1984). Heating and roasting have been shown to significantly decrease AF content in corn (Conway et al., 1978; Hale and Wilson, 1979). A review of several studies, however, suggested that processing and pasteurization of milk do not completely destroy mycotoxins (Manorama and Singh, 1995). Bentonite and aluminosilicate clays used as binding agents have been shown to reduce AF intoxication in pigs (Smith, 1980, 1984; Lindemann et al., 1993; Schell et al., 1993), cattle (Diaz et al., 1997), rats (Smith, 1980; Galey et al., 1987), and poultry (Scheideler, 1993) without causing digestive problems when mixed with AF-contaminated feeds. However, these clays are ineffective against ZEN and F, can alter the nutritional value (by binding trace minerals and vitamins and reducing their bioavailability), and produce dioxins (Devegowda and Castaldo, 2000). Esterified glucomannan (i.e. an organic compound naturally-occurring in yeasts) is a recently discovered alternative with high binding potential to mycotoxins. For example, glucomannan supplementa-tion (at 0.05% of the diet) of dairy cows consuming AF-contaminated feeds reduced AF in milk by 58% (Devegowda and Castaldo, 2000) while similar reductions in AF in milk were achieved at a much higher level (1.1% of the diet) of sodium bentonite supplementation. These data suggested the potential advantage of esterified glucomannan over clays in reducing AF toxicity. Devegowda and Castaldo (2000) have illustrated that esterified glucomannan was
successful in binding F and ZEN at high efficiency (67 and 77% of that for AF). Ammoniation of stored crops has been shown to substantially (99%) reduce AF levels by hydrolyzing the lactone ring (Phillips et al., 1994). It has also been proven effective in reducing the toxicity of OTA (Marquardt and Frolich, 1992). Recently, the potential role of dietary factors to counteract the toxic effects of mycotoxins have been reviewed (Galvano et al., 2001). The role of antioxidants (Se and Vitamins A, C, and E) and food additives were evaluated. Antioxidant defense mechanisms observed have included free radical scavenging, reduced lipid peroxidation, and general inhibition of the mutagenic process. Galvano et al. (2001) also reviewed the role of food components (fructose, phenolic compounds, coumarins, and chlorophyll) and food additives (piperine, aspartame, cyproheptadine, and allyl sulfides) in reducing the toxicity of various mycotoxins by decreasing toxin formation and enhancing metabolism. For example, phenolic compounds have been shown to metabolically enhance AFB₁ conjugation and elimination (Rompelberg et al., 1996). The antioxidant ethoxyquin has been recognized as a strong antiaflatoxigenic agent. Kensler et al. (1986) demonstrated the role of ethoxyquin in rat hepatocytes as induction of conjugating GST. Mendel et al. (1987) confirmed the enhancing effect of ethoxyquin on phase II metabolism in several subcellular components (microsomes, cytosol, and cell membrane) of the rat liver. In another study, γ-glutamyltranspeptidase was induced along with GST (Manson et al., 1997). A more recent study with marmosets has established ethoxyquin as a potential chemoprotective agent against the carcinogenic effects of AFB₁ in humans (Bammler et al., 2000). Another synthetic dietary chemoprotective agent investigated for its anti-carcinogenic activity against AFB₁ is 5-(2-pyrazinyl)-4-methyl-1, 2- dithiol-3-thione (oltipraz). Oltipraz was found to decrease the binding of AFB₁ to DNA and to increase epoxide hydrase and glucuronide and glutathione levels in rat liver and kidney (Kensler et al., 1985). Oltipraz also has been shown to decrease $P_{450}$ activation of AFB₁ and to enhance GST activity in human hepatocytes in vitro (Langouet et al., 1998).
The principle of chemoprevention is to limit the carcinogenic process subsequent to exposure such that cancer incidence is reduced. Since the discovery of aflatoxins in the early 1960s there has been tremendous progress in understanding the toxicology of these compounds (Eaton and Groopman, 1994) and this has provided a rationale both for developing biomarkers of exposure to aflatoxins and for modulating their metabolism \textit{in vivo}. These two areas of research have been integrated to permit the evaluation of chemopreventive strategies in humans exposed to dietary aflatoxins by examining the impact on aflatoxin biomarkers (Kensler \textit{et al.}, 1999).

Different animals species show a marked interspecies sensitivity to aflatoxin–DNA and protein adduct formation (Wild \textit{et al.}, 1996) and susceptibility to aflatoxin carcinogenesis (Gorelick, 1990 and Parkin, 1998). Induction of glutathione-S-transferases GST and aflatoxin aldehyde reductase reduces aflatoxin-DNA and protein adduct formation and blocks aflatoxin carcinogenicity in rats (Roebuck \textit{et al.}, 1991; Judah \textit{et al.}, 1993 and Egner \textit{et al.}, 1995). Therefore, a similar modulation of the balance between aflatoxin activation and detoxification in humans has been sought and the drug, which has been used, oltipraz, is one originally prescribed to treat schistosomiasis (Kensler \textit{et al.}, 1999).

Wang \textit{et al.}, (1996 and 1999) and Kensler \textit{et al.}, (1998) have demonstrated that when oltipraz is administered to Chinese people exposed environmentally to aflatoxin there is an increase in the level of GST conjugation of aflatoxin 8,9-epoxide but also an inhibition of cytochrome P450 1A2 activity which activates aflatoxin to this reactive epoxide. These effects were demonstrated by modulation of urinary AFM1, a product of CYP1A2 metabolism of AFB1 aflatoxin–albumin adducts in peripheral blood and urinary aflatoxin-mercapturic acid in subjects receiving the drug under different treatment protocols. As the effects on enzyme induction are more prolonged than the half-life of the drug itself this has meant that transient, intermittent administration can affect both metabolite and adduct profile. Currently, a phase 2b clinical trial with an
intervention over 1 year administering 250 or 500 mg oltipraz weekly is underway in Qidong People’s Republic of China.

The Phase 2b trial will permit selection of a safe and effective dose of oltipraz for a Phase III trial, specifically including an evaluation as to whether the minor side effects seen in the 8-week intervention of the Phase 2 trial are any more marked with long-term intervention. Specifically the adverse side effects included a syndrome involving numbness, tingling and sometimes pain, in the extremities (Jacobson et al., 1996). The phase III trial should evaluate the chemopreventive action of oltipraz against aflatoxin carcinogenesis and this would normally require disease incidence as an outcome. Unless aflatoxin exerts a hepatocarcinogenic effect late in the natural history of the disease then a long follow-up would be required to see an effect on liver cancer. Alternatively if any of the biomarkers are demonstrated to be strong predictors of cancer risk then these could be used as surrogate measures of disease outcome. It is unlikely that the transient aflatoxin adducts DNA or albumin will fulfill this requirement at the individual level; this is suggested indirectly in rats where a correlation between adducts and liver cancer occurred at the group but not individual level (Kensler et al., 1997). There is some hope that the specific p53 codon 249 mutation may be more predictive of individual risk. In this respect the re-identification of this mutation in the plasma of Gambians with liver cancer or cirrhosis is encouraging (Kirk et al., 1999).

Other enzyme inducers are likely to become available in the next few years and these may be more potent than oltipraz (Kensler et al., 1997). However, alternative rationales to chemoprevention may also be explored. The addition of mineral adsorbents e.g., aluminosilicate. Clays in animal feeds to bind aflatoxins and reduce uptake into the blood stream from the gastrointestinal GI tract is used in the USA and Southeast Asia (Phillips et al., 1994). One such clay, hydrated sodium calcium aluminosilicate, binds to aflatoxins in the GI tract reducing bioavailability and toxicity (Abdel-Wahhab et al., 1999). Whether this type of compound will have the requisite lack of toxicological and nutritional
effects to permit application to humans is still an open question. There may be other parallel approaches to prevent absorption of aflatoxins from the GI tract. For example, natural products such as chlorophyllin may be used to absorb aflatoxins and reduce the amount of toxin reaching the liver (Dashwood et al., 1998). The utilization of these compounds in humans would require careful evaluation including a consideration of the possible removal of essential nutrients from the diet.

**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-glucuronyltransferase, 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_.

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As with toxicity testing, most studies focusing on mycotoxin metabolism have been on AF. In general, great variations among species, and in some cases individual animals, exist with regard to AF metabolism. Factors influencing AF metabolism include species, sex, age, health, and diet. The pure form of AFB₁ is not mutagenic and its biotransformation in mammalian tissues is primarily accomplished by microsomal cytochrome P₄₅₀ monooxygenases.

The P₄₅₀ enzymes and their sub-families are found at different concentrations in most tissues of various animal species with abundance generally in the liver (Eaton et al., 1994). Four metabolic pathways for AF₁ include O-dealkylation to AFP₁, ketoreduction to aflatoxicol, epoxidation to AFB₁ -8,9-epoxide (acutely toxic, mutagenic, and carcinogenic), and hydroxylation to AFM₁ (acutely toxic), AFP₁, AFQ₁, or AFB₂a (all relatively non-toxic). Similar to the pathways identified for polycyclic aromatic hydrocarbons (PAH), bioactivation of AFB₁ to AFB₁ -8,9-epox-ide has been linked to prostglandin-H-synthase and cytosolic lipooxygenases in lipid-hydroperoxide dependent reactions as well as P₄₅₀ enzymes (Harvey et al., 1995). Various forms of P₄₅₀ serve different biotransformation capacities depending on the animal species. In general, activation of AFB₁ -8,9-epoxide is accomplished by enzymes in the P₄₅₀ subfamilies 1A, 2B, 2C, and 3A. The isoform CYP1A2, which is PAH-inducible, is demonstrated to have the greatest binding affinity in humans (Massey et al., 1995).

Detoxification of AFB₁ -8,9-epoxide and AFM₁ in mammalian tissues is carried out via conjugation by glutathione (GSH), and catalyzed by GST (Massey et al., 1995; Longouet et al., 1998). Alternatively AFB₁ -8, 9- epoxide is hydrolyzed to a dihydrodiol (Massey et al., 1995; Longouet et al., 1998). Activation and detoxification efficiencies in an animal species determine individual AF toxicities. Activation of AFB₁ has been shown with high capacity in mixed function oxidase systems of the nasal and tracheal mucosa of swine (Larsson and Tjalve, 1996). However, studies with hogs in AF-contaminated regions of the US resulted in a low
occurrence of AF in detectable amounts in hepatic tissues (Honstead et al., 1992), which explained the occurrence of upper respiratory cancers as opposed to hepatocellular carcinomas in AF-exposed porcines.

In another study (Kuilman et al., 1998), bovine hepatocytes metabolized AFB₁ to predominately AFM₁ but there were also measurable amounts of AFM₁ epoxide, AFB₁ dihydrodiol, and AFB₁ -GSH conjugates. Aflatoxicol was not detected in the hepatocyte cultures although it was detected in earlier studies in cows’ plasma, erythrocytes, and milk which was probably a result of the ruminal microbial degradation. Studies have shown that the GSH-GST detoxification mechanism is relatively low in humans compared with rats, mice, or rabbits (Edrington et al., 1995; Massey et al., 1995). Further studies have demonstrated variations in P₄₅₀ levels, P₄₅₀ isoform function, and GST activity among species and within tissues of a single species (Ball and Coulombe, 1991). The AFM₁ was carried from AFB₁ into milk at a conversion rate ranging from 0.5 to 5% when cows consumed AFB₁ -contaminated feed (Applebaum et al., 1982; Bodine and Mertens, 1983; Skrinjar et al., 1992; Veldman et al., 1992; Manorama and Singh, 1995; and Chopra et al., 1999).

Biotransformation of AFB₁ in the cow’s liver and the corresponding AFM₁ levels in the milk depend on several factors including milk yield, microsomal mixed function oxidase activity, and presence or absence of bacterial mastitis in the udder (Chopra et al., 1999). According to two studies (Veldman et al., 1992; Chopra et al., 1999), normal carry-over was about 0.4–0.6% and daily AFB₁ intakes of 70 g in cows resulted in greater than the regulatory limit (0.05 g/l of AFM₁) in milk accepted in most countries. There have been attempts to demonstrate that AF detoxification in ruminants may be enhanced by altering the diet in ruminants with different specific protein sources (e.g. fish meal) or supple-mental amino acids (e.g. methionine) to enhance metabolism. However, the adverse effects of AF on lambs were not altered when soybean meal was replaced with fish meal in the diet (Edrington et al., 1994).
**Aflatoxin B<sub>1</sub> 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<sub>1</sub> from the cell (Hayes *et al*., 1991a). The involvement of these two pumps in AFB<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> from mammalian cells. The major adduct formed is the chemically unstable AFB<sub>1</sub>-N<sup>7</sup>-guanyl adduct, which is lost spontaneously (when mutations are likely to arise) from DNA to yield apurinic sites. The other two adducts (AFB<sub>1</sub> FAPY and AFB<sub>1</sub> III) are not lost spontaneously and may be catalytically removed by DNA repair enzymes (Hayes *et al*., 1991a). Ball *et al*. (1990) have shown significant interspecies differences in repair capacity (AFB<sub>1</sub>-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<sub>1</sub>-N<sup>7</sup>-guanine adduct is removed spontaneously and enzymatically in fibroblasts, probably by nucleotide excision repair mechanisms. The AFB<sub>1</sub>-N<sup>7</sup>-guanine adduct, however, may be converted to non-repairable, persistent AFB<sub>1</sub>-formamidopyrimidine lesions.

Following a single dosage with AFB<sub>1</sub>, maximum liver DNA adduct levels were measured after 2 hr. By 24 hr, 88% of the AFB<sub>1</sub>-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<sub>1</sub> for the induction of turnouts.
Thus, susceptibility and resistance to the toxic and carcinogenic effects of AFB$_1$ may depend on several factors: expression of bio-activating cytochrome P$_{450}$s, detoxifying cytochrome P$_{450}$s, 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 	extit{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.

2.4. SPICES

According to the International organization for Standardization (ISO) there is no marked distinction between spices and condiments. The term spice is used for aromatic plant products or mixtures thereof, either as whole or in ground form. It is rather loosely applied to an assortment of dried barks, roots, seeds fruits and flower parts. Spices which impart aroma, flavour and piquancy to food are generally tropical in origin (Samba Murty and Subrahmanyam, 1989).

Condiments on the other hand are spices that are usually added to food after cooking. In contrast, when the aromatic vegetable product comes from a temperate plant it is considered as culinary herb (non-woody) as in case of bay leaves, coriander, fennel, mustard etc. (Samba Murty and Subrahmanyam, 1989).

Herbs make all the difference to food; the cusinie of a region is characterized as much by the herb it uses as by the staple foods. Fragrant mixtures have come to characterize the cooking of certain regions- ‘bouquet garni’ (parsely, thyme and bay leaf) in France; ‘garam masala’ (cumin, coriander seeds, cardamom, cloves, mace, cinnamon, bay leaf, black pepper) in the Northern India and five-spice powder (star anise, sichuan pepper or fagra cassia, fennel seeds and cloves) throughout China and Vietnam are very popular. Mint, an uncompromising flavour, affects the taste buds very differently in Moroccan mint tea, mint sauce (an accompaniment for roast lamb), mint
julep (a sweet drink), harissa (a Tunisian paste made from mint, chilies, cumin, coriander, caraway seeds and garlic), tabbouleh (a Middle Eastern salad of mint, parsley and bulghur wheat) and tzatziki (a mint, cucumber and yogurt dip). Thus some herbs and spices are almost universally popular. Pepper, ginger, cinnamon, nutmeg, cloves and garlic are beloved of most cuisines (Bown, 1995).

1.4.1. HISTORY OF SPICES

The history of spices’ is one of the most spicy chapters in the history of the plant kingdom. Historically, spices have been responsible for the rise and fall of empires and the great sea voyages to explore the distant corners of the globe. In fact, spices have played an important role in shaping the course of history; they have been connected with adventure, conquest, exploration etc., around the world. In the latter half of the fifteenth century, both Portugal and Spain explored sea routes to the spice islands (Moluccas). Christopher Columbus sailed West from Spain in 1942, hoping to reach the spice islands ahead of the Portuguese, but he failed in his primary mission. Instead, he discovered America and also helped in the discovery of two of the three important New World spices, all spice (*Pimenta officinalis*) and red pepper (*Capsicum* spp). The third important New World Spice is vanilla (*Vanilla planifolia*). In the early part of the eighteenth century spices were smuggled away and planted around the world, especially in the West Indies. Now a days, substantial plantations are grown in America. However, the vast majority of spices are still obtained from the wetter parts of the tropics, chiefly Asia (Pruthi, 1992).

1.4.2. INTAKE OF SPICES

Spices and condiments have played a prominent part in all the civilization of antiquity, in ancient India and China. They were among the first objects of commerce between the East and the West. Population-wide average dietary intake of common spices has been estimated at 0.5g/person per day in Europe and 1.0g/person per day in New Zealand.
According to the American Spice Trade Association, per capita spice consumption in the United States was ~4g/person per day (3.6lb/person per year) in 1998, and hot spices such as black and white pepper, red pepper, and mustard seed account for 41% of US spice usage. In contrast, on the Indian subcontinent, turmeric consumption alone has been estimated at 1.5g/person per day. Generally, cuisines that traditionally do not include much meat use a wider variety of spices for seasoning. These include cuisines in areas of the world where vegetarianism has existed for centuries, such as among followers of Hinduism and Buddhism. Nearly all spices important in cooking to day are of Asian origin, with the exception of all spices, vanilla and chili. Thus, globally, the amounts and types of spices used vary widely (Pruthi, 1992).

1.4.3. DISTRIBUTION OF INDIAN SPICES

Most of the spices are grown in India because of the varied climate (tropical, subtropical and temperate). Out of the 109 spices listed by ISO only 63 are grown in the country. Commercial cultivation is limited to about a dozen of spices which attain importance in the internal and international market. In India, the major spices produced are pepper, cardamom, ginger, turmeric and chilies. Black pepper is one of the most important India spices and known as the ‘King of Spices’ or it is called the black gold of India. While next comes cardamom called the’ Queen of the spices’ through which India earns lot of foreign exchange. Some other important spices grown in India include ajowan, aniseed, caraway, celery, coriander, cumin, dill, fennel, fenugreek, garlic, onion, saffron and vanilla. Spices are grown mainly in Kerala, Karnataka, Tamil Nadu, Andra Pradesh, Maharashtra, Orissa, Rajasthan and Bihar. Each of these states and union territories grows one or more spices in abundance (Kochhar, 1998).

The world’s romance with Indian spices continues unabated. India is the largest producer, consumer and exporter of spices in the world. The international spice scenario depicts a quantum leap over the past
decade to more than 4.5 million tones, valued at US $ 1,5000 million. India’s share of the world spices trade is estimated as 45-50 per cent by volume and 25 per cent by value. The present annual production of spices in the country is 3.0 million hectares. The lion’s share (90%) of spices produced in India is absorbed in the domestic market and only 10 per cent is exported to over 150 countries. About 8.5 per cent of India’s export earnings from agricultural and allied products come from spices which constitute 1.24 percent of the total export earnings during 1999-2000.

A new group of value added products such as spice oils and oleoresins have come to stay in recent years in production and export (Peter and Nybe, 2002).

<table>
<thead>
<tr>
<th>Spices</th>
<th>Area (hectare)</th>
<th>Production (tones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black pepper</td>
<td>192.3</td>
<td>58.3</td>
</tr>
<tr>
<td>Cardamom(small)</td>
<td>72.4</td>
<td>9.3</td>
</tr>
<tr>
<td>Chilli</td>
<td>915.2</td>
<td>1018.0</td>
</tr>
<tr>
<td>Ginger</td>
<td>77.6</td>
<td>263.2</td>
</tr>
<tr>
<td>Turmeric</td>
<td>161.3</td>
<td>653.2</td>
</tr>
<tr>
<td>Clove</td>
<td>3250.00</td>
<td>2940.0</td>
</tr>
<tr>
<td>Coriander</td>
<td>546.5</td>
<td>290.0</td>
</tr>
<tr>
<td>Cumin</td>
<td>264.0</td>
<td>108.7</td>
</tr>
<tr>
<td>Garlic</td>
<td>114.4</td>
<td>495.3</td>
</tr>
<tr>
<td>Other Seed Spices</td>
<td>84.7</td>
<td>99.4</td>
</tr>
<tr>
<td>Tree Spices</td>
<td>28.4</td>
<td>11.1</td>
</tr>
<tr>
<td>Others</td>
<td>43.2</td>
<td>16.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5750.0</strong></td>
<td><strong>5963.2</strong></td>
</tr>
</tbody>
</table>

Export of spice oils, oleoresins and curry powder alone accounted for Rs.135 crores out of the total export of Rs.607 crores during 1994-95. The export of spice oil and oleoresins has increased from 2.6 tonnes
valued at Rs.92,000 in 1971-72 to 251.9 tonnes valued Rs.7.2 crores during 1992-93 and that of oleoresins from 700 kg worth Rs.48,00 to 1008.7 tones worth Rs.59.4 crores rupees during the above period (Edison, 1995).

Exploration of medicinal and nutraceutical properties of our traditional spices will pave a long way in the development of India spice Industry.

1.4.4. BENEFICIAL EFFECTS OF SPICES

Medicinal meals remain important in traditional Chinese and Ayurvedic practice. In the Siddha system practiced in Tamil Nadu, every meal must contain some forms of medicinal ingredients too in order to avoid the consumption of medicines separately. Plants have the capacity to synthesize a diverse array of chemicals, and understanding how phytochemicals function in plants may further our understanding of the mechanism by which they benefit humans.

In warm countries, the effects of eating hot spices, such as chili, ginger and pepper, are to raise the metabolic rate, increasing perspiration, effectively cooling the body, and speeding the excretion of toxins. Spices act also as preservatives, of great importance in warm regions where food deteriorates rapidly (Bown, 1995).

Herbs add colours as well flavour. Soups made from pale ingredients are much more appetizing when flecked with finely chopped parsley or chives. Yellow is an especially appetizing colour-most subtle in saffron, and brightest in turmeric. Though seldom seen in the form of seeds, annatto is a colouring appreciated every day in butter, margarine, and “red” cheeses, which would otherwise be cream-coloured. Paprika gives a glorious brick-red colour to dishes such as goulash. It is made from dried, powdered red peppers (*Capsicum annuum*), as is cayenne, but can be used in much larger quantities (Bown, 1995).

Peppermint has a soothing, mildly anaesthetic effect on the digestive tract, hence the popularity of after dinner mints and peppermint tea. Perilla, which is used with raw fish dishes in Japan, contains...
antidotes to seafood poisoning. Garlic is an excellent gastric disinfectant, well worth taking in capsule form, as well as in food, while travelling to prevent bouts of diarrhoea and vomiting. The therapeutic side to culinary herbs was once more popular than it is today (Bown, 1995).

1.4.5. CHEMICAL PROPERTIES OF SPICES AND THEIR SIGNIFICANCE

Essential oils are the key components in both the flavour and beneficial effects of herbs and spices, and many are strongly antiseptic, protecting against harmful microorganisms. Fennel, dill and caraway contain carminative oils that almost instantly relieve gas. They are particularly good with foods that many people find indigestible – fennel with oily fish, dill with cucumbers, and caraway with coleslaw or rich meats (Bown, 1995).

Herbs and spices will also increase the vitamin and mineral content of the food, and improve digestion. Garlic is rich in germanium, which has beneficial effects on the circulation. The bitter element in herbs and spices serves to prime the digestive system, stimulating the liver and gall bladder, improving digestion, especially of fats, and helping the elimination of toxins. Aperitifs with a hint of bitterness, and raw foods such as salads and ‘crudités’, are traditionally eaten for this purpose (Bown, 1995).

In plants, these compounds function to attract beneficial and repel harmful organisms, serve as photoprotectants and response to environmental changes. In humans, they can have complementary and overlapping actions, including antioxidant effects, modulation of detoxification enzymes, stimulation of the immune system, reduction of inflammation, modulation of steroid metabolism and antibacterial effects. Embracing a cuisine rich in spice, as well as in fruit and vegetables, may further enhance the chemopreventive capacity of one’s diet (Lampe, 2003).
1.4.6. PHYTOCHEMISTRY OF SPICES

Over millions of years, plants have developed the capacity to synthesize a diverse array of chemicals. In generally, these phytochemicals function to attract beneficial and repel harmful organisms, serve as photoprotectants, and respond to environmental changes. For example, numerous classes of phytochemicals, including the isoflavones, anthocyanins, and flavanoids, function as phytoalexins, substances that assist a plant to resist pathogens. Various glycosides of these also render plants unpalatable and thereby reduce intake by animals. Paradoxically, carotenoids aid in light collection under conditions of low light or help to dissipate excess absorbed energy as heat under conditions of high sun exposure; most plants have the flexibility to alter their carotenoid composition in response to growth under deep shade or full sunlight (Demmig-Adams et al., 1996). Understanding how phytochemicals function in plants may further our understanding of mechanisms by which they may benefit humans. Paradoxically, spices are grown as food enhancers, despite the intention of these tasty constituents to discourage consumption of the plant (Lampe, 2003).

Only a small number of primary compounds serve as precursors of the large array of phytochemicals produced by higher plants, and most of these are obtained from the early products of photosynthesis (Conn, 1995). The constituents responsible for the flavor properties of spices are products of secondary metabolism in plants: that is, they are not vital for plant tissue synthesis or energy production and storage, but their production is essential to the viability of the plant. Most known phytochemicals arise from 3 well-recognized metabolic pathways: the shikimate pathway, the cinnamic acid pathway, and the isoprenoid pathway. The shikimate pathway is a major source of carbon for many compounds (Bentley, 1990), in part because the 3 aromatic amino acids formed as end products: phenylalanine, tryptophen, and tyrosine, are important precursors that feed into several synthetic pathways. For
example, phenylalanine is the starting material for the cinnamic acid pathway, which produces numerous phenolic acids, coumarins, flavonoids, isoflavonoids, and lignans. These 3 amino acids, in addition to others, also provide the carbon atoms for production of glucosinolates in Brassicaceae. Pyruvic acid, an early product of photosynthesis, is the starting point for the isoprenoid pathway, from which families of carotenoids, terpenes, saponins, and so forth are derived.

Despite the large number of phytochemical classes, most plants contain only a few of them, and botanically related plants often contain similar or even the same constituents. As a result, spices tend to cluster in certain plant families, while other families do not contain any aromatic plants (Katzer, 2002). The major phytochemical classes associated with spices are outlined in Table 5 and include a diverse array of compounds of varying molecular weight and structure. The terpenes and terpene derivatives are probably the most important class of aroma compounds, with monoterpenes contributing to the fragrance of 90% of spices (Katzer, 2002). Monoterpenes occur in many different plants, and the characteristic aroma of a spice results from a specific mixture of monoterpenes and not a specific compound (Katzer, 2002).

Many phytochemicals are present in plants as glycosides (ie., with a sugar moiety attached). Generally, glycosides are non-volatile and lack fragrance. Cleaving the glycosidic bond yields the aglycon, which itself may be volatile and fragrant. For example, glucosinolates of the cabbage family (Brassicaceae) are hydrolysed by the plant enzyme thioglucosidase (myrosinase) when cells are damaged (eg., cut or chewed), yielding the pungent isothiocyanates, and vanillin is released from a glycoside precursor during drying, a step in the processing of vanilla beans. Thus, how a spice is processed will also determine the amount and form of its constituents (Lampe, 2003).
2.5. CLOVE

Clove – *Syzygium aromaticum* (Linn) Merr.perry

Synonym – *Eugenia caryophyllata* L.

Eng. – Clove

Hindi – Laung

Family – Myrtaceae

2.5.1. Distribution and description

Clove (Plate 1) is one of the most ancient and valuable spices of the orient and holds a unique position in the international spice trade. Native to Moluccas, the so called “Spice Islands” in the East Indian Archipelago, this spice was first introduced in India around 1800 AD by the East Indian Company. The company’s spice garden in Courtallam in Tamil Nadu was then established to cultivate clove and nutmeg as the principal spice crops. Induced by the success of its cultivation in Courtallam, cultivation of clove was extended during the period after 1850 A.D. to Nilgris (Burliar) in Tamil Nadu, Southern regions of Travancore and also to Cochin state on the slopes of Western Ghats. The important clove growing regions in India now are Nilgris, Tenkasi hills and Kanyakumari districts of Tamil Nadu and Kotayam and Quilon districts of Kerala (Samba Murty and Subrahmanyam, 1989).

Clove is the dried flower-bud of *Eugenia caryophyllata*, Thunb., an evergreen tree indigenous to the Molucca islands and cultivated there, as well as on the Malay peninsula, in Zanzibar and Pemba, Java etc. As the buds develop they assume a green and then crimson colour, when they are collected and dried. During the drying the crimson colour changes to reddish brown. The dried buds are about 15 millimeters long, and of a rich, reddish-brown colour.

Each flower bud consists of a fleshy sub-cylindrical lower portion crowned by four thick calyx teeth, and four, yellowish-brown, imbricated petals; the latter enclose numerous statements and a short, thick style. A transverse section of the fleshy, lower portion exhibits numerous oil glands near the periphery. The calyx and petals also contain oil glands,
but they are not so numerous. Cloves of good quality should have a strong aromatic odour and taste; they should be plump and of a bright reddish-colour, sink in water, and readily exude oil when intended.

The stalks upon which the cloves buds are borne are also collected and exported in considerable quantities. They are brown and woody, about 1 to 2 millimeters thick and 1 to 3 centimeters long. They yield about 5 or 6 per cent, of volatile oil, which is less agreeable aromatic than that of the cloves, although resembling it in specific gravity (1.040 to 1.065, oil of cloves 1.045 to 1.070) and optical rotation ([α]D, = up to 10°, oil of cloves the same), and containing rather more eugenol.

The nearly ripe fruits are also exported under the name of mother cloves (anthrophylli): they are dark brown ovoid, one-seeded berries, crowded by the remains of the calyx teeth. More cloves contain but little oil. Both clove stalks and mother cloves have been used to adulterate ground cloves. The former may be detected by the presence of numerous, nearly isodiametric and sclerenchymatous cells and the latter by the large starch grains which the presence of numerous characteristic, nearly isodiametric, sclerenchymatous cells; both petals and the stamens have been broken off, leaving the thick portion of the clove crowded by the somewhat persistent calyx teeth. Clove dust often consists largely of the broken stamens.

1.5.2. Soil and Climate

Deep and rich loams with high humus content are best suited for clove cultivation. In India, clove has developed well in the open sandy loams and the laterite soils of South Kerala region. But the best growth is seen in black loams of the semiforest regions. Clove abhors waterlogging and, therefore, perfect drainage is essential.

Clove is strictly a tropical plant and it requires a warm humid climate. Humid atmospheric condition and an annual rainfall of 150-250 cm are the ideal requirements of the crop. Clove thrives well in all situations ranging from sea level to an altitude of 800 to 900 meters (Kochhar, 1998).
1.2.3. Propagation

Clove is propagated through seeds. Usually the seeds become available for sowing from August to October. The seeds lose their viability and hence it is necessary to sow with or without the fruit coat. Raised nursery beds are prepared in a shady place and the seeds are sown in rows adopting a spacing of about 12 cm. The seeds begin to germinate in four to five weeks after sowing. The seedlings are slender and delicate and grow very slowly. Watering is necessary throughout the nursery period. The seedlings after about six months of nursery life are transferred to baskets made of bamboo or mud pots and nurtured properly under the shade till they attain an age of 12 to 18 months. Clove can also be propagated vegetatively by grafting on its own stock (Samba Murty and Subrahmanyan, 1989).

2.5.4. Harvesting and Curing

Clove tree begins to yield from the seventh or eighth year after planting. The full bearing stage is attained after about 15 to 20 years. The flowering season is September-October in the plains and December-January in high altitudes. The buds are ready for harvest in about four months.

The harvested buds are spread to dry in the sun either on grass-mats or on cement drying floor. During nights the buds should be stored under cover, lest they re-absorb moisture. Normally it is possible to dry the cloves in four or five days under direct sun and in about four hours when they are heated in zinc trays over a regulated fire. Fully dried buds develop the characteristic dark brown colour and are crisp. If the produce is uniformly good, approximately 8,000 to 10,000 cloves would weigh one kilogram. Clove is graded according to its appearance and impurity content. Good quality clove should be brownish black in colour, with full and plump crown, somewhat rough to the touch and without wrinkles and it should not contain more than 16% moisture and 5% foreign matter. Also it should have fine aroma and flavour and should readily exude oil when the stem is pressed with the finger nail (Kochhar, 1998).
2.5.5. Yield

There is considerable variation in the yield of clove. Under favourable conditions, well-grown trees may yield as much as 4 to 8 Kg of clove. The average yield from a bearing tree in a well maintained plantations in India is reported to be about 2.5 kg considering that the percent of bearing trees will be around 60, one hectare of plantation containing about 250 trees will yield about 375 kg. of cloves (Samba Murty and Subrahmanyam, 1989).

2.5.6. Uses

Clove is very aromatic and fine flavoured and imparts warming qualities. In all Indian homes, it is used as a culinary spice as the favour blends well with both sweet and savory dishes. Clove is used for flavouring pickles, curries, ketchup and sauces. It is highly valued in medicine as a carminative, aromatic and stimulant. Clove has stimulating properties and is one of the ingredients of betel chewing. In Java, clove is used in the preparation of a special brand of cigarette for smoking. The essential oil which is obtained by distilling clove with water or stream has even more uses. It is sued medicinally in several ways. The chief constituent of the oil, Eugenol, is extracted and used as an imitation carnation in perfumes (Kochhar, 1998).

2.5.7. Clove requirement in India

The requirement of clove in India is being met through imports. We have been importing large quantities of clove in the past. The average annual imports of clove were about 2,840 tones valued at about Rs.183 lakhs in the 1950’s. However, owing to the restrictions imposed from time to time, it has been possible to cut down the imports considerably and in the 1960’s, the annual import was of the order of 500 tones valued at about Rs.47 lakhs.

The chief clove producing countries are Zanzibar, which grows 90% of the total output, followed by Pemba, Madagascar, Indonesia,
Mauritius, and the West Indies. It is also grown in Malaysia, Sri Lanka, India and Haiti (Pandey and Anitha, 1988).

2.6. EUGENOL

Alkenyl benzenes are an important group of naturally occurring food flavouring compound in herbs and spices. Human exposure to eugenol (4-allyl-1-hydroxy-2-methoxy benzene) occurs by virtue of its presence in clove oil, which is used as a food, flavouring agent and as a tobacco additive (Phillips, 1990). Eugenol is also present in the essential oils of many other plants, such as cinnamon, basil and nutmeg. Clove and thulasi are known for their medicinal properties in India and other countries. The essential oil of clove is obtained from its buds and that of thulasi from its leaves by steam distillation. Eugenol is present in the higher boiling fraction of clove oil (Sen et al., 1992).

\[
\text{OH} \quad \text{OCH}_3 \\
\text{CH}_2\text{CH=CH}_2
\]

Eugenol is considered to be non-mutagenic, non-carcinogenic and generally recognized as safe (GRAS) by the FDA (Opdyke, 1975). The joint experts committee on food additives established an acceptable daily intake of eugenol of upto 2.5 mg/kg body weight for humans (IARC monographs, 1985). The daily per capita consumption is estimated to be 0.6 mg although the intakes are bound to show wide variations (FAO/WHO, 1982). Eugenol at levels of 0.3-1.25% in the diet did not show any adverse toxic effects on prolonged feeding to rats for 103 weeks (National Toxicology Program, 1983).

In contract to many other constituent of essential oils, eugenol can be analyzed by HPLC with UV-detection. For the extraction of eugenol, a
phenol with a non-polar side chain different organic solvents have been used (Hashimoto et al., 1981). The best results were obtained with n-hexane. The sensitivity depended on the selection of the wavelength of 220 nm. The UV spectrum of eugenol showed a maximum at 220-230 nm and a smaller one at 278 nm (Fiskher and Dengler, 1990).

In view of its analgesic and local anesthetic properties, eugenol is commonly used (with Zinc oxide) as a component of several dental materials, such as dental cements, impression pastes and surgical pastes (Paffenbarger and Rupp, 1979).

In traditional medicine, eugenol has been used in the treatment of flatulent colic, chronic diarrhea and other gastrointestinal disorders (FDA, 1979).

Eugenol is reported to show high antimicrobial activity at a level of 550 ppm or less (Paffenbarger and Rupp, 1979: Shapiro, 1994).

### 2.6.1. Antioxidant activity of Eugenol

Eugenol inhibits cellular lipid peroxidation, as revealed by decrease in the cellular production of TBA-reactive substances (Jeng et al., 1994). There are two possible explanations for this,

(a) Phenolic compounds have been shown to have the properties of antioxidant and free oxygen radical scavengers (Kuehl et al., 1977). A free phenolic hydroxy group is essential for scavenging free oxygen radical. Thus the reactive oxygen produced in the cell could be captured by eugenol.

(b) The second possibility is that eugenol could inhibit cellular free radical producing enzymes xanthine/xanthine oxidase as a well-established free radical generating system in the cell. It was found that eugenol inhibits xanthine oxidase activity (Jeng et al., 1994).

Eugenol by acting as a free radical scavenger, actively restores the tissue glutathion levels (Parasakthy et al., 1993).
Eugenol inhibits lipid peroxidation at the level of initiation, propagation or both. Eugenol, incorporated into the membrane due to its hydrophobic nature appears to play a major role in inhibiting non enzymatic lipid peroxidation by radical quenching. The eugenol retained in mitochondria (3 nmol approximately) is likely to chelate the excessive amount of Fe$^{2+}$ in the incubation mixture discounting chelation as a possible mechanism of inhibition. Eugenol inhibited Ca$^{2+}$ H$_2$O$_2$ catalyzed lipid peroxidation in human erythrocyte membrane (Nagashima, 1989). Eugenol did not undergo any perceptible chemical change during the course of peroxidation. (Nagababu and Lakshmia, 1992).

2.6.2. Antiulcer activity
Eugenol pretreatment at a concentration of 10-100 mg/kg significantly and dose-dependently reduced the gastric ulcers induced by administration of two ulcerogenic agents, i.e. platelet activating factor (PAF) and ethanol. Eugenol reduces not only the number of ulcers but also the gravity of lesions (Capassoa et al., 2000).

2.6.3. Anti microbial property
Eugenol shows antimicrobial activity at a level of 500 ppm or less (PaffenBarger and Rubb, 1979; Shapiro et al., 1994). Eugenol was found to be sensitive against organisms like E.coli, Enterobacter and Klebsiella pneumonia which are resistant to antibiotics like ampicillin, erythromycin and sulphamethizole (Suresh et al., 1992).

2.6.4. Hepatoprotective action of eugenol
CCL$_4$ is a well known hepatotoxic agent. The principle damage caused to liver by CCL$_4$ is via peroxidation of poly unsaturated fatty acids, leading to irreparable breakdown of membrane structure (Plaa and witsch, 1976). Eugenol demonstrated a significant hepatoprotective effect against CCL$_4$ poisoning. Eugenol has been found to decrease serum LDH, ALP, ACP and SGOT (Parasakthi et al., 1993).
2.6.5. Anti mutagenic/anti Carcinogenic property of eugenol

Many naturally occurring dietary constituents are being studied for their anti mutagenic or anti carcinogenic activity (Parasakthi et al., 1993).

There appears to be a relationship between the ability of certain xenobiotics to act as anti carcinogens and their ability to induce a set of enzymes involved in carcinogen detoxification and excretion (Yokota et al., 1988). These include non oxidative enzymes UDP-Glucuronyl transferase, GST and DT- diaprase-I induction of these enzymes by eugenol is an indication that it possesses anti mutagens/anti carcinogenic properties (Zheng et al., 1992). Eugenol inhibited the mutagenecity of Aflatoxin B$_1$ and N-methyl N$_1$-nitroso guanidine in *Salmonella typhimurium* test strain TA 100 (Rampelberg et al., 1993). Mutagenicity of benzene (a) pyrene [B(a)P] in the Ames test using liver S-9 or microsomes from rats treated with eugenol was suppressed in comparison with B(a)P induced mutagenecity (Yokota et al., 1986). Eugenol inhibited the microsomal activation dependent mutagenecity of 2-acetamidofluorine. It ameliorates the effect of environmental mutagens especially present in food (Saudamin and Unni Krishnan, 1995).

2.6.6. Toxicity

Alkenyl benzenes occur naturally in plants and their essential oils are used often as flavoring agents in foods and pharmaceutical preparations. Some allylbenzene derivatives such as safrol, estragole and methyl eugenol are toxic or carcinogenic in rodents (Miller et al., 1983). The carcinogenicity of these compounds is dependent on their metabolism to electrophilic intermediates. In contrast, eugenol is considered to be non-mutagenic and non-carcinogenic (Delforge et al., 1980: Miller et al., 1983). Eugenol lacks DNA binding activity in a number of animal experiments (Mauro et al., 1989; Phillips, 1990).

Even at the highest dose levels of 1000 mg/kg body weight, no toxic effects were observed (Rompelberg et al., 1993). Eugenol at a lower concentration (less than 1 nmol/L) protects cells from the genetic attack
of ROS produced endogenously and exogenously, because of low frequency, duration and dosage, clinical usage of eugenol is generally safe for humans and even has beneficial effects (Jeng et al., 1994).

2.6.7. Metabolism of Eugenol

Eugenol was rapidly absorbed and metabolized after oral administration and was almost completely excreted in urine within 24hr. Unmetabolized eugenol excreted in urine amounted to less than 0.1% of the dose. The urine contained conjugates of eugenol and of nine metabolites. Some 95% of the dose was recovered in the urine, most of which (greater than 99%) consisted of phenolic conjugates. 50% of the conjugated metabolites were eugenol glucuronide and sulphate. Other metabolic routes observed were, the expoxide diet pathway, synthesis of a thiophenol and of a substituted propionic acid, allyl oxidation and migration of the double bond (Klun gsoyr and Scheline, 1983; Fischer et al., 1990a).

Eugenol in body fluids (serum, urine & bile amounts in the range 0.02-100 μg per ml. Eugenol undergoes pronounced first phase effect (metabolism of drugs before they reach the systemic circulation). In serum, unconjugated eugenol was not detected after an oral dose of 150 mg. More than 80% of the dose was excreted within 6 hr after oral administration (Fischer and Dengler, 1990).

2.6.8. The fate of eugenol in rats

The intragastric administration of eugenol at the dose of 0.5-100 mg/kg body wt. to wister albino rats resulted in rapid and extensive excretion of (14C metabolites, predominantly in the urine (75-80% of dose) and about 10% in the faces (Sutton et al., 1985).

Eugenol has a free hydroxyl group which leads to its rapid elimination by conjugation with sulphate and glucuronic acid (Plea and Witsch, 1976; Boutin et al., 1983). The metabolism also includes demethylation of the allyl double bond (Boutin et al., 1983).
Several allylbenzene derivatives are known to form 1-hydroxy metabolites which lead to the formation of electrophilic species which are responsible for the toxicity or carcinogenicity (Miller et al., 1979). These compounds include safrol, estrogol and methyl eugenol (Sotheim and Scheline, 1976., Swanson et al., 1981).

Eugenol may form 1" hydroxy eugenol (in cytochrome p-450 dependent oxidation during microsomal incubation) initially in the reaction and then rearrange (through loss of water) to form the quinona methide. Alternatively the quinone may be formed directly.

The formation of quinone methide would lead to the conjugation with glutathione. Thus no genotoxic metabolites were formed (Thompson et al., 1990).

2.7. Silymarin

2.7.1. Synonyms

Silybin, Apihepar, Laragon, Pluropon, Silarine, Silepan, Silliver, Silmar.

2.7.2. Biological Source

Silybin is obtained from the seeds of milk thistle, Silybin marianum (L.) Gaertn. (Carduus marianus L.) belonging to the natural order Asteraceae.

2.7.3. Chemical Constituents

The seeds of milk thistle is chiefly comprised of three isomers, namely: silidinanin, silicristin and the major component silybin (formerly known as silymarin). It has been more or less established beyond any reasonable doubt that silybin is produced in the plant by means of a radical coupling of a flavonoid and coniferyl alcohol (Wagner et al., 1968 as cited by Kar, 2003).
2.7.4. Isolation

A crude mixture of antihepatotoxic principle was first isolated from the plant (milk thistle) and designated as silybin (Wagner et al., 1968 as cited by Kar, 2003).

2.7.5. Characteristic Features

The anhydrous silybin has mp 158°C and it decomposes at 180°C. Its physical characteristics are as follows: \([\alpha]_{D}^{20} + 11°\) (c=0.25 in acetone+alcohol). Its \(\text{uv}_{\text{max}}\) (in methanol): 288 nm (log \(\varepsilon\) 4.33). It is soluble in acetone, ethyl acetone, methanol, ethanol and found to be sparingly soluble in chloroform. It is practically insoluble in water. It also occurs as the monohydrate crystals from a mixture of acetone and petroleum ether having mp 167°C (decomposes at 180°C); and from a mixture of methanol and water having mp 180°C.

2.7.6. Mode of Action

1. Silybin is most importantly and widely employed as a therapeutic agent for protecting liver cells \textit{in situ} or cell not yet irreversibly damaged by acting directly on the cell membranes
(i.e. the targeted site) so as to prevent the entry of toxic substances (Dehmlow et al., 1996).

2. It also augments and stimulates the ‘protein synthesis’ i.e., anabolism of protein, thereby accelerating the process of regeneration and the production of hepatocytes (Kenneth Flora et al., 1998).

3. It has also been experimentally proven that silybin binds specifically to a regulative subunit of the DNA-dependent RNA polymerase-I at a particular site ley mimicking a natural steroidal effector and thereby causing an activation of this enzyme. Consequently, the synthetic rate of ribosomal RNAs is increased considerably, thus leading to an enhanced formation of intact ribosomes that ultimately gives rise to an increased protein synthesis (Jacobs et al., 2002).

4. Silybin may also be employed as a supportive treatment for the management and cure of chronic inflammatory liver conditions and cirrhosis (Ferenci et al., 1989).